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Growth Factor Delivery From Fibrin Matrics Containing Affinity-Based Delivery Systems To Treat Peripheral Nerve Injury

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GROWTH FACTOR DELIVERY FROM FIBRIN MATRICS CONTAINING AFFINITY-BASED DELIVERY SYSTEMS TO TREAT PERIPHERAL NERVE INJURY

by

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ABSTRACT OF DISSERTATION

GROWTH FACTOR DELIVERY FROM FIBRIN MATRICS CONTAINING AFFINITY-BASED DELIVERY SYSTEMS TO TREAT PERIPHERAL NERVE INJURY

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This thesis work sought to develop a biomaterial to further the understanding of affinity-based delivery and to serve as a potential treatment for peripheral nerve injury. The use of an affinity-based delivery system (ABDS) with growth factors in a nerve guidance conduit (NGC) was hypothesized to promote nerve regeneration and functional recovery following a critical nerve defect. Evaluation of affinity-based delivery using peptides with varying binding affinity for heparin determined that peptide binding affinity for heparin affected the release rate and biological activity of nerve growth factor (NGF) *in vitro*. The ABDS presented biologically active NGF, which promoted neurite extension regardless of peptide binding affinity for heparin. The efficacy of the ABDS in vivo to promote nerve regeneration in a rat sciatic nerve critical defect was determined through histomorphometric outcomes. The ABDS with any affinity peptide and NGF was similar to the isograft in aspects of nerve regeneration including: fiber density, nerve regeneration quality, fiber maturity, and the fiber organization of the regenerating nerve 6 weeks after treatment. Alternatively,

the ABDS effectively sequestered and slowed the release of glial-derived growth factor (GDNF) and promoted neurite extension *in vitro*. The efficacy of the ABDS and GDNF *in vivo* to promote nerve regeneration in a rat sciatic nerve critical defect was determined through histomorphometric outcomes. Histomorphometric measures revealed that the ABDS and GDNF promoted nerve regeneration similar to the isograft 6 weeks after treatment in measures of fiber density, nerve regeneration quality, fiber maturity, and the fiber organization of the regenerating nerve. Functional recovery and modality specific nerve regeneration were studied with the ABDS and growth factor in a rat sciatic nerve critical defect 12 weeks after treatment. Behavioral outcomes and electrophysiological responses including evoked motor responses were similar to functional outcomes in the isograft with the delivery of NGF, but superior to the isograft with the delivery of GDNF. Both GDNF and NGF delivery supported the regeneration of motor and sensory neurons equivalent to the isograft, as assessed by retrograde labeling. Overall, this work indicates that affinity-based growth factor delivery from fibrin matrices enhances nerve regeneration.

To my parents, who have always supported me in my endeavors

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Chapter 1

Introduction

1.1 Overview

This work seeks to develop a biomaterial to serve as a potential treatment for peripheral nerve injury. This work also seeks to further the understanding of affinity-based delivery to treat peripheral nerve injury. The overall objective of this research was to evaluate the effect of growth factor delivery from fibrin matrices containing an affinity-based delivery system (ABDS) on peripheral nerve injury. Fibrin is a natural material and provisional extracellular matrix (ECM) involved in wound healing, is biocompatible, and is capable of promoting cell migration and axonal growth. Sakiyama-Elbert and Hubbell previously designed an ABDS containing a bi-domain peptide capable of covalent incorporation during fibrin polymerization and interaction with heparin, which can interact with growth factors (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b). Further research from Sakiyama-Elbert and colleagues developed bi-domain peptides that could be covalently incorporated during fibrin polymerization and could interact with heparin with varying affinity to modulate growth factor release rates. The heparin-binding domain of these bi-domain peptides was identified from an increasing step gradient of sodium chloride concentration to modulate interaction with heparin (Maxwell, Hicks et al. 2005). These peptides that vary in binding affinity for heparin were utilized for a portion of this thesis work to assess the role of peptide binding affinity for heparin and release rate on nerve regeneration. Additionally, growth factors were used in the ABDS to

promote nerve regeneration and potentially target different neuronal populations during regeneration. The choice of growth factor was based on its ability to target sensory and motor neuron populations.

The first study of this thesis work assessed the role of peptide binding affinity for heparin in modulating the release rate and biological activity of nerve growth factor (NGF). The ABDS incorporating a peptide with high binding affinity for heparin and NGF previously demonstrated enhanced biological activity (Sakiyama-Elbert and Hubbell 2000a). To explore the role of binding affinity, peptide sequences were previously identified that exhibited "high", "medium", and "low" affinity for heparin using an increasing step gradient of sodium chloride concentration (1.0 M, 1.5 M and 2.0 M NaCl were used to elute the low, medium and high affinity peptide display phage from a heparin affinity column, respectively) (Maxwell, Hicks et al. 2005). These peptides were synthesized containing the identified heparin binding domains along with a transglutaminase substrate to allow crosslinking into fibrin matrices (Maxwell, Hicks et al. 2005). The first study in this thesis work utilized these peptides to assess the role of peptide binding affinity for heparin with this ABDS and NGF. The ratio of peptide to heparin and the peptide binding affinity for heparin were varied to assess differences in NGF release rates through mathematical modeling and *in vitro* experiments. The ratio of peptide to heparin and the peptide binding affinity for heparin were also modulated to assess differences in biological response. Chick embryo dorsal root ganglia (DRG) were implanted into fibrin matrices with ABDS to assess the biological activity of delivered NGF through neurite extension.

The second study extended the previous work of the first study to provide insight into the effect of peptide binding affinity for heparin on nerve regeneration *in vivo*. To assess

how peripheral nerve regeneration *in vivo* was affected, a rat sciatic nerve critical defect (13 mm) was bridged with a nerve guidance conduit (NGC) containing the ABDS with heparin binding peptides and NGF previously utilized in the first study. To determine the effect of modulating peptide binding affinity for heparin on nerve regeneration, regenerated tissue was harvested after 6 weeks for histomorphometric analysis and compared to the clinical equivalent in a rat model (isograft).

The third study characterized GDNF delivery from the ABDS *in vitro* utilizing the heparin binding peptide that maximized nerve extension *in vitro* and best promoted nerve regeneration *in vivo* based on the results of first and second studies. Release rates were characterized for varying concentrations of delivery system components to determine which conditions resulted in sustained growth factor delivery. Dose response studies were performed to determine the appropriate concentration of GDNF to maximize neurite extension from chick embryo DRG and determine if the ABDS can enhance neurite extension.

The fourth study extended the results of the third study to assess the role of affinity-based delivery of GDNF *in vivo* to promote nerve regeneration. A rat sciatic nerve critical defect (13 mm) was bridged with a NGC containing the ABDS and GDNF to determine its efficacy in promoting peripheral nerve regeneration. The effectiveness of nerve regeneration and histomorphometric measures were assessed on regenerating tissue harvested after 6 weeks following treatment. The results were compared to the clinical equivalent in a rat model (isograft).

The final study assessed the role growth factors in an ABDS have on peripheral nerve regeneration modalities (sensory versus motor axonal regeneration) and functional

recovery. A NGC containing the ABDS and growth factors were used to bridge a rat sciatic nerve critical defect (13 mm). The growth factors included were known to target different neuronal populations. Behavioral and electrophysiological measures of regenerating nerves and innervated muscle were measured. The regenerating nerves were also retrograde labeled to assess sensory and motor nerve regeneration and compared with the functional recovery results.

This introduction will discuss characteristics of peripheral nerve injury and factors known to influence nerve regeneration to better elucidate the reasoning for the studies described in this thesis work. Additionally, current and past treatments for peripheral nerve injury including biological grafts, nerve guidance conduits, and growth factor delivery will be discussed to establish the state of the field.

1.2 Peripheral Nerve Injury and Regeneration

The peripheral nervous system (PNS) facilitates the extension of signals from the central nervous system (CNS) to limbs and other organs. The PNS is susceptible to injury particularly due to the long axonal processes that extend throughout the body. The most common cause of these injuries are motor vehicle accidents, stabbing and gun shot wounds, and stretch and compression related injuries due to falling and represent a large number of repair procedures performed annually (Kouyoumdjian 2006). Damage to the nervous system is catastrophic and results in impaired motor and/or sensory function at denervated end-organs. The PNS is capable of limited regeneration; however, axonal damage still

remains clinically challenging to treat. This section will describe peripheral nerve injury and regeneration and issues involved in nerve regeneration.

1.2.1 Characteristics of Injury and Regeneration

Peripheral nerves consist of fascicles containing a mixture of sensory and motor axons, some myelinated by Schwann cells (SCs). The nerve fascicle itself is made up of connective tissue layers: the inner endoneurium, the perineurium surrounding individual fascicles, and the epineurium that bundles the fascicles into a nerve. Injuries to the peripheral nervous system can involve secondary damage to any one of these layers, or more severe injuries such as compression or lacerations resulting in nerve lesions or complete nerve transection (Burnett and Zager 2004).

Following injury to the nerve, Waller determined that the nerve undergoes pathological changes due to the separation of the axon from the cell body. The degeneration of the separated axonal portion and myelin (distal stump) has thus been named Wallerian degeneration (Waller 1850). Initially, the loss of a significant portion of the axon can result in ionic imbalances and influx. This imbalance and other pathological conditions result in chromatolysis or possibly cell death. Cellular death after nerve transection can be as high as 30 – 35% in dorsal root ganglia (Otto, Unsicker et al. 1987; McKay Hart, Brannstrom et al. 2002). However, neurons can survive nerve injury and cell death by upregulating genes responsible for growth and survival. These genes are signaled through trophic support provided by neighboring regions to the cell body and within the cell itself due to the injury (Costigan, Befort et al. 2002).

Besides the resulting neuronal changes, the distal stump undergoes many significant changes that prepare it for regenerating axons from the proximal stump to enter. The first of which is the invasion of glial cells to facilitate phagocytosis of myelin and axonal debris in the distal stump that are inhibitory to axonal regeneration. These glial cells phagocytose the axonal and myelin debris in a process that takes approximately a week (Waller 1850; Bruck 1997). Experiments by Friede and colleagues determined that macrophages are the major cell involved in the phagocytosis of myelin and myelin debris, while SCs play a role in digesting myelin debris, but to a lesser extent (Beuche and Friede 1984; Scheidt and Friede 1987).

Closely following axonal debris clearance SCs proliferate in part due to signaling from axonal membrane and myelin debris (Salzer and Bunge 1980) but also due to stimulation by macrophages. Macrophages *in vitro* produce a medium that is mitogenic for SCs (Baichwal, Bigbee et al. 1988), and *in vivo* macrophages accumulate in the distal stump prior to SC proliferation possibly indicating a role in signaling SC proliferation (Williams and Hall 1971). These proliferating SC align themselves to the remaining basal lamina and endoneurial tubes in the distal stump. These aligned SC tubes are known as the bands of Büngner and act as natural support for sprouting axons during regeneration in order to facilitate guidance back to end-organ targets (Waller 1850; Burnett and Zager 2004). Thus, the distal stump acts as a naturally beneficial scaffold to the regenerating axons.

Following nerve transaction numerous sprouts from the original axon grow toward the distal stump along the basal lamina and SCs in the endoneurial tubes (Haftek and Thomas 1968). These sprouts or neurites are guided by cell adhesion molecules and ECM, which neurites bind via cell-surface receptors. SCs are responsible for the production and

replacement of basal lamina, which principally consists of laminin, type IV collagen and fibronectin (Rogers, Letourneau et al. 1983; Fawcett and Keynes 1990). These neurites are also guided by diffusible trophic factors, primarily secreted by SCs and to an extent by their end-organ targets, which promote neurite survival, migration , and synapse formation at the end-organ (Reichardt and Tomaselli 1991). Regenerating axons become myelinated shortly after migrating through the endoneurial tubes in the distal stump. These axons can reestablish connections with end-organ targets; for example in the case of muscle, motor axons reform neuromuscular junctions at the motor endplates. Axons reach maturity with increased myelination after reinnervation with their appropriate end-organ targets.

1.2.2 Challenges in Peripheral Nerve Regeneration

Although the PNS is capable of regeneration, axonal regeneration generally does not occur for large defects separating the proximal and distal nerve ends if not treated clinically. More importantly, axonal regeneration does not necessarily lead to restored function, and the degree of motor versus sensory regeneration varies based on nerve injury treatment (Fawcett and Keynes 1990). Consequently axonal migration may follow endoneurial tubes that lead to incorrect end-organs or the correct end-organ such as muscle, but the incorrect particular muscle (Wigston and Donahue 1988; Kingham and Terenghi 2006). Alternatively, axons may innervate muscle endplates that they did not previously innervate. As already mentioned, numerous sprouts from each axon extend into the distal stump, and these branches typically are eliminated when improper connections with an end-organ target form (Aitken 1949); however, muscle endplates can be reinnervated by different motor fibers than originally found. This improper innervation can result in loss of coordination or complete

improper functioning of the muscles associated due to insufficient muscle contraction (Kingham and Terenghi 2006). Additionally, the original arrangement of fast and slow twitch muscle fibers is no longer preserved as it was before in normal muscle (Burnett and Zager 2004). Furthermore, even with appropriate muscle reinnervation, full functional recovery outcomes are difficult due to the lack of proprioception provided by proper sensory reinnervation of the muscle (Burnett and Zager 2004).

Several theories on motor versus sensory nerve regeneration and axonal guidance exist. One hypothesis considered is that end-organs, such as muscle, provide primary support for appropriate axonal pathfinding through diffusible cues or tropic support (Madison, Robinson et al. 2007). Another theory suggests that SCs and endoneurial tubes provide guidance cues to axons (Politis 1985; Brushart 1988; Wigston and Donahue 1988). Regenerating axons in general follow a chemotropism where they grow preferentially toward the distal nerve segment instead of other tissue (Fawcett and Keynes 1990). Robinson, Madison and colleagues have extended the role of chemotropism to describe how axons migrate to their correct end-organ pathways. They demonstrated that motor axons rely on guidance cues in the form of trophic support, primarily diffusible factors derived from their end-organ targets, to properly find their reinnervation targets. These discoveries were demonstrated through a series of experiments in rat femoral nerve by modulating the distance axons travel to their end-organ after injury (Robinson and Madison 2004; Madison, Robinson et al. 2007; Uschold, Robinson et al. 2007). This theory is also supported by the observation that motor and sensory axons contain different cell-surface receptors for these diffusible factors (Boyd and Gordon 2003). Alternatively, the SCs or endoneurial tubes may be a guiding force for correct axonal pathfinding. Some evidence for this is based on nerve crush injuries, which differ from complete or partial transection injuries as the axons that

regenerate typically remain in their parent endoneurial tubes and reestablish high levels of functional recovery following injury (Haftek and Thomas 1968). Based on these results, it may be that the disruption of the endoneurial tubes in traumatic injury leads to inappropriate target reinnervation and incomplete regeneration following injury. Additionally, research in preferential motor reinnervation in the rat femoral nerve model led Brushart and colleagues to hypothesize that the endoneurial tubes themselves or the Schwann cells within the tubes may direct axons down their correct pathways (Brushart 1988). Overall, axonal guidance is likely directed to a degree by all these theories. Therefore, therapies to treat injuries should consider the inclusion of these components to lead to better functional recovery following injury.

1.3 Treatment Therapies

As previously mentioned, damage to peripheral nerve can result in a defect disconnecting the proximal and distal nerve stumps leading to loss of motor or sensory function. The rate of axonal regeneration can vary but on average is around 1 mm/day making regeneration a slow process for injuries far from the innervation site (Evans 2001; Burnett and Zager 2004). However, neural outgrowth cannot occur when a significant defect separates the proximal stump from the distal stump, and surgical intervention is often required (Lundborg 2000). In order to promote nerve regeneration, clinical strategies for repair involve bringing the damaged nerve ends together in order to promote migration of glial cells and surviving axons to grow from the proximal stump to the distal stump. The most common method of nerve repair is direct suture of the two severed nerve ends together; however, this is often not feasible if the defect between the two ends is too large,

as excessive tension produced by directly reconnecting the two ends would disrupt regeneration (de Medinaceli, Wyatt et al. 1983). Therefore, a bridge or scaffold must serve to reconnect the proximal and distal stumps. This section describes materials used to serve as the bridge between the nerve stumps.

1.3.1 Biological Grafts

Biological grafts using microsurgical techniques to bridge critical nerve defects were first performed in the 1960's (Millesi 1973). Despite significant advances in nerve reconstruction, the nerve autograft still remains the clinical standard of care for critical, long, peripheral nerve defect repair. The autograft provides a scaffold and trophic support in the form of basal lamina, endoneurial tubes, and SCs for the regenerating axons guiding nerves to the distal stump (Belkas, Shoichet et al. 2004). Donor nerves that are commonly used as autografts include the sural (sensory) nerve or other cutaneous nerves (Meek and Coert 2002). However, disadvantages of autografts include loss of feeling and possible pain or itching at the donor site due to scarring and morbidity, insufficient donor tissue availability, risk of disease spread, secondary deformities, and less than optimal dimensions (diameter and/or length) of the donor tissue to span the injury site (Evans 2001; Belkas, Shoichet et al. 2004). Additionally, functional recovery with autografts varies. For example, less than 25% of patients who received autograft repair of the median nerve at the wrist level regained full motor function and only 1-3% recovered normal sensation after 5 years (Beazley, Milek et al. 1984; Dellon and Mackinnon 1988). Even with appropriate matching of fascicles during autograft surgery, axonal guidance to the original fascicles is not guaranteed, which can lead to incorrect innervation of muscles (Gordon, Sulaiman et al. 2003). Although the autograft

needs improvements, the less than optimal results with autografts may be attributed in part to the donor nerve material. For example, the repair of motor nerve defects with motor grafts was found to be superior to sensory grafts in measures of nerve density, percent nerve, and total fiber number, independent of graft cross-sectional area (Brenner, Hess et al. 2006). Also, the repair of a mixed nerve defect with a mixed or motor graft resulted in superior regeneration than compared to sensory grafts with respect to nerve fiber number, percent nerve, and nerve density (Nichols, Brenner et al. 2004). However, donor motor nerves are difficult, if not impossible, to obtain due to their invaluable current function; therefore, alternative biological grafts have been considered.

Nerve allografts have been considered as one such alternative; however, their use requires immunosuppression to avoid rejection and nerve regeneration failure due to their foreign nature (Evans, Midha et al. 1994). The Mackinnon lab has given considerable study to the use of nerve allografts as alternatives to autografts. They have tested antibodies to cell-adhesion molecules to induce antigen-specific tolerance, which allow the allografts to perform as well as isografts in nerve histomorphometry measures in mice (Nakao, Mackinnon et al. 1995) and rats (Nakao, MacKinnon et al. 1995). They also utilized a systemic treatment of anti-CD40 ligand monoclonal antibody in mouse (Brenner, Tung et al. 2004) and primate (Brenner, Jensen et al. 2004) models, which resulted in nerve regeneration in allografts that were similar to autografts during the drug treatment; however, after the drug treatment was stopped, the allografts were rejected by the body (Brenner, Jensen et al. 2004; Brenner, Tung et al. 2004). To avoid immune suppression, the company Axogen, Inc developed technology to decellularize allografts taken from human cadaver tissue. The Mackinnon lab performed studies with the grafts compared to isografts to determine its potential effectiveness in peripheral nerve injury. They demonstrated that decellularized

nerve allografts do not perform as well as isografts in nerve histology, electrophysiology, and effectiveness in bridging a 14 mm critical defect length. The enhanced nerve regeneration in the isograft was attributed in part to SCs retained in isografts promoting nerve regeneration, which are lost in the decellularization process (Whitlock, Tuffaha et al. 2009). Others researchers have also studied acellular nerve grafts as alternatives and similarly concluded that the lack of cellular support for long nerve defects resulted in nerve regeneration inferior to autografts (Gulati 1988).

Other biological tissues to serve as grafts have been investigated due to their greater availability and dimensions (diameter and/or length). Vein or artery has been used a biological substitute to nervous tissue due to its similarities to a conduit, which can encapsulate the regenerating nerves and naturally degrades over time (Foidart-Dessalle, Dubuisson et al. 1997). Consequently, veins and arteries can present a major obstacle to regeneration because their mechanical properties are not ideal, where the thin walls can lead to collapse and excess pressure on regenerating nerves, and the tissue is mismatched potentially leading to scarring (Belkas, Shoichet et al. 2004). Muscle tissue offers a better alternative to vein or artery due to basal lamina arrangement that mimics the endoneurial tubes contained in peripheral nerve and contains collagen and laminin to promote nerve outgrowth (Belkas, Shoichet et al. 2004). Mechanical dissimilarities and scarring due to tissue mismatch have been observed when it is used as a graft (Meek and Coert 2002). More promising results with both vein and muscle grafts have been demonstrated with isogenic SCs injected into either graft as treatment for nerve injury (Fansa, Keilhoff et al. 1999a; Fansa, Keilhoff et al. 1999b; Fansa and Keilhoff 2004). Either donor tissue loaded with SCs performed as well as the isograft in histomorphometric measures of nerve density and g-ratios while the tissues without cells were inferior in nerve regeneration (Fansa and Keilhoff

2004). However, the donor tissue would still need to be harvested from the recipient in order to avoid adverse foreign tissue responses (Fansa, Schneider et al. 2002; Belkas, Shoichet et al. 2004). Therefore, other materials, such as tubular repair using nerve guidance conduits (NGCs), have been considered.

1.3.2 Nerve Guidance Conduits (NGCs)

NGCs have been studied for many years as a potential "off the shelf" alternative to nerve grafts for the treatment of nerve gaps. NGCs typically consist of a hollow conduit that can be filled with an ECM scaffold. Efforts have been focused on the construction of biodegradable conduits, which may avoid biocompatibility issues present with permanent material placement in the body. The main advantage of using conduits is that they isolate the environment where regeneration is occurring and allow controlled presentation of cues to examine their effects on nerve regeneration.

Lundborg and colleagues considered using a tube or conduit to bridge a nerve defect; however, their motivation initially was to study peripheral nerve regeneration, not as alternative treatment for bridging the nerve defect. They used a psuedosynovial neural sheath to bridge a nerve defect in the rat sciatic nerve (Lundborg and Hansson 1979; Lundborg and Hansson 1980), which was shortly followed by silicone conduits. Both materials led to a convenient method to encapsulate trophic factors associated with nerve regeneration and to study the time course of nerve regeneration. These experiments provided knowledge that a cellular scaffold of natural proteins and glial cell migration followed axonal nerve sprouting into an empty tube, indicating that even in the absence of a cellular scaffold peripheral nerves are capable of producing their own to support

regeneration (Lundborg and Hansson 1979; Lundborg and Hansson 1980; Lundborg, Dahlin et al. 1982a; Lundborg, Dahlin et al. 1982b).

Clinically, silicone conduits have been used in the treatment of small nerve defects in humans (Lundborg, Dahlin et al. 1991; Lundborg, Rosen et al. 1997; Lundborg, Rosen et al. 2004) due to their biocompatibility and mechanical stability, although there have been reports of associated morbidities. Silicone conduits have been reported to cause chronic nerve compression and irritation at the implantation site requiring removal (Merle, Dellon et al. 1989; Danielsen, Dahlin et al. 1993; Dellon 1994; Battiston, Geuna et al. 2005). The long-term presence of a material surrounding nerve may have presented the pathology; therefore, alternatives have been suggested to alleviate the permanence of the material, such as a biodegradable conduit.

To this end numerous groups have constructed and examined the properties of conduit materials such as poly-L-lactic acid (Hadlock, Elisseeff et al. 1998), poly(lactic-co-glycolic acid) copolymer (Hadlock, Elisseeff et al. 1998), poly(L-lactide–co-6-caprolactone) (Nicoli Aldini, Perego et al. 1996) and vinylidenefluoride-trifluoroethylene copolymer (Fine, Valentini et al. 1991). These polymer materials were selected due to their range of degradation, mechanical stability, and in the latter instance, its piezoelectric properties that may be beneficial to nerve regeneration. Alternatively, natural materials can serve as biodegradable conduits substitutes to polymers, offer permeability to oxygen and nutrients and may have biocompatible advantages over polymers. The repair of peripheral nerves with a conduit fabricated from collagen has demonstrated promise in terms of its ability to promote nerve regeneration and still provide structural support during the regeneration process. In a rodent and primate short nerve defect model, these conduits demonstrated

effectiveness in promoting functional recovery in electrophysiology measures compared to direct suture repair and autologous nerve graft repair (Archibald, Krarup et al. 1991). These collagen conduits also worked as effectively as nerve autografts in terms of physiological recovery of motor and sensory responses in primates in further follow-up studies (Li, Archibald et al. 1992). Alternatively fibronectin has been oriented into mats in order to produce conduits that can promote nerve regeneration (Whitworth, Brown et al. 1995), and more recently, fibrin conduits were constructed that promoted nerve regeneration (Kalbermatten, Pettersson et al. 2009).

Overall, NGCs provide valuable insight into the nerve regeneration process as well as potential to treat peripheral nerve defects. Polymer and natural NGC materials generally support axonal regeneration for small defects, and in some cases large defects. However, NGCs can be further improved by design of a cellular scaffold to encourage and promote SC and axonal growth into the conduit.

1.3.3 Scaffolds for a Nerve Guidance Conduit

Stimulation of regeneration through NGCs for large nerve defects (> 3 cm) has proven difficult, so a number of materials have been studied for use as luminal conduit fillers (Schmidt and Leach 2003). Initial research with NGCs demonstrated that a scaffold for cellular migration proceeds glial cellular infiltration and axonal growth into a conduit; therefore, providing a scaffold for cellular migration could circumvent the need for the body to construct its own scaffold before nerve regeneration and accelerate the regeneration process. The focus of this thesis work was directed toward a luminal component for NGCs.

The ideal scaffold would consist of proteins that naturally reside at the nerve defect site. SCs' major function is to produce extracellular matrix, where they provide the basal lamina for the cell (Bunge, Bunge et al. 1986), therefore besides providing a scaffold for axonal migration, the support of SC proliferation and migration would also be ideal. *In vitro* studies comparing various ECM proteins in axonal development have not demonstrated dramatic differences due to the material protein used. For example, laminin and type IV collagen substrates have both increased neurite outgrowth over uncoated surfaces (Sanes 1989; Isahara and Yamamoto 1995; Venstrom and Reichardt 1995). Therefore, *in vivo* studies have focused on various natural proteins that could encourage nerve regeneration.

The nerve graft itself is primarily made up of basal lamina protein consisting of laminin, which is produced by SCs. Laminin for this reason has been incorporated into conduits to test its capacity on nerve regeneration. Madison *et al.* increased the rate of axonal growth in a short rat sciatic nerve defect within a silicone or degradable conduit by the inclusion of laminin gels (Madison, da Silva et al. 1987). Furthermore, they later followed this work to determine that the inclusion of collagen or laminin gels enhanced nerve regeneration and the effectiveness in bridging long nerve defects (> 20 mm) (Madison, Da Silva et al. 1988).

Fibrin is a provisional ECM and a natural material that has been considered for nerve regeneration. Initial experiments using a 10 mm gap in the rat sciatic nerve contained within a silicone conduit revealed that at one week a fibrin matrix spanned the chamber. At two weeks, SCs, fibroblasts, and endothelial cells had migrated into the matrix and into both stumps, and axons reached the distal stump by three weeks with a proximal gradient of myelin advancing (Williams, Longo et al. 1983). Therefore, it was postulated that pre-filling

a conduit with fibrin could accelerate the regeneration process. Indeed, filling a silicone conduit with plasma resulted in increased axonal regeneration and SC migration into the conduit for a rat critical defect (Williams 1987). Besides being native to the injury site, fibrin also contains two RGD binding sites that can facilitate binding of cells via integrin receptors (Thiagarajan, Rippon et al. 1996). SCs contain a specific integrin receptor for binding RGD on fibrin ($\alpha_V\beta_8$), which can facilitate their migration through fibrin (Chernousov and Carey 2003).

Other natural protein matrices have been investigated for use as a scaffold to fill the lumen of a NGC. For example, the Bellamkonda lab demonstrated that agarose gels containing laminin and NGF were comparable to autografts in functional measures in a 10 mm rat sciatic nerve defect (Yu and Bellamkonda 2003). Collagen-glycosaminoglycan copolymers are an ECM analog and can encourage rat sciatic nerve regeneration across a 10 mm defect (Chamberlain, Yannas et al. 1998). Additionally, alginate can act as a scaffold to bridge a 2 – 4 cm gap in the rabbit peroneal nerve (Mohanna, Terenghi et al. 2005), which can also be covalently modified to incorporate heparin and growth factors for delivery to treat rat sciatic nerve injuries (Ohta, Suzuki et al. 2004).

While luminal scaffolds have focused on natural and ECM materials, synthetic polymers have been considered as they offer more degrees of freedom for construction and chemistry. In particular, much research in synthetic materials has involved the use of aligned or parallel fibers within a conduit to better represent the organized endoneurial tubes normally present. Synthetic longitudinally arranged polymer filaments have included: polyamide (Dahlin and Lundborg 1999; Arai, Lundborg et al. 2000), catgut (Dahlin and Lundborg 1999; Arai, Lundborg et al. 2000), polydioxanone (Arai, Lundborg et al. 2000),

poly-glycolide (Lietz, Dreesmann et al. 2006) and polyglactin (Arai, Lundborg et al. 2000), which all supported nerve regeneration across a nerve gap of 15 mm better than an empty conduit alone. However, the biocompatibility of such polymer filaments may present a problem as large numbers of macrophages were found on catgut and polyglactin filaments (Terada, Bjursten et al. 1997). Furthermore, the biodegradability of polymers is an issue as polyamide is non-resorbable, while the others investigated are biodegradable but may still break down into acidic byproducts that may inhibit the nerve regeneration process and present safety issues, especially in long-term implantation situations (Terada, Bjursten et al. 1997).

An additional aspect of the design of all scaffolds is the dependence of the concentration of the natural or ECM proteins or polymer stiffness used. Navarro and colleagues have examined agarose (Labrador, Buti et al. 1995), collagen and laminin gels (Labrador, Buti et al. 1998) to determine if their concentration, or density/stiffness, affected neural regeneration. Labrador *et al.* implanted conduits filled with agarose gels of varying concentrations to bridge a short defect in the rat sciatic nerve. They found that the gel concentration or stiffness affected functional recovery following nerve injury, where there was an optimal concentration that enhanced functional recovery compared to other concentrations (Labrador, Buti et al. 1995). They also performed followed-up studies using collagen and laminin gels in both short defect and critical mouse sciatic nerve defects, and again found that there was an optimal concentration or stiffness for either gel solution that maximized axonal regeneration. They also determined that there were differences in axonal regeneration between the two solutions based on the defect length; however, the mechanism and reason for the differences are not well understood (Labrador, Buti et al. 1998).

Overall, studies considering scaffolds for nerve regeneration have found that natural materials or proteins can effectively promote nerve regeneration beyond defect lengths that would normally be limited in empty NGCs. Scaffolds for nerve regeneration also are not limited to their inclusion in NGCs and may be included in other materials in future work. This thesis work utilized a fibrin matrix to act as a luminal scaffold for nerve regeneration due to its regenerative properties and its ability to be chemically modified for drug delivery, as described later.

1.4 Growth Factors

Growth factors are known to influence neural regeneration. After nerve injury growth factors, in particular neurotrophic factors, are upregulated due to a decrease in trophic support from the deinnervated end-organs (Costigan, Befort et al. 2002). These factors typically exert their effects via binding to cell membrane surface receptors which lead to a diverse array of transcriptional effects. Following activation, they regulate neurite growth, branching and synaptogenesis, as well as adult synaptic plasticity and maturation of neuronal phenotype. Most importantly, neurotrophic factors promote the survival of neurons and counteract pathological neuronal cell death. Neurotrophic factors currently are classified by three major families: the neurotrophins, including nerve growth factor (NGF); the neurokines; and the GDNF family of ligands (GFL) including, GDNF (Boyd and Gordon 2003). Biological grafts contain neurotrophic factors naturally to a degree, and the delivery of neurotrophic factors with alternative therapies, such as NGCs, can enhance and promote nerve regeneration, as will be discussed later. Two of these neurotrophic factors,

NGF and GDNF, will be described in detail in this section due to their involvement in this thesis work.

1.4.1 Nerve Growth Factor (NGF)

NGF was the first discovered neurotrophin (Levi-Montalcini and Hamburger 1951; Cohen, Levi-Montalcini et al. 1954) and was discovered in mouse sarcoma due to its effects on chick embryo ganglia (Cohen, Levi-Montalcini et al. 1954). Processed and biologically active NGF (β-NGF) takes the form of a dimer with 3 disulfide chains to stabilize itself (Sofroniew, Howe et al. 2001a). NGF's receptors include p140 or, more commonly, tyrosine receptor kinase A (TrkA) and $p75^{NTR}$ (p75 or low affinity growth factor receptor). Both are the two major known receptors for NGF in many neurons, and NGF binds to either receptor with moderate to low affinity. The co-localization of the two receptors forms an oligomer leading to a high affinity NGF binding receptor stronger than either receptor alone (Sofroniew, Howe et al. 2001a). TrkA homodimer and the combination of p75-TrkA heterdimer receptor signaling promote cell survival and differentiation, while p75 homodimer, a tumor necrosis factor receptor family member (Esposito, Patel et al. 2001), mediates apoptosis upon binding and activation with NGF (Niederhauser, Mangold et al. 2000).

NGF has a specific role in neural development where it supports the survival and maturation of a number of neuronal cells. It also is involved in nerve injury, where glial cells upregulate its expression in response to the injury in both the central nervous system and the peripheral nervous system (Sofroniew, Howe et al. 2001a). Exogenous delivery of NGF has proven beneficial in numerous studies of sciatic nerve injury. NGF delivered from a silicone

tube protected dorsal root ganglia from injury-induced death after sciatic nerve injury (Otto, Unsicker et al. 1987). NGF delivered via a subcutaneous silicone reservoir for 12 weeks after nerve injury promoted improved nerve regeneration more than epineurial repair alone (Santos, Rodrigo et al. 1998). However, NGF is limited in axonal targeting in the peripheral nervous system as sensory neurons express the receptors for NGF, mainly TrkA, while motor neurons do not express TrkA receptors (Boyd and Gordon 2003).

1.4.2 Glial Derived Neurotrophic Factor (GDNF)

GDNF was the first discovered of the GDNF family of ligands (Lin, Doherty et al. 1993; Baloh, Enomoto et al. 2000). GDNF was initially identified for its ability to promote the survival of dopaminergic neurons (Lin, Doherty et al. 1993). Following peripheral nerve injury its expression is upregulated in SCs (Hoke, Gordon et al. 2002; Boyd and Gordon 2003; Zhao, Veltri et al. 2004), while its receptors are upregulated in motor neurons (Boyd and Gordon 2003). GDNF is also upregulated in skeletal muscle after injury (Nagano and Suzuki 2003; Zhao, Veltri et al. 2004) and regulates presynaptic differentiation and neuromuscular junction connections (Nagano and Suzuki 2003; Yang and Nelson 2004). GDNF binding is facilitated through a receptor unit, where the primary receptor subunit is GDNF receptor α-1, which elicits signaling through its linked partner subunit, c-Ret receptor tyrosine kinase (Baloh, Enomoto et al. 2000).

Exogenous GDNF has promoted neurite outgrowth and neuronal survival *in vitro* (Trupp, Ryden et al. 1995; Matheson, Carnahan et al. 1997; Bennett, Michael et al. 1998; Gavazzi, Kumar et al. 1999; Tucker, Rahimtula et al. 2006; Leclere, Norman et al. 2007) and promotes survival of axotimized sciatic neurons *in vivo* (Matheson, Carnahan et al. 1997).

While GDNF is recognized to be beneficial to both sensory and motor neuron survival and regeneration, multiple studies have found GDNF to be a potent motor neuron trophic and survival factor *in vitro* and *in vivo* (Henderson, Phillips et al. 1994; Yan, Matheson et al. 1995; Oppenheim, Houenou et al. 2000). Studies have also shown GDNF to have increased ability to promote motor nerve regeneration compared to other neurotrophic factors such as NT-3 or NGF (Barras, Pasche et al. 2002; Fine, Decosterd et al. 2002). Furthermore, the effects of GDNF go beyond immediate nerve regeneration effects but also play a role in promoting maturation of the neuromuscular junction and functional connections. Using nerve-muscle co-cultures Wang *et al.* demonstrated that GDNF not only increased the total length of neurites in the motor neurons, but also facilitated aggregation of synaptic vesicles in the presynaptic terminals, as well as increased acetylcholine receptors clustering in the postsynaptic terminal (Wang, Yang et al. 2002). Nguyen *et al.* used transgenic mice that overexpress GDNF in muscle to show that increased exposure of GDNF not only decreased spinal motor neuron death, but also led to hyperinnervation of the neuromuscular junctions (Nguyen, Parsadanian et al. 1998). Therefore, GDNF appears to play a significant role in motor nerve regeneration and the reestablishment of neuromuscular junctions in skeletal muscle at denervation.

1.5 Controlled Protein Delivery

Growth factors can promote nerve regeneration; however, injected and ingested drugs or proteins have associated problems such as low stability due to degradation, rapid clearance of the drug, which may affect its potency at the injury site, and more importantly, systemic effects, which may not be desirable due to adverse side effects in other tissues.

Osmotic pumps and other implantable reservoirs can effectively produce localized delivery but can have inflammatory problems due to their permanent nature (Schmidt and Leach 2003). An alternative degradable delivery system can be achieved with NGCs, which can delivery proteins and other soluble molecules using controlled release mechanisms. The delivery using NGCs can occur from both the conduit walls and materials within the lumen of the conduit. Different strategies have been considered for drug delivery where the most common mechanism is through diffusion-based release. Diffusion-based systems release drug or protein by modulating the diffusion coefficient of the drug within the material. The primary control of this modulation is through altering the pore size of the material (Langer and Folkman 1976; Saltzman and Langer 1989).

Numerous NGCs have employed diffusion-based release systems. Aebischer and colleagues have constructed ethylene vinyl acetate copolymer (EVA) conduits that prevent the diffusion of growth factors from the outer portion of the conduit while permitting release to the inner portion that contains the nerve stumps (Aebischer, Salessiotis et al. 1989). The release of growth factors has an initial drug burst but is followed by weeks of linear growth factor release (Aebischer, Salessiotis et al. 1989). These conduits have been used to treat 15 mm defects in rat sciatic nerve with basic fibroblast growth factor (bFGF) (Aebischer, Salessiotis et al. 1989), GDNF, and NGF (Fine, Decosterd et al. 2002) and to treat 8 mm defects in rat facial nerve with neurotrophin-3 (NT-3) and GDNF (Barras, Pasche et al. 2002). Alternatively, Terenghi and colleagues used a conduit constructed from a natural material, fibronectin, to delivery growth factors. Fibronectin mats impregnated with NGF and NT-3 effectively delivered either growth factor to enhance nerve regeneration in a rat sciatic nerve defect (Whitworth, Brown et al. 1996; Sterne, Brown et al. 1997).

Scaffolds for NGCs have included growth factors to promote nerve regeneration, but few have used controlled release mechanisms. Commonly the drug is loaded into the scaffold and is therefore free to diffuse away from the conduit (Yu and Bellamkonda 2003). Affinity-based delivery offers an alternative to loading free drug or growth factor into a scaffold by non-covalently sequestering the drug within the scaffold or matrix. Affinity-based release systems control release by modulating the concentration of drug or protein available for release. This thesis work utilized an ABDS to sequester and deliver growth factors to promote nerve regeneration.

1.5.1 Affinity-based Delivery

ABDSs differ from diffusion-based delivery systems in that the systems immobilize drugs within the matrix via non-covalent interactions. These non-covalent interactions allow the release to be controlled by modulating the diffusible fraction of drug. The fraction of drug available for release can be specifically modulated by degradation of the matrix that binds the drug, controlling the quantity of binding sites available for drug within the matrix, or tailoring the affinity of drug interaction with the matrix.

Heparin is a polysaccharide that is commonly used to sequester growth factors in ABDSs. Heparin protects growth factors from degradation and contains charged sulfate groups, which facilitate electrostatic interactions with a variety of growth factors via basic domains (Yamada 1983; Mach, Volkin et al. 1993; Mulloy 2005). ABDSs utilizing heparin have been employed in a variety of systems. Edelman and colleagues used heparin-conjugated Sepharose beads encapsulated in alginate to immobilize bFGF and protect it from degradation. They achieved controlled growth factor release which could be

modulated by enzyme degradation (Edelman, Mathiowitz et al. 1991). This delivery system was effective enough *in vivo* to reach human clinical trials for the treatment of myocardium revascularization (Laham, Sellke et al. 1999). Another system was developed using collagen matrices that immobilized heparin through crosslinking the collagen. The immobilized heparin in turn sequestered bFGF within the matrices allowing enhanced endothelial cell proliferation and reducing the minimum cell seeding density needed for proliferation (Wissink, Beernink et al. 2000a; Wissink, Beernink et al. 2000b). More recently, heparin was methacrylated and copolymerized with dimethacrylated poly(ethylene glycol) monomers to yield hydrogels that sequester bFGF (Benoit and Anseth 2005).

Sakiyama-Elbert and Hubbell developed an ABDS that sequesters proteins in a fibrin matrix using non-covalent interactions (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b). This system utilizes a bi-domain peptide that is uniquely able to incorporate into a fibrin matrix and still interact with molecules on its other domain. Fibrinogen is normally cleaved at acceptor sites by thrombin which permits interactions at the acceptor sites with other fibrinogen proteins resulting in a non-covalent fibrin mesh. This is further stabilized by the formation of covalent bonds within the mesh due to transglutaminase substrates contained on fibrinogen facilitated through the transglutaminase Factor XIIIa (Loewy, Dunathan et al. 1961). In the ABDS, one domain of the bi-domain peptide consists of a transglutaminase substrate, based on α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983; Kimura, Tamaki et al. 1985), which allows it to be crosslinked into fibrin matrices during polymerization. The other domain consists of a modified version of the heparin-binding domain from antithrombin III (Tyler-Cross, Sobel et al. 1994; Tyler-Cross, Sobel et al. 1996; Sakiyama, Schense et al. 1999) allowing non-covalent interactions of heparin to peptide crosslinked within a fibrin matrix. The heparin-binding domain has the

capability to sequester various neurotrophic factors due to their ability to interact with heparin to varying degrees.

1.6 Concluding Remarks

Previously, the aforementioned fibrin-based ABDS was applied to treat peripheral nerve injury using the delivery of NGF (Lee, Yu et al. 2003a). This study demonstrated the efficacy of this ABDS to treat peripheral nerve injury; however, additional aspects of the delivery system were considered of interest: what role peptide binding affinity for heparin might perform in peripheral nerve injury treatment and whether other growth factors such as GDNF could be delivered to treat nerve injury, particularly due to the ability of GDNF to target motor axons which cannot be specifically targeted with NGF due to the lack of receptors. Therefore, this thesis work first examined the role of each component *in vitro* and then applied each to a rat animal model of peripheral nerve injury to assess the role of each in nerve regeneration.

The rat sciatic nerve has been classically used to study peripheral nerve injury due to its availability, cost, and nerve size. The rat sciatic nerve has a fair degree of sensitivity to histological measures of nerve regeneration, as at mid-thigh level the sciatic nerve contains a mixture of sensory and motor axons (approximately 28,000 axons); of these axons, 8000 are myelinated and 4000 – 5000 of these myelinated axons are motor fibers (Schmalbruch 1986; Schmalbruch 1987a; Schmalbruch 1987b; Mackinnon and Dellon 1988). Furthermore, rat nerve morphology bears similarities to human nerve morphology (Mackinnon and Dellon 1988). Additionally as already discussed, the placement of a silicone conduit between nerve

stumps results in nerve regeneration in a rat (Williams, Longo et al. 1983); however, the repair of the rat sciatic nerve with a silicone conduit resulted in nerve regeneration only when the gap length was 10 mm or less. The use of a defect greater than 10 mm resulted in dramatically fewer instances of nerve regeneration (Lundborg, Dahlin et al. 1982a). Therefore, a silicone conduit filled with the ABDS can serve as useful injury model to observe improvements in nerve regeneration and was thus chosen as the *in vivo* injury model used in this thesis work.

This thesis work utilized affinity-based delivery to delivery neurotrophic factors to promote peripheral nerve regeneration. The first and second studies of this thesis work utilized peptides with varying binding affinity for heparin to determine whether peptide binding affinity for heparin or release rate affected peripheral nerve regeneration both *in vitro* and *in vivo*. The third study investigated whether the growth factor GDNF could be sequestered by the ABDS and how the release rates could be modulated. It also determined how the ABDS and GDNF might be directed to promote *in vivo* nerve regeneration by assessing *in vitro* neurite extension. The fourth study considered the efficacy of the ABDS and GDNF in promoting peripheral nerve regeneration following injury in a short-term animal model through histology measures. The final study utilized the ABDS with both NGF and GDNF to determine whether the ABDS could promote functional recovery following injury in a long-term animal model and whether there were differences in sensory versus motor nerve regeneration. Overall, this thesis work represents an effort toward designing a material for the potential treatment of peripheral nerve injury.

Chapter 2

Release rate controls biological activity of nerve growth factor released from fibrin matrices containing affinity-based delivery systems*

2.1 Abstract

Previously, combinatorial techniques were used to identify peptide sequences exhibiting high, medium, and low affinity for heparin. Bi-domain peptides were synthesized containing a transglutaminase sequence for one domain and one of the heparin affinity sequences for the other domain. A delivery system was made consisting of bi-domain peptides, heparin, and nerve growth factor (NGF), which binds to heparin with moderate affinity. The goal of this research was to determine if peptide affinity for heparin and the molar ratio of peptide to heparin affected the release rate of NGF from the delivery system and the biological activity of NGF release. This study also explored whether peptide affinity modulated biological activity independent of release rate. Mathematically modeling the delivery system confirmed that release could be controlled by both peptide affinity and molar ratio of peptide to heparin. Experimentally the rate of NGF release from the delivery system was found to be affected by the affinity and molar ratio. The delivery system presented biologically active NGF as assayed by embryonic chick dorsal root ganglia (DRGs) neurite extension, where extension was similar to or increased for DRGs grown in fibrin matrices containing the delivery system compared to DRGs grown with NGF in the culture media. Furthermore, by modulating the molar ratio of peptide to heparin in the delivery

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system, similar release rates of NGF were obtained for different affinity peptides and these conditions promoted similar levels of neurite extension, demonstrating that release rate appears to be the main mechanism controlling the biological activity of released NGF.

2.2 Introduction

The peripheral nervous system is capable of limited regeneration after injury, however in the case of large nerve gaps, surgical intervention is often required (Lundborg 2000). Nerve autografts are commonly used to repair these larger gaps but their limitations suggest that an alternative is needed (Staniforth and Fisher 1978; Lundborg 2000). Biomaterial matrices and neurotrophins, such as nerve growth factor (NGF), have been implicated as potential therapeutics for peripheral nerve injury (Schmidt and Leach 2003; Bellamkonda 2006), where NGF has been shown to promote neurite extension *in vitro* (Conti, Fischer et al. 1997; Macias, Battocletti et al. 2000; Sakiyama-Elbert and Hubbell 2000a; Xu, Yu et al. 2002) and nerve regeneration *in vivo* when combined with a biomaterial scaffold (Xu, Yu et al. 2002; Lee, Yu et al. 2003b; Yu and Bellamkonda 2003). A nerve guidance conduit filled with a biomaterial scaffold and NGF could be used as potential treatment for peripheral nerve injury, providing a physical bridge between the severed nerve ends and cues for neuronal survival. In order to maximize their effect on regeneration, controlled release of the growth factors is needed.

Diffusion-based release from biomaterial scaffolds is a common mechanism of drug delivery, where release of the drug is controlled by the diffusion coefficient of the drug within the material. By modulating the pore size of the material, the diffusion coefficient of

the material can be altered (Langer and Folkman 1976; Saltzman and Langer 1989; Burdick, Ward et al. 2006). Alternatively, affinity-based delivery systems have been studied, which differ in that the delivery systems immobilize drugs within the matrix via non-covalent interactions allowing the release to be controlled by the modulating the fraction of the drug in the diffusible form.

Affinity-based delivery systems utilizing heparin have been used for a variety of applications and take advantage of the ability of the sulfated groups on heparin to interact with proteins, such as growth factors, via basic domains (Mach, Volkin et al. 1993; Mulloy 2005). One such system was developed by Edelman and coworkers and used heparin-conjugated Sepharose beads encapsulated in alginate to immobilize basic fibroblast growth factor (bFGF). This system allowed the growth factors to be released in a controlled process over time and protected them from degradation within the alginate matrix (Edelman, Mathiowitz et al. 1991; Laham, Sellke et al. 1999). Another system was developed using heparinized collagen matrices, made by crosslinking collagen and covalently immobilizing heparin within the matrices. This system was able to sequester bFGF within collagen matrices allowing enhanced endothelial cell proliferation and reducing the minimum cell seeding density required for proliferation (Wissink, Beernink et al. 2000a; Wissink, Beernink et al. 2000b). More recently, heparin was copolymerized with poly(ethylene glycol) (PEG) dimethacrylate to yield hydrogels that sequestered bFGF (Benoit and Anseth 2005). While another group used hyaluronan, gelatin, and heparin modified with thiol groups and crosslinked these components with PEG diacrylate to make hydrogels that sequestered vascular endothelial growth factor or bFGF (Pike, Cai et al. 2006).

We have developed an affinity-based delivery system that sequesters proteins within a fibrin matrix using non-covalent interactions (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b). This system contains a bi-domain peptide, consisting of a transglutaminase substrate from α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983; Kimura, Tamaki et al. 1985), and a heparin-binding domain. Based on the transglutaminase substrate the peptide is crosslinked into fibrin matrices during polymerization by Factor XIIIa, leaving the other domain free to interact with heparin. This work demonstrated that this delivery system is capable of delivering NGF through heparin interactions with NGF, which binds with modest affinity, creating a ternary complex (peptide, heparin, and NGF) within the fibrin matrix (Sakiyama-Elbert and Hubbell 2000a).

To modulate the affinity of the heparin-binding domain for heparin, combinatorial techniques were used to identify peptides with varying affinity for heparin. Peptide sequences were identified exhibiting "high", "medium", and "low" affinity for heparin using an increasing step gradient of sodium chloride concentration (1 M, 1.5 M and 2.0 M were used to elute the low, medium and high affinity peptide, respectively). Peptides were synthesized containing the identified heparin binding domains along with a transglutaminase substrate to allow crosslinking into fibrin matrices and to bind to heparin (Maxwell, Hicks et al. 2005).

This system allows the release rate of NGF to be modulated by changing the molar ratio of peptide to heparin in the delivery system (thus modulating the fraction of NGF in the diffusible form) or the peptide affinity for heparin. This allows us to test whether peptide affinity affects NGF biological activity independent of release rate because molar ratios of peptide to heparin can be identified for two different affinity peptides that result in

similar release rate. The notion of biological activity being independent of release rate may seem unconventional; however, in nature there are examples of growth factors with varying affinity for ECM or cell surface receptors that demonstrate different biological activity. For example, the different isoforms of transforming growth factor-β demonstrate different affinity for heparin sulfate and exhibit different biological responses in wound healing (Lyon, Rushton et al. 1997). Additionally, the neurotrophin NGF binds with different affinity to its receptors, TrkA and p75, where the ratio of each receptor activated can lead to different cell responses (survival vs. apoptosis) (Bothwell 1995; Sofroniew, Howe et al. 2001b; Kuruvilla, Zweifel et al. 2004). Thus biological activity may be affected by affinity in controlled release systems as well.

The focus of this study was to assess the release rate and biological activity of β-NGF released from fibrin matrices containing a heparin-binding delivery system (HBDS) with peptides of varying affinity for heparin. The release of NGF from matrices was modeled mathematically as a function of peptide affinity for heparin and the molar ratio of peptide to heparin to determine if similar release rates of NGF could be obtained for peptides with different heparin affinity. Additionally, the release of NGF was measured experimentally over 7 days and the ability of the delivery system to present biologically active NGF was analyzed using chick embryonic DRGs.

2.3 Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

2.3.1 Mathematical modeling

A mathematical model was developed to understand how varying peptide affinity for heparin and the molar ratio of peptide to heparin affected the concentrations of species containing NGF bound within the fibrin matrix at equilibrium. Additionally, the release of NGF from fibrin matrices to aqueous media was modeled over 24 h to determine the role of peptide affinity for heparin and molar ratio of peptide to heparin and to reveal if similar release rates of NGF could be obtained for peptides of different heparin affinity. Therefore, the equilibrium and release of species containing NGF from fibrin matrices was modeled after situations similar to those described in the experimental methods below, where a closed system of reacting species were allowed to reach equilibrium followed by the passive release of species to aqueous media.

The delivery system was modeled with nine species (five of which contained NGF) given by: peptide, either matrix bound (P_B) or unbound (free, P_U) to the fibrin matrix, heparin (H), NGF (G), peptide-heparin complex, either matrix bound (PH_B) or unbound (PH_U), heparin-NGF complex (free, HG), and peptide-heparin-NGF complex, again matrix bound (PHG_B) or unbound (PHG_U). This model explored the effects of having both bound (cross-linked to the fibrin matrix) and unbound peptide present (and capable of binding to) heparin and in turn bind NGF. These species reacted according to the following chemical equations governing the network, where all possible complexes between the species (P, H, and G) were considered:

(2.1) $$P_B + H \underset{\kappa_r}{\overset{\kappa_f}{\rightleftharpoons}} PH_B$$

(2.2) $$P_U + H \underset{\kappa_r}{\overset{\kappa_f}{\rightleftharpoons}} PH_U$$

(2.3) $$H + G \underset{k_r}{\overset{k_f}{\rightleftharpoons}} HG$$

(2.4) $$PH_B + G \underset{k_r}{\overset{k_f}{\rightleftharpoons}} PHG_B$$

(2.5) $$PH_U + G \underset{k_r}{\overset{k_f}{\rightleftharpoons}} PHG_U$$

(2.6) $$P_B + HG \underset{\kappa_r}{\overset{\kappa_f}{\rightleftharpoons}} PHG_B$$

(2.7) $$P_U + HG \underset{\kappa_r}{\overset{\kappa_f}{\rightleftharpoons}} PHG_U .$$

It was assumed that the kinetic rates of heparin binding to NGF and peptide binding to heparin were independent of previous interactions with another species. Additionally, peptide-containing and heparin-containing species were assumed to contain only one binding site for interaction with heparin-containing species and NGF-containing species, respectively. Others have found that heparin can bind multiple sites on acidic fibroblast growth factor with high affinity interactions due to interactions of basic residues of the growth factor with the anionic regions of heparin (Mach, Volkin et al. 1993). However, since a molar excess of heparin was present in all model calculations, it was assumed that NGF would primarily occupy only one binding site on heparin. The kinetic rate constants for peptide interacting with heparin were κ_f for the association rate constant, κ_r for the dissociation rate constant, and $K_{D,\,PH}$ for the equilibrium dissociation constant. The kinetic

rate constants for heparin interacting with NGF were k_f for the association rate constant, k_r for the dissociation rate constant, and $K_{D, HG}$ for the equilibrium dissociation constant.

At the start of release experiments fibrin matrices were assumed to be at equilibrium in a closed system. Nine equations governing the concentrations of the species at equilibrium (eqns. 2.8 – 2.12), found from considering the reactions of the species (eqns. 2.1 – 2.7) and the concentrations due to the conservation of mass for a closed system (eqns. 2.13 – 2.16), were evaluated to obtain the starting conditions for release. The equations consisted of the following:

(2.8) $$K_{D,\,PH} = \frac{[P]_B[H]}{[PH]_B}$$

(2.9) $$K_{D,\,PH} = \frac{[P]_U[H]}{[PH]_U}$$

(2.10) $$K_{D,\,HG} = \frac{[H][G]}{[HG]}$$

(2.11) $$K_{D,\,PH} = \frac{[P]_B[HG]}{[PHG]_B}$$

(2.12) $$K_{D,\,PH} = \frac{[P]_U[HG]}{[PHG]_U}$$

(2.13) $$[P]^{EQ}_{B,\,Total} = [P]^{EQ}_B + [PH]^{EQ}_B + [PHG_n]^{EQ}_B$$

(2.14) $$[P]^{EQ}_{U,\,Total} = [P]^{EQ}_U + [PH]^{EQ}_U + [PHG_n]^{EQ}_U$$

(2.15)

$$[H]_{Total}^{EQ} = [H]^{EQ} + [PH]_{B}^{EQ} + [PH]_{U}^{EQ} + [HG]^{EQ} + [PHG]_{B}^{EQ} + [PHG]_{U}^{EQ}$$

(2.16)

$$[G]_{Total}^{EQ} = [G]^{EQ} + [HG]^{EQ} + [PHG]_{B}^{EQ} + [PHG]_{U}^{EQ}$$

(2.17)

$$\alpha = \frac{\frac{1}{2}[G]^{EQ} + \frac{1}{2}[HG]^{EQ} + \frac{1}{2}[PHG]_{U}^{EQ} + [PHG]_{B}^{EQ}}{[G]_{Total}^{EQ}} \quad ,$$

where the values for the total concentrations of P, H, and G at equilibrium were the same as those used for experiments (see below). Equations 2.13 and 2.14 assume that not all the peptide was cross-linked into the fibrin matrix, and matrix bound peptide was estimated to be approximately 8 moles of peptide per mole of fibrinogen (Sakiyama, Schense et al. 1999; Schense and Hubbell 1999). Equation 2.17 describes the results of the *in vitro* equilibrium study (see below), where α represents the fraction of [G] retained within the matrix. Equations 2.8 – 2.17 were entered into MATLAB (Mathworks, Inc., Novi, MI) and solved using a non-linear equation solver to obtain the dissociation constant ($K_{D, HG}$) for the interaction of heparin with NGF (the average value for $K_{D, HG}$ is given in Table 2.1) and by omitting equation 2.17 ($K_{D, HG}$ was kept at the average shown in Table 2.1) to determine the fraction of bound growth factor as the heparin concentration varied.

Table 2.1: Constants employed in math model

Constant	**Value**
$K_{D, HG}$	$1.7 \pm 2.6 \times 10^{-6}$ M
$K_{D, PH}$ (Low affinity peptide)	9.0×10^{-5} M (Maxwell, Hicks et al. 2005)
$K_{D, PH}$ (Medium affinity peptide)	6.1×10^{-5} M (Maxwell, Hicks et al. 2005)
$K_{D, PH}$ (High affinity peptide)	3.8×10^{-5} M (Maxwell, Hicks et al. 2005)

$K_{D, PH}$ (ATIII peptide)	8.78×10^{-8} M (Olson, Srinivasan et al. 1981; Kridel, Chan et al. 1996)
$D_{P, W}$ (MW ~ 2200 Da.)	1.9×10^{-2} mm^2/min
$D_{H, W}$	9.3×10^{-3} mm^2/min
$D_{G, W}$	1.0×10^{-2} mm^2/min
$D_{PH, W}$	8.9×10^{-3} mm^2/min
$D_{HG, W}$	7.7×10^{-3} mm^2/min
$D_{PHG, W}$	7.6×10^{-3} mm^2/min
$D_{P, M}$	1.9×10^{-2} mm^2/min
$D_{H, M}$	9.1×10^{-3} mm^2/min
$D_{G, M}$	9.7×10^{-3} mm^2/min
$D_{PH, M}$	8.6×10^{-3} mm^2/min
$D_{HG, M}$	7.5×10^{-3} mm^2/min
$D_{PHG, M}$	7.3×10^{-3} mm^2/min

The release of species from fibrin matrices was described by the mass balances for the species given by partial differential equations (PDEs) describing the kinetics of binding and dissociation and the diffusive transport of the species in the fibrin matrix and in the aqueous media with time. The delivery system was considered with the following equations:

(2.18) $$\frac{\partial [P]_B}{\partial t} = -\kappa_f ([P]_B[H] + [P]_B[HG]) + \kappa_r ([PH]_B + [PHG]_B)$$

(2.19) $$\frac{\partial [P]_U}{\partial t} = D_{P,j}\nabla^2[P]_U - \kappa_f([P]_U[H] - [P]_U[HG]) + \kappa_r([PH]_U + [PHG]_U)$$

(2.20) $$\frac{\partial [H]}{\partial t} = D_{H,j}\nabla^2[H] - \kappa_f([P]_U[H] - [P]_B[H]) + \kappa_r([PH]_B - [PH]_U) + k_r[HG]_U - k_f[H]_U[G]_U$$

(2.21)

$$\frac{\partial[G]}{\partial t} = D_{G,j}\nabla^2[G] - k_f([H][G] + [PH]_B[G] + [PH]_U[G]) + k_r([HG] + [PHG]_B + [PHG]_U)$$

(2.22) $$\frac{\partial[PH]_B}{\partial t} = \kappa_f[P]_B[H] - \kappa_r[PH]_B - k_f[PH]_B[G] + k_r[PHG]_B$$

(2.23) $$\frac{\partial[PH]_U}{\partial t} = D_{PH,j}\nabla^2[PH]_U[H]_f \kappa_U[PH] - k_f[PH]_U[G]_f k_U[PHG]_r U$$

(2.24) $$\frac{\partial[HG]}{\partial t} = D_{HG,j}\nabla^2[HG] + k_f[H][G]([R]_j[HG] - [P][HG]) + U$$
$$\kappa_r([PHG]_B[PHG])_U$$

(2.25) $$\frac{\partial[PHG]_B}{\partial t} = k_f[PH]_B[G] - k_r[PHG]_B[HG]_f k_B[PHG]_r B$$

(2.26) $$\frac{\partial[PHG]_U}{\partial t} = D_{PHG,j}\nabla^2[PHG]_U + k_f[PH]_U[G][P] - [HG][PHG]_U[PHG]_U - r U,$$

where $[i]$ is the concentration of the species i given by P_B, P_U, H, G, PH_B, PH_U, HG, PHG_B, and PHG_U, j is the material in which the species is diffusing, and t represents time. The mass balances for species not cross-linked to the fibrin matrix included a term for diffusive transport as well as kinetic terms, while the mass balances for matrix bound species included only kinetic terms because the peptide was assumed to be immobilized in the fibrin matrix. The equilibrium dissociation constants (used to estimate the kinetic rate constants) and diffusion coefficients ($D_{i,j}$) are given in Table 2.1 where i is the species and j is the material in which the species is diffusing (matrix (M) or aqueous media (W)).

The diffusion coefficients for the species diffusing through the aqueous media (water) were found by the Stokes-Einstein Equation (Einstein 1906; Saltzman 2001) :

$$D_{A,W} = \frac{k_B T}{6\pi\mu a}, \tag{2.27}$$

where k_B is Boltzmann's constant, T is the temperature, μ is the viscosity of water, and a is the hydrodynamic radius of the species A. The diffusion coefficients for the species diffusing through the fibrin matrix were found by the Ogston Fiber Matrix Model (Ogston 1958; Ogston, Preston et al. 1973; Saltzman 2001) :

$$D_{A,M} = D_{A,W} \exp\left(-\Phi^{1/2} \frac{a}{r}\right), \tag{2.28}$$

where Φ is the volume fraction occupied by the fibers (~ 5% (Carr and Hermans 1978; Diamond 1999; Guthold, Liu et al. 2004)) and r is the radius of the fibrin fibers (~ 5 nm (Galanakis, Lane et al. 1987; Diamond 1999; Guthold, Liu et al. 2004)). The delivery system was modeled in one-dimension as a 2 mm fibrin matrix in contact with 5 mm aqueous media. The interface of the fibrin matrix and the aqueous media were coupled by two boundary conditions where it was assumed that the concentrations and fluxes of the species were equal. Since the fibrin matrix was > 95% water, the partition coefficient between the two interfaces was assumed to be ~ 1, and the mass transfer area between the two interfaces was equal. At the other end of the fibrin matrix, the flux of species was zero. The other end of the aqueous media was assumed to have a constant concentration of zero for all species.

The results of the equilibrium modeling for the fibrin matrices were used as the initial conditions (concentrations of the species in the fibrin matrix), and the concentration of all species in the aqueous media was assumed to be zero initially. The equations (2.18 –

2.26), constants, and initial conditions for passive release of NGF were solved using FEMLAB (COMSOL, Inc., Burlington, MA), a numerical PDE solver. The model was run replacing the aqueous medium with new aqueous medium, where the concentration of the species in the entire medium were initially zero, over the course of 24 h in a manner similar to that described in the experimental methods for the *in vitro* release assay. While the aqueous medium were cleared of species at specific times, the concentration of species in the fibrin matrix remained the same as concentrations in the fibrin matrix before the aqueous medium species were cleared, similar to the *in vitro* release assay.

2.3.2 Peptide synthesis

Peptides of varying heparin-binding affinity were synthesized by standard solid phase Fmoc chemistry (amino acids from Nova Biochem, San Diego, CA; peptide synthesis solvents from Applied Biosystems, Foster City, CA) using an ABI433A peptide synthesizer (Applied Biosystems) and were based on amino acid sequences identified previously (Table 2.2) (Tyler-Cross, Sobel et al. 1994; Sakiyama, Schense et al. 1999; Maxwell, Hicks et al. 2005). The peptides denoted high, medium, and low affinity were based on previously identified 12-mer peptides found from screening a phage display library with heparin-Sepharose chromatography (Maxwell, Hicks et al. 2005), and the peptide denoted ATIII was based on a modified version of the antithrombin III-heparin binding domain (Tyler-Cross, Sobel et al. 1994; Sakiyama, Schense et al. 1999). After synthesis, the peptides were cleaved from the resin with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane by volume for 2-3 h using 10 mL of cocktail per 1 g of resin. The mixture was filtered through glass wool to remove the resin then precipitated in cold diethyl ether. The crude peptide

filtrates were dried under vacuum and then purified by standard C18 reverse phase liquid chromatography (Shimadzu, Columbia, MD) and lyophilized. The identity of purified peptides were verified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

Table 2.2: Heparin-binding peptides synthesized

(Transglutaminase substrate is in Italics)

Peptide Name	**Amino Acid Sequence**
Low affinity	*NQEQVSP*GALPNSSKLAPSR (Maxwell, Hicks et al. 2005)
Medium affinity	*NQEQVSP*GSSANGKKPSTRR (Maxwell, Hicks et al. 2005)
High affinity	*NQEQVSP*GNSAHRTRGRQRS (Maxwell, Hicks et al. 2005)
ATIII	(*Ac*G)*NQEQVSP*K(βA)FAKLAARLYRKA (Sakiyama, Schense et al. 1999)

2.3.3 Fibrin matrix preparation

Fibrin matrices were prepared as previously described (Schense and Hubbell 1999) by mixing the following components (final concentrations given): human plasminogen-free fibrinogen containing Factor XIII (4.0 mg/mL), bovine thrombin (2 NIH units/mL), and $CaCl_2$ (2.5 mM, Fisher Scientific, Pittsburgh, PA). The delivery system was prepared by incorporating the following additional components: peptide (0.25 mM high, medium, low affinity, or ATIII, to obtain ~ 8 moles of peptide cross-linked per mole of fibrinogen (Sakiyama, Schense et al. 1999; Schense and Hubbell 1999)), heparin (sodium salt from porcine intestinal mucosa 18,000 average MW) added at 62.5, 6.25, and 1.25 μM to obtain 4:1, 40:1, and 200:1 molar ratios of peptide to heparin, respectively, and human β–NGF (100 ng/mL, Peprotech Inc., Rocky Hill, NJ and R&D systems, Minneapolis, MN for the *in vitro*

biological activity and release assay, respectively). The components were polymerized in 24-well tissue culture plates for 60 min at 37°C and 5% CO_2 yielding 400 μL matrices.

2.3.4 *In vitro* release assay

Fibrin matrices were prepared as described above then incubated with an aqueous wash consisting of Tris-buffered saline (TBS, 137 mM NaCl, 2.7 mM KCl, 33 mM Tris, pH 7.4, Fisher Scientific) containing 1% bovine serum albumin (BSA) at 37°C. Equilibrium studies were performed where 400 μL washes were added to the matrices and then collected after 48 h, which was previously determined to be sufficient time for the NGF concentration to reach equilibrium between the matrix and wash phases (Willerth, Johnson et al. 2007). Additionally, a 7-day release study was performed where 1 mL washes were added and collected 5 times in the first 24 h, followed by collection subsequently every 24 h for the next 6 days. For both studies all washes were collected in siliconized tubes to reduce NGF loss due to adsorption on tube walls and stored at -20°C. Upon completion of the release studies, the remaining NGF was extracted from the fibrin matrices by cutting them into 1 mm cubes and placing them in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4, Fisher Scientific) containing 0.56 mM heparin, an additional 2 M NaCl (Fisher Scientific), 0.01% Triton-X, and 1% BSA at 4 °C for 48 h, after which the mixture was stored at -20°C until analysis was performed.

The amount of NGF released and remaining in the fibrin matrices was quantified by an enzyme-linked immunosorption assay (ELISA) for human β-NGF according to the manufacturer's instructions (R&D systems). The absorbance was read at 450 nm with an optical subtraction at 650 nm using a multi-well plate spectrophotometer (MultiSkan RC,

Labsystems), and sample concentrations were calculated from a standard curve of known β-NGF concentrations. Control matrices were made by omitting the peptide, heparin, and/or NGF from the fibrin matrices.

2.3.5 *In vitro* biological activity assay

DRGs were dissected from day-10 white leghorn chick embryos (Specific Pathogen Free, Sunrise Farms, Catskill, NY). Media consisted of modified neurobasal media (NBM, Invitrogen, Carlsbad, CA) containing the following: insulin (5 g/mL), transferrin (100 g/mL), progesterone (6.4 ng/mL), putrescine (16.11 g/mL), selenite (5.2 ng/mL), and BSA (0.1%, Sigma-Aldrich). A positive control for neurite extension consisted of an unmodified fibrin matrix (no peptide, heparin, or NGF) with NBM containing 20 ng/mL of NGF, previously found to be an optimal dose (Sakiyama-Elbert and Hubbell 2000a). Fibrin matrices were prepared as described above then washed 5 times in the first 24 h with 1 mL of TBS for the first 4 washes and the last wash consisting of 1 mL of modified NBM media (20 ng/mL of NGF added for the positive control). One DRG was implanted per matrix using dissection forceps and allowed to adhere to the fibrin matrix for 1 h at 37°C and 5% CO_2. One mL of modified NBM was added after 1 h and left on the fibrin matrices for the remainder of the experiment.

DRGs were allowed to grow and extend neurites for 48 h upon which images were captured. Brightfield images with a 2x objective using a CCD camera (Magnifire, Olympus) were collected and analyzed using Image-Pro Express software (MediaCybernetics, San Diego, CA) to determine the average neurite extension. The average neurite extension was calculated as the radius of an annulus between the DRG body and the outer halo of

extending neurites, as described previously (Herbert, Bittner et al. 1996). All average neurite extension was normalized to the average neurite extension of the positive control from each experiment. Additional control matrices were made by omitting the peptide, heparin, and/or NGF from the fibrin matrices.

2.3.6 Statistical analysis

Statistical analysis was performed using Statistica (Statsoft) with comparative analysis using Scheffe's F post-hoc test by analysis of variance at a 95% confidence interval ($\alpha = 0.05$). The release assays were performed with 3 matrices per replicate; the biological activity study was performed with 6 matrices per replicate. All studies were performed in at least triplicate, and reported values are given as mean ± standard deviation (S.D.).

2.4 Results

2.4.1 Mathematical modeling

To explore the effect of varying the peptide affinity for heparin and the molar ratio of peptide to heparin, the equilibrium concentrations of matrix bound species containing NGF were calculated for the delivery system, consisting of nine interacting species in a fibrin matrix, at varying heparin concentrations (while holding peptide and NGF concentrations constant) (Figure 2.1). At high molar ratios of peptide to heparin (e.g. above 100:1), the amount of bound NGF decreased because the ratio of heparin to NGF was reduced by the low heparin concentration required to obtain high peptide to heparin ratios. The amount of

bound NGF in the delivery system increased to a maximum value as the molar ratio of peptide to heparin decreased to an optimal molar ratio of peptide to heparin (1:1 – 4:1). As the molar ratio of peptide to heparin decreased past this optimal molar ratio (<1:1), the amount of NGF that was bound in the delivery system decreased rapidly. The rapid decrease was due to an increase in unbound heparin, which resulted in more unbound heparin-NGF complex formation and the saturation of binding sites on the peptides for heparin. Additionally, the affinity of the peptide for heparin affected the equilibrium concentration of bound species. The maximum amount of bound NGF in the delivery system increased as peptide affinity for heparin increased. Thus, at equilibrium a biphasic response of bound NGF was observed for all peptides, where an increase or decrease in the molar ratio of peptide to heparin from the optimal ratio resulted in a decrease of matrix bound NGF.

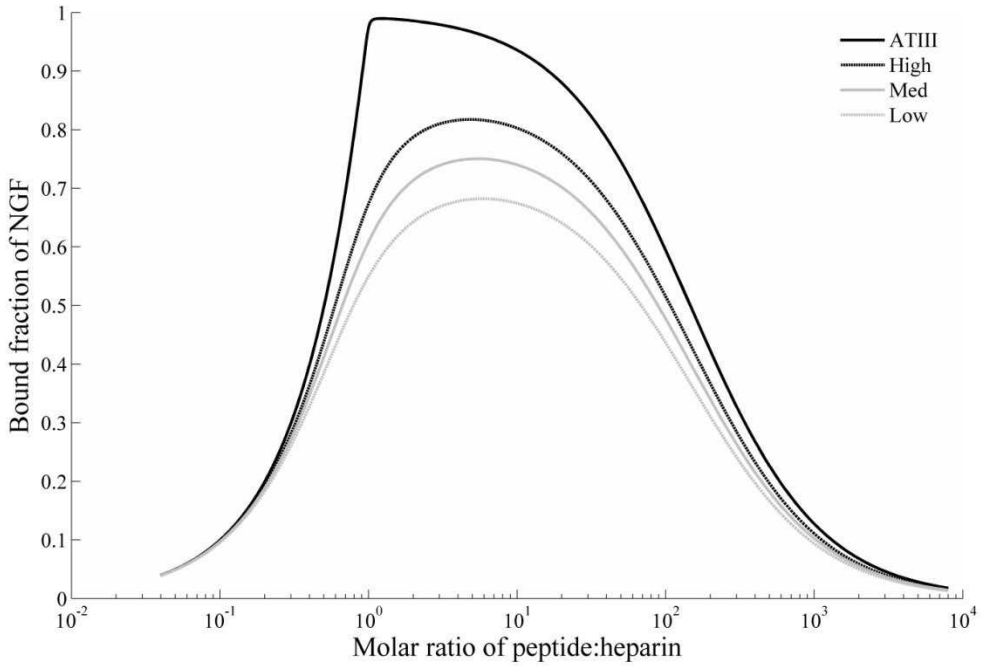

Figure 2.1. Effect of molar ratio of peptide to heparin on the fraction of NGF in the bound state. At a state of equilibrium, mathematical modeling of the delivery system in the fibrin matrix demonstrated that the amount of bound NGF was dependent upon the affinity of the peptide for heparin and the molar ratio of peptide to heparin. The curves displayed a biphasic nature where there was an optimal molar ratio of peptide to heparin to obtain the maximum amount of available bound NGF, regardless of affinity.

Using the equilibrium concentrations as the initial conditions within the fibrin matrix, the amount of NGF released to aqueous media over 24 h was simulated in a manner similar to the experimental *in vitro* release study. The presence of the delivery system resulted in a decrease in NGF release regardless of peptide affinity for heparin or molar ratio of peptide to heparin (Figure 2.2). At similar molar ratios of peptide to heparin, the total amount of NGF released decreased as peptide affinity for heparin increased. By changing the molar ratio of peptide to heparin for the affinity peptides, NGF release was modulated resulting in increased NGF release as the molar ratio of peptide to heparin increased.

Additionally, this result demonstrated that peptides with different heparin affinity are capable of releasing NGF over 24 h at similar rates if the appropriate molar ratios of peptide to heparin are used. Therefore, the modeling demonstrates the feasibility of using this delivery system to test whether delivery system affinity affects biological activity independent of release rate.

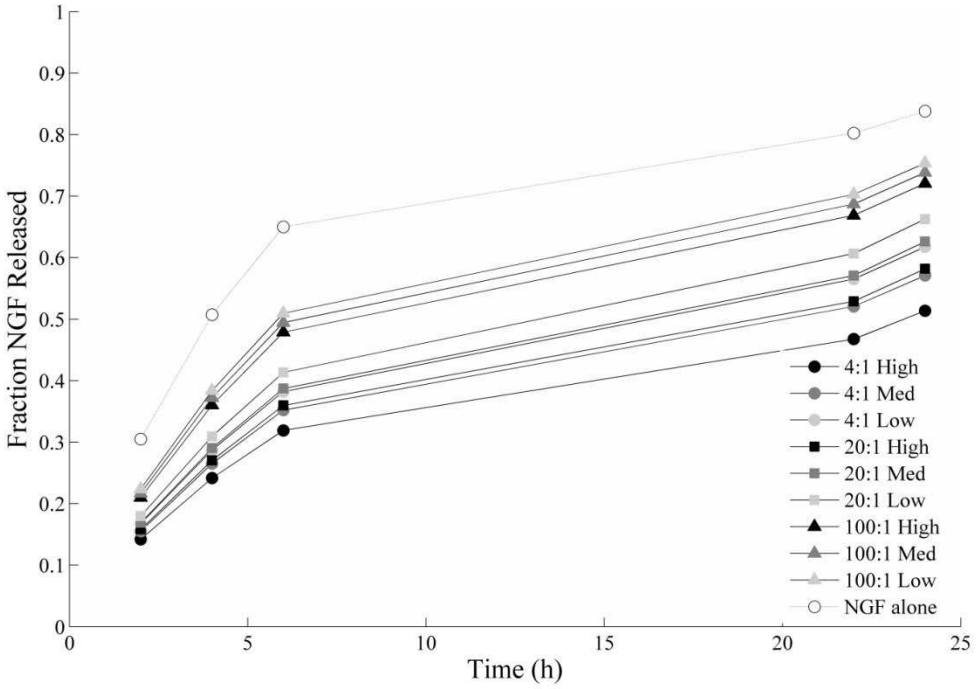

Figure 2.2. Fraction of NGF released at 24 h depends on the peptide affinity for heparin and the molar ratio of peptide to heparin in the delivery system. The amount of NGF released over 24 h was modeled mathematically to mimic the experimental *in vitro* release study. The presence of the delivery system resulted in reduced NGF release. Unique release profiles were obtained by varying the peptide affinity for heparin and the molar ratio of peptide to heparin. Additionally, different affinity peptides had the ability to retain similar levels of NGF after 24 h (at different peptide to heparin ratios) demonstrating the feasibility of the in vitro biological activity assay.

2.4.2 *In vitro* release assay

The ability of the delivery system to immobilize NGF within fibrin matrices was assessed in equilibrium release studies by measuring the amount of NGF present within the matrices after 48 h (previously found to be sufficient time for the NGF concentration to reach equilibrium) (Willerth, Johnson et al. 2007). The retention of NGF within fibrin matrices at equilibrium was compared to the amount of NGF present in their corresponding aqueous washes (equal volumes). The amount of NGF present in fibrin matrices and the washes without the complete delivery system (fibrin alone, NGF with peptide but no heparin, or NGF with heparin but no peptide) were equal at ~50-51% of the NGF initially added to the matrix, demonstrating equal partitioning of NGF in the absence of delivery system (Table 2.3). However, the presence of the delivery system enhanced the amount of NGF retained within the fibrin matrices, where the level of enhancement depended upon the molar ratio of peptide to heparin and the peptide affinity for heparin. The delivery system containing the low affinity peptide retained at most 55 ± 3% of the initial NGF at all molar ratios of peptide to heparin, similar to unmodified fibrin. The presence of the delivery system employing the ATIII, high affinity, or medium affinity peptides increased retention of NGF in fibrin matrices compared to fibrin alone at peptide to heparin molar ratios of 4:1 and 40:1. The medium affinity peptide retained 64 ± 5% and 68 ± 1%, the high affinity peptide retained 73 ± 8% and 73 ± 1%, and the ATIII peptide retained 78 ± 3% and 76 ± 1% of the initial NGF (4:1 and 40:1 molar ratios of peptide to heparin, respectively). However, at a peptide to heparin molar ratio of 200:1 only the delivery system employing the ATIII or the high affinity peptide retained more NGF than unmodified fibrin, where the high affinity peptide retained 63 ± 1% and the ATIII peptide retained 71 ± 3% of the initial NGF.

Table 2.3: Mass retention of NGF in fibrin matrices with HBDS

* indicates statistical significance compared to fibrin matrices containing only NGF

	Molar ratio of peptide to heparin			
Peptide	**4:1**	**40:1**	**200:1**	**No heparin**
None	50 ± 4	51 ± 1	51 ± 1	50 ± 1
Low	53 ± 1	55 ± 3	53 ± 1	51 ± 2
Medium	64 ± 5 *	68 ± 1 *	56 ± 1	51 ± 1
High	73 ± 8 *	73 ± 1 *	63 ± 1 *	51 ± 2
ATIII	78 ± 3 *	76 ± 1 *	71 ± 3 *	51 ± 1

The effect of varying the affinity of heparin-binding peptides for heparin and the molar ratio of peptide to heparin was investigated by measuring the cumulative release of NGF from fibrin matrices over 7 days. The release of NGF from fibrin matrices (without peptide or heparin) was rapid with a large initial burst at 2 h (38 ± 3%) followed by the loss of 92 ± 3% of the NGF over 24 h (Figure 2.3). By day 7, the delivery system retained only a fraction of the initial NGF (< 1%). Fibrin matrix controls consisting of any of the peptides with NGF (but no heparin) were similar to unmodified fibrin matrices with NGF (Figure 3D).

The presence of the delivery system slowed the release of NGF and retained a greater fraction of NGF over 7 days depending on the peptide affinity for heparin and molar ratio of peptide to heparin. The initial release (2 h) of NGF was statistically decreased for the delivery systems incorporating the medium affinity, high affinity, and ATIII peptides at a 4:1 molar ratio of peptide to heparin compared to unmodified fibrin matrices (Figure 2.3A). A greater fraction of NGF was also retained at 24 h for the medium affinity (35 ± 3%), high affinity (44 ± 1%), and ATIII (50 ± 4%) peptides. Release began to level off after 6 days

with < 2% loss per day, while still retaining more NGF than unmodified fibrin matrices or fibrin matrices containing an incomplete delivery system (heparin but no peptide) (12 ± 1% medium affinity, 23 ± 1% high affinity, and 33 ± 1% ATIII). The delivery system incorporating the low affinity peptide did not show a difference versus unmodified fibrin matrices or a delivery system incorporating heparin (but no peptide) at 2 h, 24 h, or day 7 (2 ± 1% NGF retained).

When the molar ratio of peptide to heparin was increased to 40:1, the initial release of NGF was decreased compared to unmodified fibrin matrices or a delivery system incorporating heparin (but no peptide) (Figure 2.3B). Additionally, a higher fraction of NGF was retained after 24 h for all peptides (29 ± 3% low affinity, 45 ± 1% medium affinity, 49 ± 1% high affinity, and 55 ± 6% ATIII) compared to unmodified fibrin matrices or a delivery system incorporating heparin (but no peptide). Release from delivery systems containing any peptides at the 40:1 molar ratio began to level off after 6 days with < 3% loss per day, however these systems still retained greater levels of NGF versus unmodified fibrin matrices or a delivery system incorporating heparin (but no peptide), even in the case of the low affinity peptide, (9 ± 1% low affinity, 14 ± 1% medium affinity, 20 ± 2% high affinity, and 32 ± 2% ATIII).

Additionally, when the molar ratio of peptide to heparin was 200:1, an initial burst release of NGF was observed, similar to that observed for unmodified fibrin matrices that lacked the delivery system (Figure 2.3C). However, at a 200:1 molar ratio of peptide to heparin all peptides had similar NGF retention levels at 24 h (27 ± 3% low affinity, 29 ± 2% medium affinity, 29 ± 1% high affinity, and 30 ± 2% ATIII), which was greater than the level retained in unmodified fibrin matrices. At day 7, a greater fraction of NGF remained

for the ATIII (11 ± 1%) peptide compared to unmodified fibrin matrices or a delivery system incorporating heparin (but no peptide).

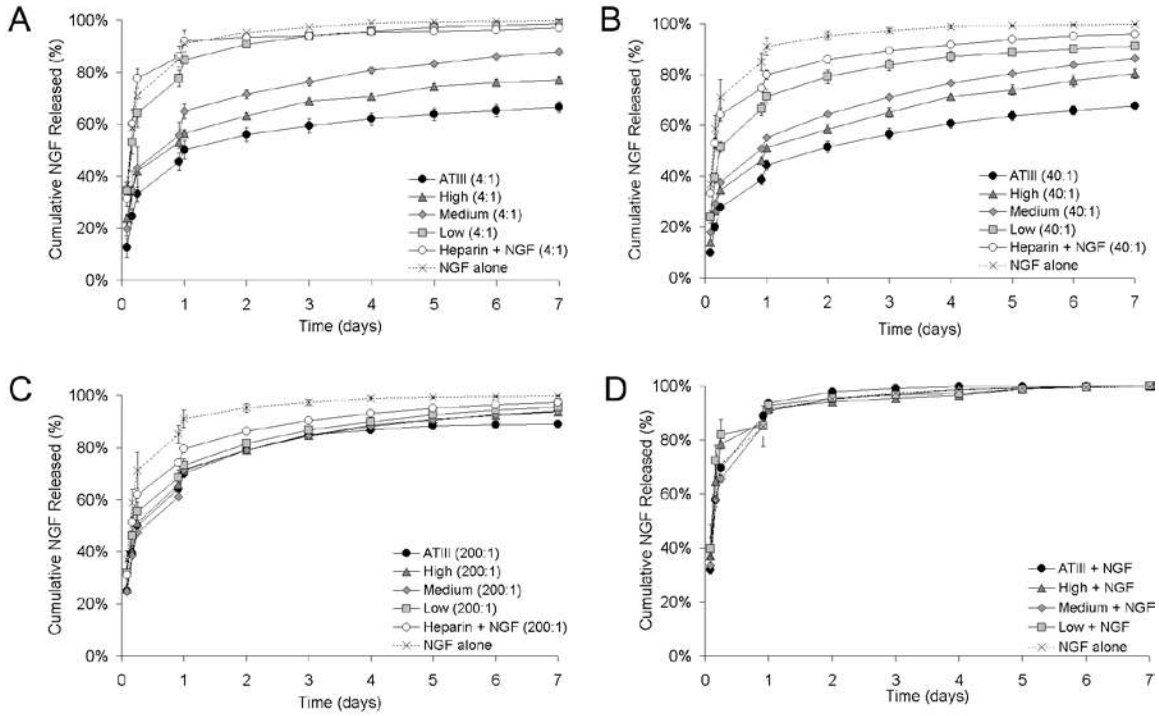

Figure 2.3. NGF release *in vitro* over 7 days depends on the peptide affinity for heparin and the ratio of peptide to heparin in the delivery system. NGF was released over 7 days from fibrin matrices with and without the delivery system at a various molar ratios of peptide to heparin containing varying affinity peptides for heparin. At the 4:1 molar ratio (A), the delivery system incorporating the medium affinity, high affinity, and ATIII peptides retained higher levels of NGF after 2 h, 24 h, and 7 days. At the 40:1 molar ratio (B), the delivery system incorporating any peptide retained higher levels of NGF after 2 h, 24 h, and 7 days. At the 200:1 molar ratio (C), only the ATIII peptide retained higher levels of NGF after 7 days. The peptides alone were not able to affect the retention of NGF (D). Data ($n \geq 3$) represented by mean ± SD and statistical significance was considered $p<0.05$ compared to fibrin matrix alone or delivery system incorporating only heparin at a similar concentration.

2.4.3 *In vitro* biological activity assay

In order to assess the biological activity of NGF released by the delivery system, embryonic chick DRGs were implanted into fibrin matrices with and without the delivery system, and the average neurite extension was measured. Neurite extension was normalized

to neurite extension from DRGs grown in unmodified fibrin matrices with NGF in the culture media at an optimal dose (20 ng/mL) (Sakiyama-Elbert and Hubbell 2000a). Fibrin matrices containing the delivery system with any affinity peptide at all molar ratios of peptide to heparin tested exhibited neurite extension that was equivalent to or greater than that observed for fibrin matrices with NGF in the media (Figure 2.4A). Varying the peptide affinity for heparin resulted in different biological responses, where a 4:1 molar ratio of peptide to heparin showed increased neurite extension for the medium affinity (23 ± 17%), high affinity (19 ± 14%), or ATIII (29 ± 20%) peptides versus NGF in the media, while the low affinity peptide was similar to NGF in the media.

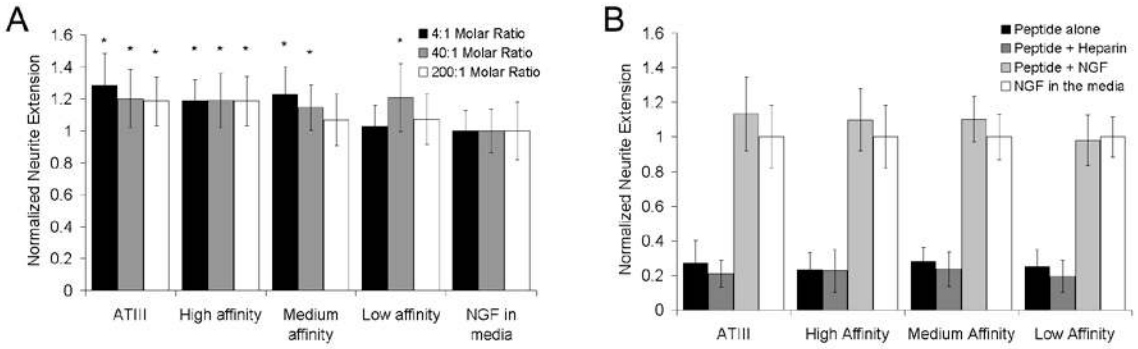

Figure 2.4. Effect of peptide affinity and peptide to heparin molar ratio on DRG neurite extension *in vitro*. Normalized neurite extension from chick DRGs was affected by varying the peptide affinity or the molar ratio of peptide to heparin in the delivery system. All DRG neurite extension was normalized to the average neurite extension for unmodified fibrin matrices with 20 ng/mL of NGF added to the media for the same experiment. The delivery system incorporating any peptide with 100 ng/mL of NGF caused increased or equivalent neurite extension compared to NGF in the media (A). Additionally, the entire delivery system was required to increase normalized neurite extension (B). Data ($n \geq 18$) represents mean ± S.D. and an asterisk indicates statistical significance ($p<0.05$) compared to NGF in the media.

Additionally, the effect of varying the molar ratio of peptide to heparin was explored. At a molar ratio of 40:1 peptide to heparin, all peptides exhibited increased neurite extension versus NGF in the media (21 ± 21% low affinity, 15 ± 14% medium affinity, 19 ± 17%

high affinity, and 20 ± 18% ATIII). In particular, greater neurite extension was observed for the low affinity peptide found at this molar ratio. Conversely, neurite extension was not enhanced at the 4:1 molar ratio. However, at a molar ratio of 200:1 peptide to heparin, only the high affinity (18 ± 15%) and ATIII (19 ± 15%) peptides displayed increased neurite extension versus to NGF in media, while the medium and low affinity peptides were similar to NGF in the media.

To assess the contribution of the other components in the delivery system, DRGs were implanted into fibrin matrices lacking the complete delivery system and the average neurite extension was measured. The absence of NGF (with heparin at any concentration and/or any of the peptides) did not promote robust neurite extension and was statistically decreased from DRGs grown with NGF in the media by ~70% (Figure 2.4B). When NGF was included, incorporating any of the peptides, heparin at any concentration (data not shown), or neither peptide nor heparin resulted in neurite extension that was similar to DRGs grown with NGF in the media.

Additionally, the effect of similar release regimes for peptides of different heparin binding affinity on the biological activity of NGF was assessed. The NGF release rates of the low, medium, and high affinity peptides at a 40:1 molar ratio of peptide to heparin were each compared to other peptides and molar ratios of peptide to heparin with similar NGF release over the first 24 h. For each peptide at the 40:1 molar ratio of peptide to heparin, other peptides and molar ratios of peptide to heparin with similar NGF retention at 24 h are grouped and listed in Table 2.4, where the NGF retained after 24 h was presented as the average of the group members for each similar release group. Since DRGs were implanted in fibrin matrices after 24 h of washing during the *in vitro* biological activity assay, neurite

extension was compared for all members within a similar release group to assess the effect of release on biological activity. Neurite extension between members within the same release group demonstrated similar means, where the average increase in neurite extension (versus NGF in the media) for each release group is given: low affinity (16 ± 7%), medium affinity (21 ± 6%), and high affinity (20 ± 5%) peptide groups. This result indicates that the ability of the delivery system to retain NGF affected biological activity, and there was no effect of peptide affinity independent of release rate.

Table 2.4: Similar mass retention of NGF at 24 h results in similar normalized neurite outgrowth regardless of peptide affinity

(Neurite outgrowth was normalized to its respective positive control)

Peptide group	**Similar peptide groups**	**Mass retention at 24 h**	**Average mass retention**	**Normalized neurite outgrowth**	**Average normalized neurite outgrowth**
Low 40:1	Low 40:1	29 ± 3 %	30 ± 3 %	121 ± 21	116 ± 7
	Med 4:1	35 ± 3 %		123 ± 17	
	ATIII 200:1	30 ± 2 %		119 ± 15	
	High 200:1	29 ± 1 %		118 ± 15	
	Med 200:1	29 ± 2 %		107 ± 16	
	Low 200:1	27 ± 3 %		107 ± 16	
Med 40:1	Med 40:1	45 ± 1 %	47 ± 3 %	115 ± 14	121 ± 6
	High 4:1	44 ± 1 %		119 ± 14	
	High 40:1	49 ± 1 %		119 ± 17	
	ATIII 4:1	50 ± 4 %		129 ± 20	
High 40:1	High 40:1	49 ± 1 %	49 ± 5 %	119 ± 17	120 ± 5
	Med 40:1	45 ± 1 %		115 ± 14	
	High 4:1	44 ± 1 %		119 ± 14	
	ATIII 4:1	50 ± 4 %		129 ± 20	
	ATIII 40:1	56 ± 2 %		119 ± 15	

2.5 Discussion

The drug delivery system investigated in this study used a rational approach to delivery system design to develop a biomaterial scaffold to sequester and slow the release rate of NGF. Other affinity-based drug delivery systems incorporating components that reversibly bind the drug of interest, such as heparin within fibrin (Jeon, Kang et al. 2005; Jeon, Ryu et al. 2005), collagen (Wissink, Beernink et al. 2000a), and polymer matrices (Benoit and Anseth 2005; Pike, Cai et al. 2006) can modulate the release of drug through the strength of affinity interactions. The delivery system investigated in this study demonstrates two methods to modulate the rate of drug release, the peptide affinity for heparin and the molar ratio of peptide to heparin, as verified through mathematical modeling and experimental data.

The mathematical modeling performed predicted that more NGF could be sequestered within fibrin matrices at equilibrium by increasing peptide affinity for heparin. Additionally, it predicted that the greatest amount of NGF could be sequestered by optimizing the molar ratio of peptide to heparin for each peptide. The key component that emerged from the model results was the fraction of matrix bound heparin, which implied that the rate of NGF release could be modulated by varying the concentration of heparin (or the molar ratio of peptide to heparin). To explore this implication, NGF release was modeled with the removal all species from the aqueous wash media (similar to the changing of the wash solutions in the experimental studies). This approach allowed us to estimate what range of molar ratios of peptide to heparin could yield different release rates for each affinity peptides and similar release rates between different affinity peptides. Based on these results, the experimental range of molar ratios of peptide to heparin was chosen.

Experimentally, peptide affinity for heparin and molar ratio of the peptide to heparin was found to modulate the release rates of NGF, resulting in different release rates for the delivery system, as predicted by the math model. As the molar ratio of the peptide to heparin increased from 4:1 or 40:1 to 200:1, NGF release occurred more rapidly for all of the peptides. Furthermore, as peptide affinity for heparin increased, NGF release was slowed for all molar ratios of peptide to heparin. The ability of the delivery system to modulate the release rate through these two parameters provides two mechanisms for controlling release rate.

NGF released from the delivery system induced neurite extension that was similar to or greater than that induced by NGF in the media, suggesting that the delivery system did not have a negative impact on the biological activity of the NGF. Previously, others have found that localized delivery of neurotrophic factors can enhance neurite extension more than neurotrophic factors in the media (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b; Taylor, McDonald et al. 2004). This enhancement could be due to the formation of concentration gradients within the fibrin matrices as extending neurite locally degrade the fibrin matrix and release the matrix bound growth factor. NGF has been shown to guide and enhance neurite extension through concentration gradients, but only if the steepness of the gradient was above a critical value (Cao and Shoichet 2001; Cao and Shoichet 2003; Rosoff, Urbach et al. 2004). In this study, enhanced neurite extension compared to NGF in the media was observed for the medium and high affinity and ATIII peptides at 4:1 and 40:1 molar ratios of peptide to heparin, where enhanced NGF retention was observed as well. However, enhanced neurite extension compared to NGF in the media was only observed for the low affinity peptide at a molar ratio of 40:1 as opposed to the 4:1 molar ratio. This result could be due to the larger amount of NGF retained after 24 h for

the 40:1 molar ratio of peptide to heparin versus the 4:1, establishing the minimum retention level needed in the fibrin matrices to elicit enhanced neurite extension.

As mentioned previously, the NGF remaining after 24 h for the low affinity peptide at a 4:1 molar ratio of peptide to heparin was comparable to unmodified fibrin matrices, while more NGF remained for all the other peptides (medium, high and ATIII), which resulted in increased neurite extension for the other peptides. When the molar ratio of peptide to heparin was increased to 40:1, all of the peptides retained more NGF than unmodified fibrin, and neurite extension was increased for delivery systems containing any of the peptides (and heparin) indicating that NGF retention was critical for neurite extension. To confirm that the delivery system components were all necessary to modulate the release rate of NGF (and thus to elicit neurite extension), one or more components of the delivery system were removed, which resulted in lower NGF retention and the loss of enhanced neurite extension.

Apart from modulating neurite extension through altering the release rate of NGF, the delivery system allowed the ability to test whether the delivery system affinity could affect biological activity independent of release rate. To test this hypothesis, different affinity peptides with similar release rates after 24 h (selected molar ratios of peptide to heparin) were compared. It was found that the neurite extension was similar for all group members within a group of delivery system conditions that elicited similar rates of NGF release. These results suggest that when two peptides of different heparin affinity were combined with heparin concentrations such that they exhibited similar release rate, the resulting neurite extension (biological activity) elicited by the released NGF was similar, thus there was no effect of affinity independent of release rate.

The ability of the delivery system to present biologically active NGF *in vitro* suggests it may be useful for the treatment of nerve injury *in vivo*. In particular, NGF has been delivered locally to treat peripheral nerve injury (Lee, Yu et al. 2003b), but not in a way that tests at what rate NGF should be administered. Dinbergs and coworkers discovered that not all growth factors are best delivered slowly and locally. They found bFGF enhanced endothelial and smooth muscle cell proliferation through sustained release compared to bolus administration while transforming growth factor-β1 inhibited endothelial cells more efficiently in bolus form compared to slow release (Dinbergs, Brown et al. 1996). This delivery system has the unique ability to allow us to study the effects of how the rate of growth factor release affects nerve regeneration.

Chapter 3

Heparin-binding affinity-based delivery systems releasing nerve growth factor enhance sciatic nerve regeneration*

3.1 Abstract

The controlled delivery of nerve growth factor (NGF) to the peripheral nervous system has been shown to enhance nerve regeneration following injury, although the effect of release rate has not been previously studied with an affinity-based delivery system (DS). The goal of this research was to determine if the binding site affinity of the DS affected nerve regeneration *in vivo* using nerve guidance conduits (NGCs) in a 13 mm rat sciatic nerve defect. These DSs consisted of bi-domain peptides that varied in heparin-binding affinity, heparin, and NGF, which binds to heparin with moderate affinity. Eight experimental groups were evaluated consisting of NGF with DS, control groups excluding one or more components of the DS within silicone conduits, and nerve isografts. Nerves were harvested 6 weeks after treatment for analysis by histomorphometry. These DSs with NGF resulted in a higher frequency of nerve regeneration compared to control groups and were similar to the nerve isograft group in measures of nerve fiber density and percent neural tissue, but not in total nerve fiber count. In addition, these DSs with NGF contained a significantly greater percentage of larger diameter nerve fibers, suggesting more mature regenerating nerve content. While there were no differences in nerve regeneration due to varying peptide

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affinity with these DSs, their use with NGF enhanced peripheral nerve regeneration through a NGC across a critical nerve gap.

3.2 Introduction

Despite significant advances in nerve reconstruction, the autograft remains the clinical standard of care for long gaps in critical peripheral nerves; however, functional outcomes are still suboptimal and drawbacks exist including donor site morbidity (Lundborg 2000; Schmidt and Leach 2003). Because of these disadvantages, alternatives have been sought including NGCs (Lundborg 2000; Schmidt and Leach 2003; Bellamkonda 2006). The use of a NGC offers the advantage of being able to control the microenvironment for regeneration by modulating the composition and luminal contents of the conduit, in particular through the inclusion of growth factors and extracellular matrix (ECM) molecules (Lundborg 2000; Schmidt and Leach 2003; Bellamkonda 2006).

Much of the research has focused on the design of biodegradable conduits (Widmer, Gupta et al. 1998; Piquilloud, Christen et al. 2007; Pfister, Alther et al. 2008). However, the ultimate design goal of NGCs may also include a drug delivery matrix within a biodegradable conduit. Growth factor delivery from biomaterial matrices is often controlled by diffusion (Yu and Bellamkonda 2003; Mohanna, Terenghi et al. 2005), however, one shortcoming of this approach is that the release rate cannot be modulated or controlled by cells during regeneration. One alternative is to use an *affinity-based* DS that allows the release of growth factors to be controlled by cell-based degradation of the DS. This study is focused on the role of affinity-based drug delivery from the conduit lumen.

Previously, we developed an affinity-based DS incorporating heparin that sequesters proteins within a fibrin matrix based on non-covalent interactions (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b). Initially, this system consisted of a bi-domain heparin-binding peptide (ATIII peptide) where one domain consisted of a transglutaminase substrate, based on α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983; Kimura, Tamaki et al. 1985), allowing it to be crosslinked into fibrin matrices during polymerization by the transglutaminase Factor XIIIa. The other domain consisted of a modified version of the heparin binding domain from antithrombin III (Tyler-Cross, Sobel et al. 1994; Tyler-Cross, Sobel et al. 1996; Sakiyama, Schense et al. 1999) allowing non-covalent binding of heparin to peptide crosslinked within a fibrin matrix. With the inclusion of a protein that interacts with heparin, a ternary complex is formed consisting of peptide, heparin, and protein bound within the fibrin matrix that effectively sequesters the protein within the matrix, allowing release by cell-mediated processes.

NGF is well known for its ability to promote neurite extension *in vitro* (Conti, Fischer et al. 1997; Macias, Battocletti et al. 2000; Sakiyama-Elbert and Hubbell 2000a; Xu, Yu et al. 2002) and nerve regeneration *in vivo* (Xu, Yu et al. 2002; Lee, Yu et al. 2003a; Yu and Bellamkonda 2003). Our lab has determined that the presentation of NGF, e.g. free NGF in a biomaterial matrix or NGF bound to a biomaterial matrix (such as our DS through a strong heparin-binding peptide) can affect the biological response to NGF. We previously demonstrated that our affinity-based DS enhanced nerve regeneration when containing a strong heparin-binding peptide (ATIII peptide) compared to matrices loaded with NGF alone (Lee, Yu et al. 2003a). Moreover, others recently have shown that collagen matrices that can sequester NGF by affinity interactions are more effective than diffusion-based delivery of NGF at promoting neural regeneration (Sun, Lin et al. 2007). However, the

effect of binding affinity (the system's ability to sequester drug) of an affinity-based delivery system on nerve regeneration *in vivo* has not been elucidated. Specifically, if binding affinity and drug release rate for an affinity-based DS is modulated, how is the *in vivo* biological activity of the drug that is being released modulated.

To explore the role of binding affinity, we used combinatorial techniques to tailor the affinity of non-covalent interactions between a heparin-binding peptide and heparin (Maxwell, Hicks et al. 2005). Peptide sequences were identified that exhibited "high", "medium", and "low" affinity for heparin using an increasing step gradient of sodium chloride concentration (1.0 M, 1.5 M and 2.0 M NaCl were used to elute the low, medium and high affinity peptide display phage from a heparin affinity column, respectively) (Maxwell, Hicks et al. 2005). Peptides were synthesized containing the identified heparin binding domains along with a transglutaminase substrate to allow crosslinking into fibrin matrices (Maxwell, Hicks et al. 2005). By utilizing these peptide sequences exhibiting high, medium, and low affinity for heparin and the previously identified ATIII peptide, which has the strongest binding-affinity for heparin of the peptides in this study, our DS can release NGF at different rates. Our previous work *in vitro* demonstrated that the ability of our DS to sequester NGF was the factor controlling the ability to enhance neurite extension (Wood and Sakiyama-Elbert 2008). The current study extends the previous work and provides insight into the effect of binding affinity on nerve regeneration *in vivo*.

The focus of this study was to assess whether peptide affinity for heparin affects nerve regeneration. To test this premise, peptides that previously demonstrated the ability to modulate the release rate and *in vitro* biological activity of NGF were used in an affinity-based DS. Fibrin matrices containing the DS were placed within a silicone NGC to

examine nerve regeneration across a 13-mm rat sciatic nerve defect, which exceeds the critical defect size for spontaneous regeneration (Lundborg, Dahlin et al. 1982a). The effect of the DS on nerve regeneration was assessed through histomorphometric analysis of the nerve in the conduits after 6 weeks.

3.3 Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

3.3.1 Peptide synthesis

Peptides of varying heparin-binding affinity were synthesized by standard solid phase Fmoc chemistry (amino acids from Nova Biochem, San Diego, CA; peptide synthesis solvents from Applied Biosystems, Foster City, CA) using an ABI433A peptide synthesizer (Applied Biosystems) and were based on amino acid sequences identified previously (Table 3.1) (Tyler-Cross, Sobel et al. 1994; Sakiyama, Schense et al. 1999; Maxwell, Hicks et al. 2005). The peptides denoted high and medium affinity were based on previously identified 12-mer peptides identified by screening a phage display library with heparin-Sepharose chromatography (Maxwell, Hicks et al. 2005), and the peptide denoted ATIII was based on a modified version of the antithrombin III-heparin binding domain and has the highest binding affinity for heparin of all the peptides used in the study (Tyler-Cross, Sobel et al. 1994; Sakiyama, Schense et al. 1999). After synthesis, the peptides were cleaved from the resin with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane by volume for 2-

3 h using 10 mL of cocktail per 1 g of resin. The mixture was filtered through glass wool to remove the resin then precipitated in cold diethyl ether. The crude peptide filtrates were dried under vacuum and then purified by standard C18 reverse phase liquid chromatography (Shimadzu, Columbia, MD) and lyophilized. The identity of purified peptides were verified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

Table 3.1: Heparin-binding peptides with equilibrium dissociation constants of peptide interaction with heparin

(Transglutaminase substrate in *italics*)

Peptide Name	**Amino Acid Sequence**	**K_D ($P \longleftrightarrow H$)**
Medium affinity	*NQEQVSP*GSSANGKKPSTRR (Maxwell, Hicks et al. 2005)	6.1 x 10^{-5} M (Maxwell, Hicks et al. 2005)
High affinity	*NQEQVSP*GNSAHRTRGRQRS (Maxwell, Hicks et al. 2005)	3.8 x 10^{-5} M (Maxwell, Hicks et al. 2005)
ATIII	(*Ac*G)*NQEQVSP*K(βA)FAKLAARLY RKA (Sakiyama, Schense et al. 1999)	8.78 x 10^{-8} M (Olson, Srinivasan et al. 1981; Kridel, Chan et al. 1996)

3.3.2 Fibrin matrix preparation

Fibrin matrices were prepared as previously described (Lee, Yu et al. 2003a) by mixing the following components (final concentrations given): human plasminogen-free fibrinogen containing Factor XIII (4.0 mg/mL), bovine thrombin (2 NIH units/mL), and $CaCl_2$ (2.5 mM, Fisher Scientific, Pittsburgh, PA). Fibrin matrices including DS were prepared by incorporating the following additional components: peptide (0.25 mM of medium affinity, high affinity, or ATIII to obtain ~ 8 moles of peptide cross-linked per mole of fibrinogen (Sakiyama, Schense et al. 1999; Schense and Hubbell 1999)), heparin (Sigma-

Aldrich product no. H9399, sodium salt from porcine intestinal mucosa 18,000 average MW) added at 62.5 μM to obtain a 4:1 molar ratio of peptide to heparin, and 50 ng/mL human β-NGF (Peprotech Inc., Rocky Hill, NJ).

Silicone tubing (SF Medical, Hudson, MA; 0.058 in. inside diameter x 0.009 in. wall thickness) was cut into 15-mm segments and autoclaved. Prior to filling, the tubes were rinsed with sterile saline solution. The fibrin solution was drawn into the silicone tube using a pipette (~22 μL/conduit). The fibrin matrix was allowed to polymerize for 10 minutes prior to implantation of the conduit.

3.3.3 Experimental animals

Adult male Lewis rats (Harlan Sprague-Dawley, Indianopolis, IN), each weighing 250-300g were used in this study. All surgical procedures and peri-operative care measures were performed in strict accordance with the National Institutes of Health guidelines and were approved by the Washington University Animal Studies Committee. All animals were housed in a central animal facility, given a rodent diet (PicoLab Rodent Diet 20 #5053, PMI Nutrition International) and water ad libitum.

3.3.4 Experimental Design

Ninety-six animals were randomized into eight groups (n = 12) as shown in Table 3.2. An additional six animals served as nerve graft donors. Group I served as a negative control group and received an empty conduit. Groups II – IV were additional controls

receiving conduits containing fibrin alone, fibrin with the ATIII peptide used in the DS (but no growth factor) or fibrin with the growth factor but no DS, respectively. These groups examined the effects of the components of the DS. Groups V – VII were implanted with conduits containing complete DS with one of three varying affinity peptides and 50 ng/mL β-NGF (dose selected based on a previous study (Lee, Yu et al. 2003a)). Group VIII served as a positive control receiving reversed nerve isografts from syngeneic donor animals.

Table 3.2: Experimental Design

(n = 12 for all groups)

Group	**Group Name**	**Peptide used in delivery system (DS)**	**Nerve Growth Factor (NGF) Dose (ng/mL)**	**Fibrin**	**Rats with Regeneration (out of 12)**
I	Empty	None	0	No	3
II	Fibrin Alone	None	0	Yes	5
III	ATIII DS (no NGF)	ATIII	0	Yes	3
IV	NGF (no DS)	None	50	Yes	4
V	Med DS	Medium affinity	50	Yes	5
VI	High DS	High affinity	50	Yes	6
VII	ATIII DS	ATIII	50	Yes	7
VIII	Isograft	None	0	No	12

3.3.5 Operative Procedure

Operative procedures were performed using aseptic technique and microsurgical dissection and repairs. Using 4% isoflurane gas (Vedco Inc., St Josephs, MO) anesthesia, the hind leg of the rat was prepped and the sciatic nerve was exposed through a dorsolateral-

gluteal muscle splitting incision. A 5 mm nerve segment was excised proximal to the trifurcation of the sciatic nerve and a 15 mm silicone tube containing fibrin matrices, with or without DS and NGF, was sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end, creating a total regeneration gap of 13 mm (Figure 3.1). Four 9-0 nylon interrupted microepineurial sutures were used to secure the conduit (two per side). In animals receiving the isograft control, a 13 mm segment of sciatic nerve was harvested from a syngeneic donor animal and inserted into the recipient animal using no more than four 10-0 nylon microepineurial sutures per side to secure the graft. Wounds were irrigated with saline, dried with a q-tip, and closed with a running 5-0 vicryl suture in muscle fascia, and then interrupted 4-0 nylon skin sutures. Animals were recovered in a warm aseptic environment and returned to the housing facility.

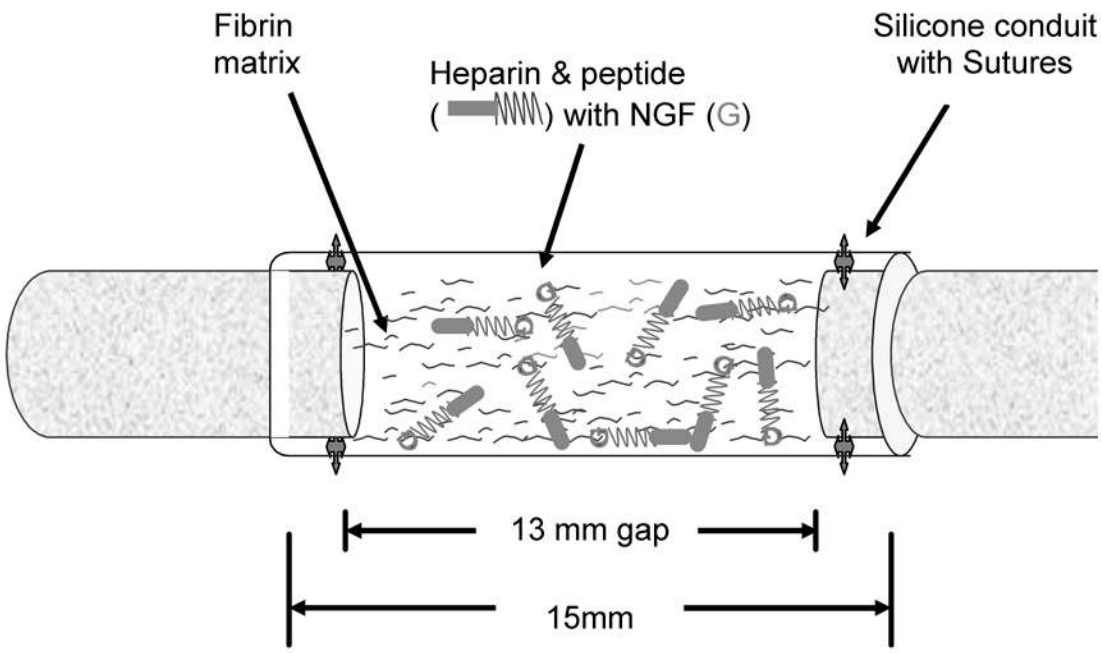


Figure 3.1. Schematic representation of surgical implantation of nerve guidance conduit containing the affinity-based delivery system. A 13 mm nerve gap was repaired with a 15 mm silicone conduit containing fibrin matrices with or without DS and growth factor and sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end. The DS consisted of a bi-domain peptide cross-linked into the fibrin matrix at one domain while the other binds heparin by electrostatic interactions. The NGF can then bind to the bound heparin, creating a matrix-bound, non-diffusible complex, which can be retained for cell mediated degradation of the fibrin matrix (modified from Wood et al. (Wood, Moore et al. 2009)).

At the 6-week end point, all animals were re-anesthetized and nerve harvests were performed by reopening the prior muscle splitting incision. The nerve conduit or graft and a 5 mm portion of native nerve both proximally and distally were harvested. The specimens were marked with a proximal suture and stored in 3% glutaraldehyde in phosphate buffer at 4°C until histomorphometric analysis was performed.

3.3.6 Histomorphometric Evaluation

Tissues, harvested and fixed as described above, were post-fixed with 1% osmium tetroxide, ethanol dehydrated and embedded in Araldite 502 (Polyscience Inc., Warrington, PA). Thin (1-μm) sections were made from the tissue using a LKB II Ultramicrotome (LKB-Produckter A.B., Broma, Sweden) and then stained with 1% toluidine blue for examination under a light microscope.

Midline cross sections from the host nerve through the conduit or graft were evaluated using methods detailed previously (Hunter, Moradzadeh et al. 2007). Briefly, at 1000X magnification, six representative fields per nerve were evaluated with an automated digital image-analysis system linked to histomorphometry software. The system digitized and displayed the microscope image on a video monitor with a resolution of 0.125 μm/pixel. Total fascicular area, fiber diameter and density were measured using at least 80% of the nerve area contained within 4 – 6 intrafascicular field areas. From these primary measurements the following morphometric indices were calculated: total number of nerve fibers (nerve fiber density x total fascicular area), nerve fiber density (fiber number/intrafascicular area), percent neural tissue (100 x neural area/intrafascicular area), and nerve fiber width. For animals with nerve regeneration, the nerve fiber width was used to assess fiber maturity for the range of fiber widths. Morphometric indices from experimental groups were compared to the isograft control.

3.3.7 Statistical analysis

All conduits and grafts were included in histomorphometric analysis, except the fiber distribution analysis, even when no axons were found in the midline cross section. All results are reported as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using Statistica version 6 (Statsoft Inc., Tulsa, OK). All data were evaluated for differences between groups using the Kruskal-Wallis analysis of variance (ANOVA) and median test. Post hoc Mann-Whitney U-tests were used for determining which groups differed with significance set at $\alpha = 0.05$. Additionally, fiber width data was evaluated for differences between groups using ANOVA and post hoc LSD tests were used for determining which groups differed with a significance set at $\alpha = 0.05$. Groups with a $p>0.05$ were considered statistically similar, while groups with a $p<0.05$ were considered statistically different. All statements in the text considering whether groups were different or similar to one another utilize these statistics to support the statements.

3.4 Results

3.4.1 Nerve guidance conduit harvest

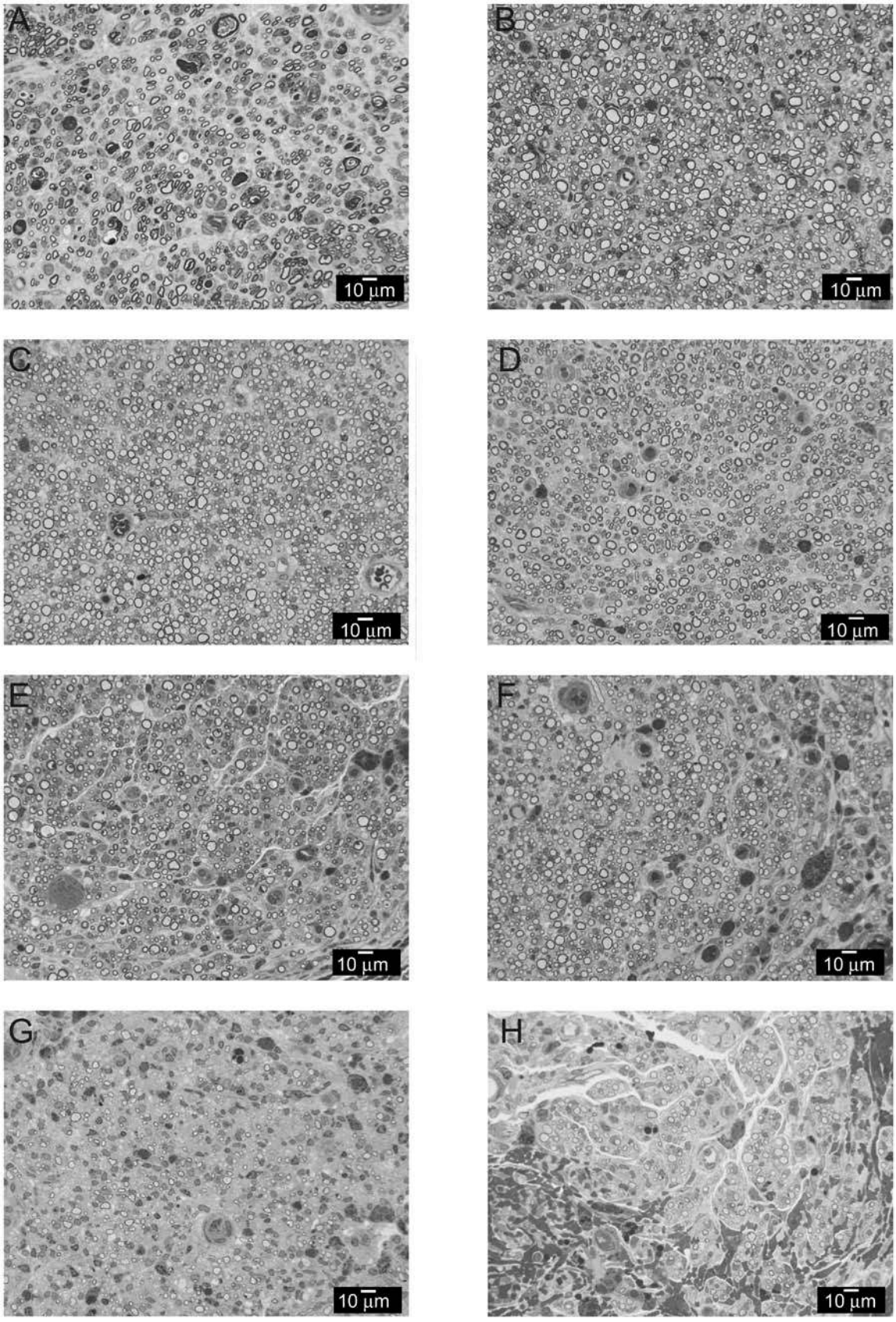
The effectiveness of the DS and/or NGF in promoting nerve regeneration across a critical nerve gap was evaluated *in vivo* after sciatic nerve transection and NGC implantation. To assess whether heparin-binding peptide affinity affects nerve regeneration, peptides with varying affinity for heparin (ATIII ($K_D = 8.78 \times 10^{-8}$ M), high ($K_D = 3.8 \times 10^{-5}$ M), and medium peptides ($K_D = 6.1 \times 10^{-5}$M), in order of highest to lowest affinity, respectively) were examined with the DS and NGF. After 42 days, groups with the DS and NGF resulted in a

higher percentage of animals with a neural cable spanning the 13 mm gap. Seven of 12 conduits utilizing the ATIII peptide (ATIII DS, group VII), 6 of 12 conduits using the high affinity peptide (High DS, group VI), and 5 of 12 using the medium affinity peptide (Med DS, group V) contained regenerated nerve cables when used in the DS with NGF (Table 3.2). Five of 12 conduits from the group containing fibrin alone (group II), 4 of 12 conduits from the group with NGF with no DS (NGF (no DS), group IV), and 3 of 12 from the DS alone with no NGF (DS alone (no GF), group III) group and the empty group (group I) contained regenerated nerve cables. All 12 animals in the isograft group demonstrated had nerve cables after 6 weeks. The gross appearance of the regenerating nerve cables in the DS with NGF groups exhibited a larger, more robust nerve cable in comparison to the other groups (lacking DS and/or NGF). The nerve cable was centered compactly in the conduit, away from the walls, in all conduit specimens. All conduit specimens had the proximal and distal sciatic nerve still sutured into the conduit at 6 weeks, regardless of the presence of a nerve cable spanning the conduit.

3.4.2 Histology

Qualitative examination of sections from the midline of the conduits or isografts by light microscopy revealed differences in nerve architecture as reflected by the arrangement of the regenerating axons (Figure 3.2). In particular, the rat sciatic nerve normally contains myelinated fibers in a packed, semi-symmetrical, uniform arrangement with fibers that are relatively similar in shape to one another. Overall, this arrangement can be described as "organized" architecture. The isograft and groups with a DS and NGF reflect this organized appearance, while the fibrin alone group demonstrated more random spacing and had less

symmetrically shaped myelinated fibers. In addition, groups incorporating DS and NGF appeared to have more tightly packed fibers than the isograft, which was likely due to the compact area for neural regeneration in the silicone tube. No inflammatory response or residual fibrin was observed.


A
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E
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H
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Figure 3.2 Histological sections of regenerating nerves at the midline of the conduit (or graft). (A) Isograft; (B) DS incorporating the ATIII peptide and NGF; (C) DS incorporating the high affinity peptide and NGF; (D) DS incorporating the medium affinity peptide and NGF; (E) fibrin with NGF alone; (F) DS with the ATIII peptide and no NGF; (G) fibrin alone; (H) empty conduit. Thin (1 μm) sections of sciatic nerve specimens were stained with 1% toluidine blue for qualitative examination of the midline of the conduits by light microscopy. Groups with NGF demonstrated more organized neural architecture, closely approximating the isograft, in comparison to the fibrin alone or empty conduit groups. Scale bar, 10 μm.

3.4.3 Histomorphometry

At six weeks, all sections from the midline of the conduits and isografts were assessed by histomorphometry, regardless of the extent of nerve regeneration. The average total myelinated fiber number, a measure of the effectiveness of neural regeneration, was 12,000 ± 600 fibers (n = 12) for the isograft, while groups receiving NGCs and NGF had ~2000 fibers (n = 12 per group) and groups receiving NGCs without NGF had ~1700 fibers (n = 12 per group). The average number of fibers in a normal rat sciatic nerve is approximately 7100 ± 400 (Mackinnon, Dellon et al. 1991). None of the conduit treated groups were able to match the isograft in total myelinated fiber numbers (Figure 3.3A) largely due to the smaller cable area (fascicular area) observed for these groups.

Nerve fiber density is another measure of nerve regeneration, and percent neural tissue is a measure that provides insight into the quality of the regenerating nerve. Neither of these measures is dependent on the area of the regenerating nerve cable. The nerve fiber density at the midline of the graft was ~20,000 fibers/mm^2 for the isograft, which was similar to groups with the DS and NGF (ATIII, High and Med DS, ~11,000-15,000 fibers/mm^2) at the midline of the conduit, but not to groups lacking the DS and/or NGF (Figure 3.3B). For normal rat sciatic nerve, the fiber density is ~12,000 fibers/mm^2 (Mackinnon, Dellon et al. 1991). The isograft also had ~19% neural tissue in the

regenerating nerve at the midline of the graft, which was similar to groups with the DS and NGF (ATIII, High and Med DS, ~12 – 16%) at the midline of the conduit, but greater than groups lacking the DS and/or NGF (Figure 3.3C). These assessments suggest that one aspect of nerve regeneration (nerve fiber density) and the quality of nerve regeneration (percentage neural tissue) was equivalent to an isograft for groups with the DS incorporating any heparin-binding affinity peptide (ATIII, High or Med) and NGF.

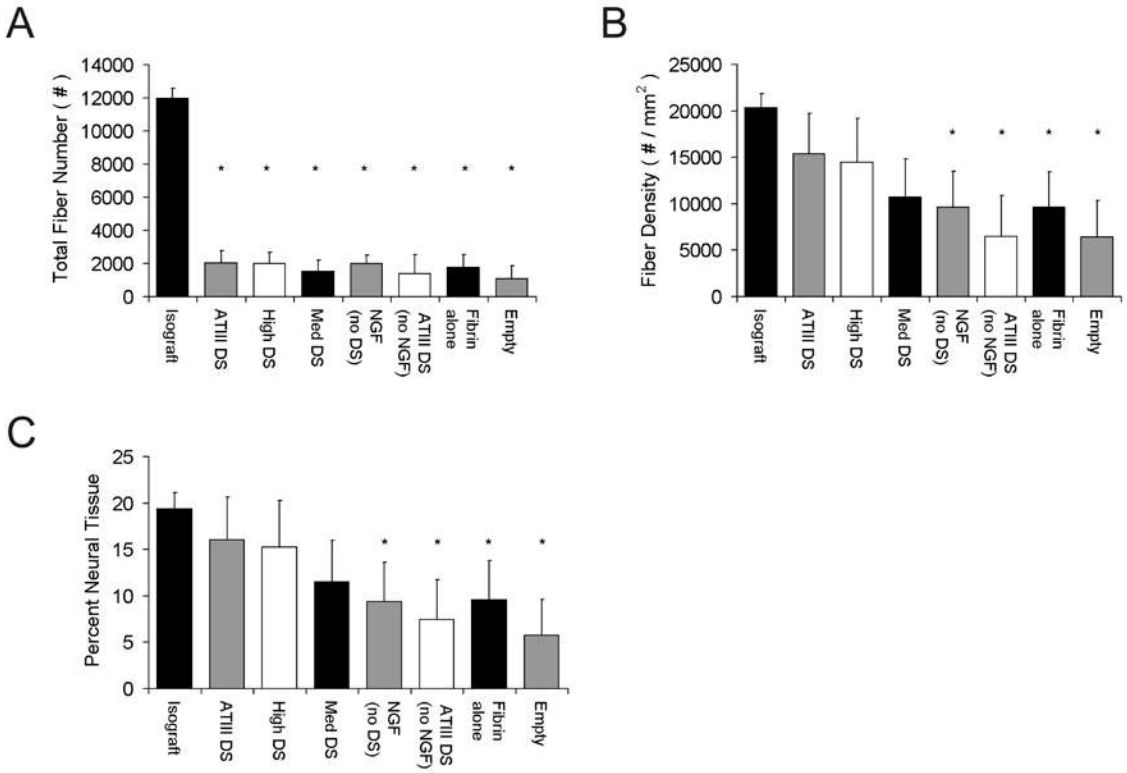

Figure 3.3. Histomorphometric analysis of nerves at the midline of the conduit (or graft). The total number of myelinated nerve fibers, density, and percent neural tissue were measured by quantitative histomorphometry. No groups approximated the nerve regeneration of an isograft (A), but groups with the DS incorporating any affinity peptide and NGF were comparable in nerve fiber density (B) and percent neural tissue (C) to an isograft. Data (n = 12) represents mean ± S.E.M. and * indicates statistical significance ($p<0.05$) compared to isograft.

The myelinated nerve fiber width was assessed as a measure of maturity of the regenerating nerve fibers for animals with nerve regeneration, and groups that were most

efficient in promoting nerve regeneration (at least 5 conduits contained nerve cables) were assessed for their fiber width distribution. All conduit groups contained fewer smaller nerve fibers (2 – 3 μm) compared to isograft controls (Figure 3.4A); furthermore, the High DS group also contained fewer nerve fibers than the isograft in the 3 – 4 μm distribution. All conduit groups demonstrated a higher percentage of larger nerve fibers (4 – 5 μm) compared to the isograft, but only groups with a DS and NGF (ATIII, High or Med DS) promoted a higher percentage of nerve fibers in the 5 – 6 μm width range ($p < 0.05$) compared to the isograft, suggesting more mature regenerating fibers. Overall, the average fiber width for conduit groups with nerve regeneration were greater than isografts (Figure 3.4B). The normal median nerve fiber width of a rat is ~6.5 μm (Mackinnon, Dellon et al. 1991). No differences between groups were observered for the 1-2 μm or the 6-7 μm range, both of which contained a low percentage of fibers in all groups. No functional gains were observed as assessed by Walking Track Analysis with Sciatic Functional Index scoring (data not shown).

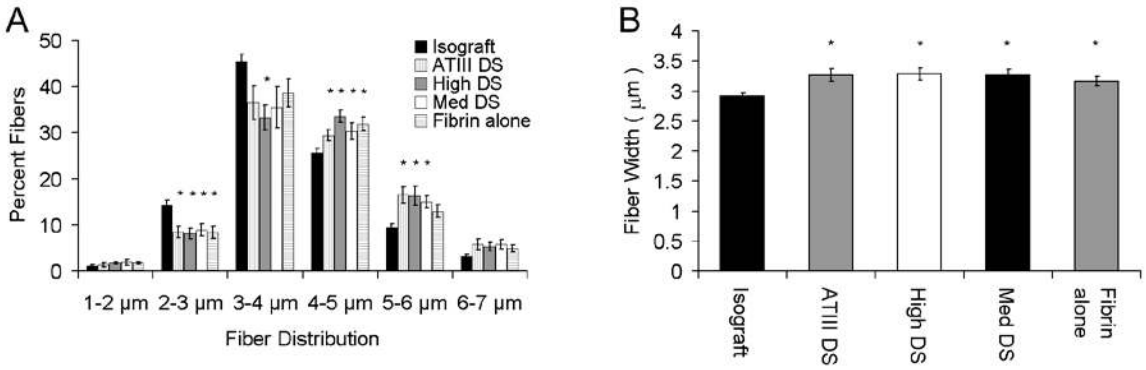

Figure 3.4. Myelinated fiber width distribution of regenerating nerves at the midline of the conduit (or graft). The nerve fiber width distributions (A) and averages (B) were measured by quantitative histomorphometry. The percentage of large regenerating nerve fibers (4 – 5 µm) was larger in the conduit groups compared to the isograft group. The DS with any affinity peptide and NGF promoted the greatest percentage of larger fibers (5 – 6 µm). Overall, average fiber width for conduit groups were greater than isografts. Data represents mean ± S.E.M. and * indicates statistical significance ($p<0.05$) compared to the isograft.

3.5 Discussion

Complete clinical recovery after peripheral nerve injury is rare suggesting that alternatives to the current standards of care are needed. One alternative examined in this study was the use of a NGC filled with a biomaterial matrix capable of delivering growth factors with an affinity-based DS. The affinity-based DS allows the release of growth factor by cells, which activate proteases allowing the degradation of the fibrin matrix and other DS components via the activation of plasminogen to plasmin (Kalderon 1984; Krystosek and Seeds 1984; Alvarez-Buylla and Valinsky 1985; Pittman and Buettner 1989; Pittman, Ivins et al. 1989; Herbert, Bittner et al. 1996).

In this study, we found that drug delivery from a fibrin matrix improved the effectiveness of nerve regeneration (the number of conduits with a nerve cable spanning the defect). A fibrin matrix naturally forms within an empty silicone conduit over a one week

period when the severed nerve ends of rat sciatic nerve are connected to the conduit (Williams, Longo et al. 1983). Therefore, we hypothesize that an exogenous fibrin matrix could facilitate bridging a critical nerve defect by eliminating the need to wait one week for the bridge to form. Fibrin has been used previously as a scaffold to support nerve regeneration within a NGC (Lee, Yu et al. 2003a; Galla, Vedecnik et al. 2004; Marcol, Kotulska et al. 2005) and can promote cell adhesion because it contains binding sites for integrins (Thiagarajan, Rippon et al. 1996) and Schwann cells (Chernousov and Carey 2003). Additionally, NGF is known to facilitate and enhance nerve regeneration (Xu, Yu et al. 2002; Lee, Yu et al. 2003a; Yu and Bellamkonda 2003), therefore, incorporation of NGF into fibrin matrices would likely have a synergistic effect. The use of fibrin was needed to improve the effectiveness of sciatic nerve regeneration. However, the addition of NGF and its method of delivery, whether free or bound in the affinity based DS, also affected its effectiveness in promoting nerve regeneration.

Simple, polymer NGCs can bridge small nerve gaps with high effectiveness; however, nerve regeneration effectiveness decreases beyond a 10 mm defect size (critical defect length) without the addition of other components, such as extracellular matrix molecules, Schwann cells, plasma, or neurotrophic factors to the conduit. Furthermore, these larger defects may require a luminal matrix that utilizes a different method of drug delivery than diffusion or loading free drug for enhanced nerve regeneration. For example, Yu *et al.* found that agarose matrices incorporating laminin or freely-diffusible NGF promoted nerve regeneration across the conduit in only half the animals (Yu and Bellamkonda 2003). When the gap length was increased past the critical defect length, Dodla *et al.* determined that agarose matrices with free or unbound ECM or growth factor were unable to effectively promote sciatic nerve regeneration across a 20 mm defect.

However, anisotropic matrices with concentration gradients of NGF and laminin, were more effective in bridging the gap and enhanced nerve regeneration compared to isotropic matrices (Dodla and Bellamkonda 2008).

Similarly in our study, a critical nerve defect was bridged more effectively with the inclusion of an affinity-based DS sequestering NGF. This result could be due to loss of NGF by diffusion from the conduit when there is no DS in the fibrin matrix, resulting in a limited supply of NGF available to promote nerve regeneration. We previously demonstrated *in vitro* that an affinity-based DS sequestered NGF. NGF release rates were observed to decrease as peptide affinity increased, which increased DRG neurite outgrowth compared to free NGF (Sakiyama-Elbert and Hubbell 2000a; Maxwell, Hicks et al. 2005; Wood and Sakiyama-Elbert 2008).

The use of an affinity-based DS and NGF promoted enhanced regeneration as assessed by one aspect of nerve regeneration, nerve fiber density, and the quality of nerve regeneration, as assessed by percent neural tissue. Both measures were found to be similar to nerve isograft. The DS and NGF did not promote total nerve fiber counts that were similar to the isograft; however, a lower fiber count was likely due to the small cross-sectional area of the regenerating nerve cables that were found in the silicone conduits. This effect of silicone conduits has been noted by others (Williams, Longo et al. 1983; Lloyd, Luginbuhl et al. 2007). Additionally, DS with NGF improved nerve fiber maturity, as assessed by the number of large diameter fibers. Nerve fiber diameter is a commonly used measure of maturity (Aitken, Sharman et al. 1947; Young 1949; Williams and Wendell-Smith 1971; Fraher and Dockery 1998; Lee, Yu et al. 2003a) and larger diameter fibers promote better conduction velocity and return of function (Williams and Wendell-Smith 1971; Fraher

and Dockery 1998). Our findings suggest that although it did not promote an equivalent fiber number, the delivery of NGF from the DS produces more mature nerve fibers and a similar nerve fiber density and neural tissue percentage compared to an isograft.

Other affinity-based drug DSs have incorporated components that reversibly bind the drug of interest, such as heparin within fibrin (Jeon, Kang et al. 2005; Jeon, Ryu et al. 2005), collagen (Wissink, Beernink et al. 2000a), and polymer matrices (Benoit and Anseth 2005; Pike, Cai et al. 2006), but have not specifically looked at how the cellular response is affected by difference in release rates in these DSs *in vivo*. Diffusion-based DSs were found to promote differences in nerve regeneration due to varied release rates of growth factor, and therefore affinity-based DSs could have similar effects due to varied binding affinity leading to different release rates. For example, when GDNF release kinetics are varied from a NGC used to treat a sciatic nerve defect, higher nerve fiber counts but lower functional recovery result with a higher GDNF release rate (Piquilloud, Christen et al. 2007). We have previously examined NGF release from this DS and found that release of NGF from the DS increased myelinated nerve fiber sprouting and outgrowth compared to free NGF and fibrin matrices alone *in vivo* (Lee, Yu et al. 2003a). Therefore, in this study, we focused on the role of heparin-binding peptide affinity on nerve regeneration by utilizing an affinity-based DS. This DS is designed to release growth factors (such as NGF) at different rates by using different peptide sequences to modulate the affinity of the heparin-binding domain (Maxwell, Hicks et al. 2005; Wood and Sakiyama-Elbert 2008). There were no statistical differences in measures of nerve regeneration when peptide affinity was varied, although we noticed a general trend toward increased nerve regeneration with increasing heparin-binding affinity. Specifically, as heparin-binding affinity increased, the efficiency of regeneration and histomorphometric measures generally increased. Previously, the DS demonstrated trends *in*

vitro where as heparin-binding affinity increased, neurite extension generally increased and the ability of the DS to retain NGF directly correlated with increased neurite extension (Wood and Sakiyama-Elbert 2008).

In our study, we used the DS within a silicone NGC. Silicone NGCs possess a degree of biocompatibility, a known critical defect length for the rat sciatic nerve injury model, and are mechanically stable. Clinically, silicone conduits are not ideal and have associated morbidities (Merle, Dellon et al. 1989; Dellon 1994; Battiston, Geuna et al. 2005), therefore, the combination of a drug DS with a biodegradable conduit would be more desirable for clinical peripheral nerve repair. Future studies directed toward this goal would be of benefit in translating our DS into clinical practice. Additionally, future work with this DS will likely focus on the use of the ATIII peptide, since it was found in previous studies to be the most effective at sequestering NGF (Wood and Sakiyama-Elbert 2008) and demonstrated the strongest trends toward enhanced nerve regeneration within this study. In nerve regeneration, the ability of an affinity-based DS to retain NGF appears to be key to enhancing regeneration (Wood and Sakiyama-Elbert 2008). However, other biomaterial applications may see a benefit in an affinity-based DS that is capable of more passive growth factor release with less growth factor sequestered, for example, to signal distant cells for recruitment to the implantation site.

3.6 Conclusions

In summary, the goal of this study was to evaluate the efficiency of our affinity-based DS *in vivo* and determine if the affinity of the heparin-binding peptide in the DS affected

nerve regeneration in a critical defect rat sciatic nerve model. We examined the effect on histological outcomes of NGF delivery and compared it to controls for the DS and to the equivalent of the clinical standard of care, an isograft. Our DS enhanced one aspect of nerve regeneration and the quality of nerve regeneration, as well as nerve fiber maturity and the organization of the regenerating nerve. We did not observe differences in nerve regeneration due to heparin-binding affinity with this DS. We hypothesize delivery of NGF from an affinity-based DS offers a potential future alternative for the treatment of peripheral nerve injuries.

Chapter 4

Controlled release of glial-derived neurotrophic factor from fibrin matrices containing an affinity-based delivery system*

4.1 Abstract

This research evaluated the controlled release of glial-derived neurotrophic factor (GDNF) from an affinity-based delivery system (ABDS) as potential treatment for peripheral nerve injury. The ABDS consisted of a bi-domain peptide containing a transglutaminase substrate, allowing crosslinking into fibrin matrices, and a heparin-binding domain based on the antithrombin-III heparin-binding domain, heparin, and GDNF, which was sequestered based on its heparin-binding affinity. The objective of this research was to determine the release rate and biological activity of GDNF released from the ABDS *in vitro*. The ratio of peptide to heparin was found to modulate the rate of GDNF release. The biological activity of GDNF released from the ABDS was assayed using chick dorsal root ganglia (DRGs) neurite extension. Neurite extension was equivalent for fibrin matrices containing the ABDS for all concentrations of GDNF tested versus DRGs grown with GDNF in the media. Furthermore, neurite extension was enhanced in fibrin matrices containing 100 ng/mL of GDNF with the ABDS versus matrices with GDNF at a simliar dose but no ABDS. These results suggest that GDNF can be retained and released in a biologically activity form from the ABDS, and thus this approach may prove useful for the treatment of peripheral nerve injury.

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4.2 Introduction

Peripheral nerve injury due to a complete transection of the nerve fibers results in loss of function and neuropathic pain (Lundborg 2000). The peripheral nervous system is capable of limited regeneration when the two ends of the severed nerve can be rejoined. In the case of clean transections or small gaps, the ends are typically sutured back together. In longer gaps, suturing would generate excess tension; therefore, the gap is typically bridged by a non-critical donor sensory nerve autograft. Despite significant advances in nerve reconstruction, autografting remains the clinical standard of care for critical long nerve gap repair. However, the functional recovery following autografting remains incomplete, and the resulting donor site morbidity has led investigators to seek alternative therapies (Lundborg 2000; Schmidt and Leach 2003). In particular, a conduit filled with a biomaterial matrix and neurotrophic growth factors has been studied as an alternative repair strategy that could serve to bridge the gap and enhance axonal regeneration (Schmidt and Leach 2003; Bellamkonda 2006).

Drug delivery from biomaterial matrices often involves diffusion-based release, where release is controlled by diffusion of the drug within the pores of the material. Alternatively, affinity-based delivery systems immobilize drug within matrices via non-covalent interactions, controlling release by limiting the fraction of drug in the diffusible form. Of the affinity-based delivery systems studied, many of them utilize heparin, taking advantage of the ability of negatively-charged sulfated groups on heparin to interact with proteins, such as growth factors, typically via exposed basic domains (Mach, Volkin et al. 1993; Mulloy 2005). Some previous methods to incorporate heparin into biomaterial matrices have included heparin-conjugated Sepharose beads encapsulated in alginate

(Edelman, Mathiowitz et al. 1991; Laham, Sellke et al. 1999), heparinized collagen matrices (Wissink, Beernink et al. 2000a; Wissink, Beernink et al. 2000b), and use of poly(ethylene glycol) (PEG) linkers in various forms to covalently incorporate heparin into the system (Benoit and Anseth 2005; Pike, Cai et al. 2006; Tae, Scatena et al. 2006).

Previously, we have developed an affinity-based delivery system incorporating heparin that sequesters proteins within a fibrin matrix using non-covalent interactions (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b). This system contains a bi-domain peptide with one domain consisting of a transglutaminase substrate, based on α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983; Kimura, Tamaki et al. 1985), allowing it to be crosslinked into fibrin matrices during polymerization by the transglutaminase Factor XIIIa. This process leaves the other peptide domain free to interact with heparin, via a modified version of the antithrombin III-heparin binding domain (Tyler-Cross, Sobel et al. 1994; Tyler-Cross, Sobel et al. 1996; Sakiyama, Schense et al. 1999). With the inclusion of a heparin-binding protein (e.g. heparin-binding growth factor), a bound ternary complex within the fibrin matrix is formed consisting of peptide, heparin, and protein, effectively sequestering the protein within the fibrin matrix leaving it available for cell-mediated processes.

This delivery system has been employed using a variety of growth factors that interact non-covalently with heparin, such as platelet-derived growth factor-BB (PDGF-BB) (Gelberman, Thomopoulos et al. 2007), neurotrophin-3 (NT-3) (Taylor, McDonald et al. 2004; Taylor and Sakiyama-Elbert 2006), and nerve growth factor (NGF) (Sakiyama-Elbert and Hubbell 2000a; Lee, Yu et al. 2003a; Wood and Sakiyama-Elbert 2008) for a variety of potential treatment applications, which include flexor tendon repair (Gelberman,

Thomopoulos et al. 2007), spinal cord injury (Taylor, McDonald et al. 2004; Taylor and Sakiyama-Elbert 2006), and peripheral nerve injury (Lee, Yu et al. 2003b). In particular for the potential treatment of peripheral nerve injury, release of NGF has been characterized with this delivery system (Sakiyama-Elbert and Hubbell 2000a; Lee, Yu et al. 2003a; Wood and Sakiyama-Elbert 2008). However, NGF has been found to promote sensory neuronal survival and recovery, and motor neurons do not express or upregulate NGF or its receptors after injury (Boyd and Gordon 2003). Therefore, additional growth factors that target motor neurons are desired for use in this delivery system.

Glial-derived neurotrophic factor (GDNF) has been implicated as a potential therapeutic agent for peripheral nerve regeneration. GDNF was initially identified for its ability to promote the survival of dopaminergic neurons (Lin, Doherty et al. 1993), but following peripheral nerve injury its expression is upregulated in Schwann cells (Hoke, Gordon et al. 2002; Boyd and Gordon 2003; Zhao, Veltri et al. 2004), while its receptors are upregulated in motor neurons (Boyd and Gordon 2003). Furthermore, GDNF promotes neurite outgrowth and neuronal survival *in vitro* (Trupp, Ryden et al. 1995; Matheson, Carnahan et al. 1997; Bennett, Michael et al. 1998; Gavazzi, Kumar et al. 1999; Tucker, Rahimtula et al. 2006; Leclere, Norman et al. 2007) and promotes survival of axotomized sciatic neurons *in vivo* (Matheson, Carnahan et al. 1997), making it a good candidate for promoting motor nerve recovery. GDNF is also ideal for controlled delivery from our delivery system because it binds to heparin with moderately high affinity (Rickard, Mummery et al. 2003; Rider 2003; Rider 2006; Silvian, Jin et al. 2006; Alfano, Vora et al. 2007), specifically at a basic amino-acid-rich sequence near the N-terminal to the first cysteine of the transforming growth factor β domain of GDNF (Alfano, Vora et al. 2007).

The focus of this study was to assess the release rate and biological activity of GDNF released from fibrin matrices containing an affinity-based delivery system (ABDS). The release of GDNF was measured over 7 days, where the delivery system component ratios and the ratio of peptide to heparin were modulated to control release. Additionally, neurite extension from chick embryonic dorsal root ganglia (DRGs) was measured to assess the presence of biologically active GDNF.

4.3 Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

4.3.1 Fibrin matrix preparation

Fibrin matrices were prepared as previously described (Schense and Hubbell 1999) by mixing the following components (final concentrations given): human plasminogen-free fibrinogen containing Factor XIII (4.0 mg/mL), bovine thrombin (2 NIH units/mL), and $CaCl_2$ (2.5 mM, Fisher Scientific, Pittsburgh, PA). For the delivery system, a bi-domain peptide (ATIII) based on a modified version of the antithrombin III-heparin binding domain (*(AcG)NQEQVSPK*(βA)FAKLAAR-LYRKA, where AcG denotes N-acetyl-glycine and the tranglutaminase substrate is given in italics) (Tyler-Cross, Sobel et al. 1994; Sakiyama, Schense et al. 1999) was synthesized as described previously (Wood and Sakiyama-Elbert 2008). Briefly, the peptide was synthesized by standard solid phase Fmoc chemistry (amino acids from Nova Biochem, San Diego, CA; peptide synthesis solvents from Applied

Biosystems, Foster City, CA) using an ABI433A peptide synthesizer (Applied Biosystems). After synthesis, the peptide was cleaved from the resin with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane by volume for 2-3 h, filtered through glass wool to remove the resin, and then precipitated in cold diethyl ether. The crude peptide filtrates were dried under vacuum, purified by standard C18 reverse phase liquid chromatography (Shimadzu, Columbia, MD) and lyophilized. The identity of the ATIII peptide was verified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

Fibrin matrices including the delivery system were prepared by incorporating the following additional components: peptide (0.25 mM to obtain ~ 8 moles of peptide cross-linked per mole of fibrinogen (Sakiyama, Schense et al. 1999; Schense and Hubbell 1999)), heparin (Sigma-Aldrich product no. H9399, sodium salt from porcine intestinal mucosa 18,000 average MW) added at 62.5 and 6.25 μM to obtain 4:1 and 40:1 molar ratios of peptide to heparin, respectively, and human GDNF (Peprotech Inc., Rocky Hill, NJ and R&D systems, Minneapolis, MN for the *in vitro* biological activity and release assay, respectively). Fibrin matrices with or without the delivery system were polymerized in 24-well tissue culture plates for 1 h at 37°C and 5% CO_2 yielding 400 μL matrices.

4.3.2 *In vitro* release assay

To measure the release of GDNF, fibrin matrices were prepared as described above then incubated with an aqueous wash consisting of Tris-buffered saline (TBS, 137 mM NaCl, 2.7 mM KCl, 33 mM Tris, pH 7.4, Fisher Scientific) containing 1% bovine serum albumin (BSA) at 37°C. Equilibrium studies were performed where 400 μL washes were added to the matrices and then collected after 48 h, which was previously determined to be sufficient time

for NGF to reach equilibrium between the matrix and wash phases in a similar experiment (Willerth, Johnson et al. 2007). Additionally, a 7-day release study was performed where 1 mL washes were added and collected 5 times in the first 24 h, followed by collection subsequently every 24 h for the next 6 days. For both studies all washes were collected in silconized tubes to reduce GDNF loss and stored at -20°C. Upon completion of the release studies, the remaining GDNF was extracted from the fibrin matrices by cutting them into 1 mm cubes and placing them in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4, Fisher Scientific) containing 0.56 mM heparin, an additional 2 M NaCl (Fisher Scientific), 0.01% Triton-X, and 1% BSA at 4 °C for 48 h, after which the mixture was stored at -20°C until analysis was performed.

The amount of GDNF released and remaining in the fibrin matrices was quantified by an enzyme-linked immunosorption assay (ELISA) for human GDNF according to the manufacturer's instructions (R&D systems). The absorbance was read at 450 nm with an optical subtraction at 650 nm using a multi-well plate spectrophotometer (MultiSkan RC, Labsystems), and sample concentrations were calculated from a standard curve of known GDNF concentrations. Control matrices were made by omitting the peptide, heparin, and/or GDNF from the fibrin matrices.

4.3.3 *In vitro* biological activity assay

Fibrin matrices were prepared as described above then washed 5 times in the first 24 h with 1 mL of TBS for the first 4 washes and the last wash consisting of 1 mL of modified neurobasal media (10 ng/mL of GDNF added for the positive control, see below). Modified neurobasal media (NBM, Invitrogen, Carlsbad, CA) consisted of the following:

insulin (5 g/mL), transferrin (100 g/mL), progesterone (6.4 ng/mL), putrescine (16.11 g/mL), selenite (5.2 ng/mL), and BSA (0.1%). DRGs were dissected from day-10 white leghorn chick embryos (Specific Pathogen Free, Sunrise Farms, Catskill, NY). One DRG was implanted per matrix using dissection forceps and allowed to adhere to the fibrin matrix for 1 h at 37°C and 5% CO_2. One mL of modified NBM was added after 1 h and left on the fibrin matrices for the remainder of the experiment. The concentrations of peptide, heparin, and GDNF and their corresponding molar ratios used in the delivery system for experiments are contained in Table 4.1.

Table 4.1: Concentrations of delivery system components employed for *in vitro* biological activity assay

GDNF (ng/mL)	**Peptide (mM)**	**Heparin (μM)**	**Molar ratio of peptide : heparin**	**Molar ratio of heparin : GDNF**
10	0.25	62.5	4	200000
25	0.25	62.5	4	80000
100	0.25	62.5	4	20000
100	0.25	6.25	40	2000
250	0.25	62.5	4	8000
500	0.25	62.5	4	4000

DRGs were allowed to grow and extend neurites for 48 h upon which images were captured. Brightfield images with a 2x objective using a CCD camera (Magnifire, Olympus) were collected and analyzed using Image-Pro Express software (MediaCybernetics, San Diego, CA) to determine the average neurite extension. The average neurite extension was calculated as the radius of an annulus between the DRG body and the outer halo of extending neurites, as described previously (Herbert, Bittner et al. 1996). All average neurite

extension was normalized to the average neurite extension of a positive control consisting of DRGs grown in 10 ng/mL of GDNF in the media (dose resulting in largest neurite extension for soluble GDNF doses tested) from each experiment. Additional control matrices were made by omitting the GDNF and/or peptide and heparin from the fibrin matrices.

4.3.4 Immunocytochemistry

DRGs were mechanically dissociated, seeded to fibrinogen-coated 24 well plates (4.0 mg/mL) and given neurobasal media supplemented with 10% fetal bovine serum (Invitrogen) and 10 ng/mL GDNF, and allowed to extend neurites for 24 h. The cells were fixed in 2% formalin (pH 7.4) in PBS for 1 h at 37°C, permeabilized with 0.1% Triton X-100 and blocked with 3% BSA in PBS. These cultures were incubated with GDNF receptor α-1 (GFRα-1) primary antibody (1 μg/mL, Neuromics, Edina, MN) at 4°C for 24 h, followed by Alexa Fluor 488 tagged secondary antibody (1 μg/mL, Invitrogen) detection for 2 h. Fluorescence light microscopy images were captured using a CCD camera, where DRGs with only the addition of the secondary antibody served as a threshold control to remove excess background staining.

4.3.5 Statistical analysis

Statistical analysis was performed using Statistica (Statsoft, Tulsa, OK) with comparative analysis using Scheffe's F post-hoc test by analysis of variance at a 95% confidence interval ($\alpha = 0.05$). The release assays were performed with 3 matrices per

replicate; the biological activity study was performed with 6 matrices per replicate. All studies were performed in at least triplicate, and reported values are given as mean ± standard deviation (S.D.).

4.4 Results

4.4.1 *In vitro* release assay

To understand how the delivery system would release GDNF over time, the system was analyzed for its potential to bind GDNF. The ability of the delivery system to sequester GDNF was assessed by incubating fibrin matrices (with or without delivery system) containing GDNF with an equal volume of wash buffer and allowing the system to come to equilibrium for 48 h (previously found to be sufficient time for growth factor concentrations to reach equilibrium between the aqueous media and matrix (Willerth, Johnson et al. 2007)). The amount of GDNF present within the matrices and the aqueous wash media was measured to determine the concentration of free and matrix bound GDNF by assuming that the concentration of free GDNF was the same in both phases. The amount of GDNF present in fibrin matrices and their aqueous media without the complete delivery system (fibrin alone, GDNF with peptide but no heparin, or GDNF with heparin but no peptide) was equal to ~50% of the GDNF initially added to the matrix, demonstrating equal partitioning of GDNF in the absence of delivery system (Figure 4.1). However, fibrin matrices with the complete delivery system at both 4:1 and 40:1 molar ratios of peptide to heparin retained more GDNF compared to matrices with no delivery system (62 ± 5% and 66 ± 1%, respectively versus ~50%).

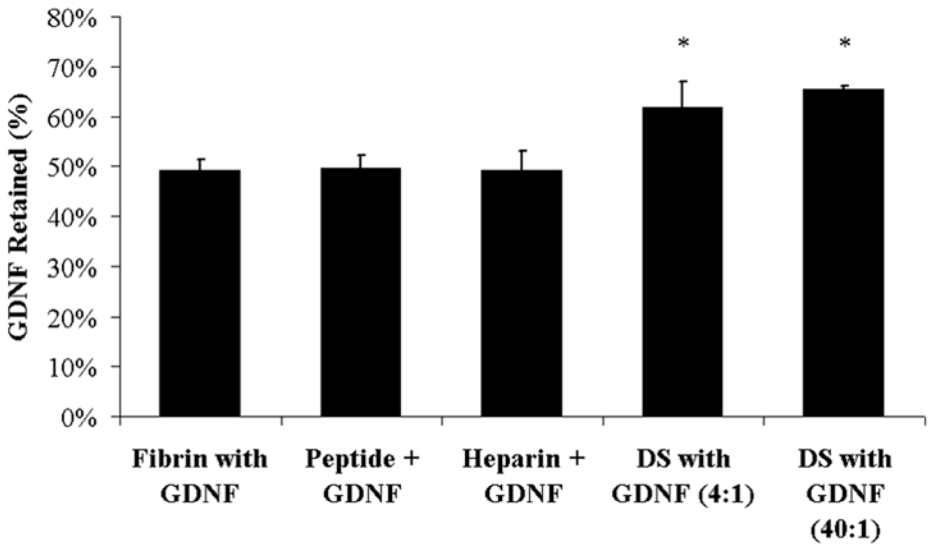

Figure 4.1. GDNF retention *in vitro* at 48 h is enhanced by the delivery system. GDNF was released from fibrin matrices with or without delivery system (DS) over 48 h to an equal volume of aqueous media to assess how much GDNF was retained in the fibrin matrices. The delivery system at either molar ratio of peptide to heparin enhanced GDNF retention in the fibrin matrices, while the inclusion of GDNF with peptide or heparin alone did not affect the retention. Data ($n \geq 3$) represented by mean ± SD where the parentheses contains the peptide to heparin molar ratio. Statistical significance was considered $p<0.05$ compared to fibrin matrix alone or fibrin matrix with either peptide or heparin.

The dynamic release of GDNF from fibrin matrices over 7 days was assessed by measuring the concentration in aqueous wash media collected 5 times in the first 24 h and once every 24 h for the following 6 days. GDNF was released rapidly from matrices lacking the complete delivery system (matrices containing only GDNF, GDNF and heparin, or GDNF and peptide), with 27-30% released in the initial burst and 79-85% released after 24 h (Figure 4.2). By day 3 all groups lacking the complete delivery system had released 90% of their initial GDNF, and at the end of the study (day 7) these matrices retained little GDNF (< 3%).

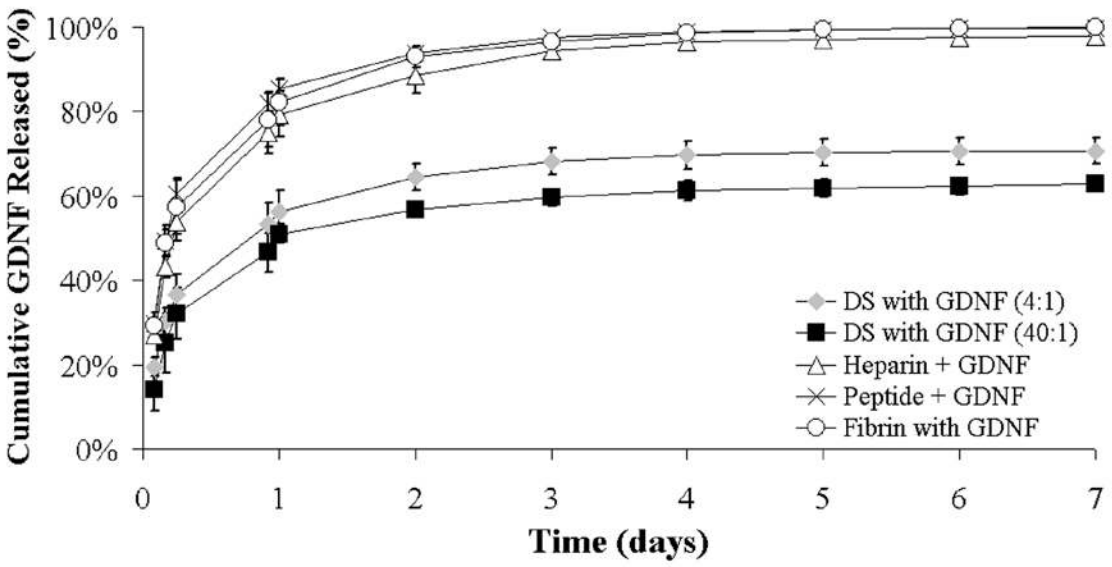

Figure 4.2. GDNF release *in vitro* over 7 days is controlled by the ratio of peptide to heparin in the delivery system. GDNF was released over 7 days from fibrin matrices with and without the delivery system (DS) at various molar ratios of peptide to heparin. At the either molar ratio, the delivery system retained a greater fraction of GDNF over all 7 days compared to no delivery system. Additionally, at the 40:1 molar ratio, a greater fraction of GDNF was retained after the initial 24 h until the end of the release at 7 days. GDNF with either peptide or heparin alone did not slow the release of GDNF. Data ($n \geq 3$) represented by mean ± SD where the parentheses contains the peptide to heparin molar ratio, and statistical significance was considered $p<0.05$.

The presence of the complete delivery system slowed the release of GDNF and resulted in the retention of a greater fraction of GDNF over 7 days. The initial burst of GDNF from matrices with the delivery system during the first 24 h was reduced compared to matrices lacking the complete delivery system, while 19 ± 2% and 14 ± 5% were released for 4:1 and 40:1 peptide to heparin molar ratios, respectively. After 24 h, the matrices containing the complete delivery system retained more GDNF than controls again with only 56 ± 5% and 51 ± 2% GDNF released (4:1 and 40:1 molar ratios respectively). By day 4 both ratios released less than 2% of the total GDNF per day demonstrating a relatively stable state of retention, and at day 7 matrices with the delivery system had retained 29 ± 3% for a 4:1 ratio and 37 ± 2% for a 40:1 ratio. Additionally, the molar ratio of peptide to

heparin in the delivery system affected the fraction of GDNF retained. The 40:1 ratio released less GDNF than the 4:1 ratio after 24 h, which was sustained for the remainder of the experiment.

4.4.2 *In vitro* biological activity assay

To assess the feasibility of DRGs for the biological activity assay, DRGs in fibrin matrices were stained for the primary ligand-binding subunit (GDNF receptor α-1), which elicits signaling through its linked partner subunit, c-Ret receptor tyrosine kinase (Baloh, Enomoto et al. 2000). While DRGs contain a majority of sensory neurons, a population of the total neurons are responsive to GDNF (Trupp, Ryden et al. 1995; Matheson, Carnahan et al. 1997; Bennett, Michael et al. 1998; Tucker, Rahimtula et al. 2006), where DRG neurite extension can be measured from treatment with GDNF (Gavazzi, Kumar et al. 1999; Fine, Decosterd et al. 2002; Tucker, Rahimtula et al. 2006; Leclere, Norman et al. 2007). Dissociated DRGs demonstrated robust staining for GDNF receptor α-1 (Figure 4.3), validating their use and sensitivity for quantitative assessment of neurite extension in response to GDNF delivered from our delivery system.

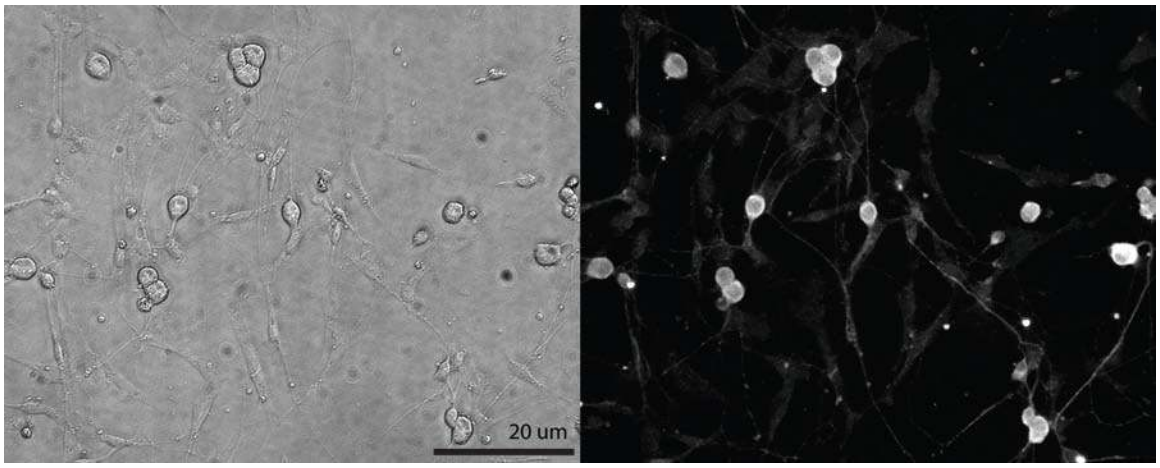

Figure 4.3. GDNF receptor α-1 is expressed by chick embryo DRGs. Dissociated DRGs plated on fibrinogen-coated wells demonstrated robust staining for GDNF receptor α-1. While the biological activity assay utilized whole DRGs implanted into 3-D fibrin matrices, this staining validates their use for quantitative assessment of neurite extension in response to GDNF delivered from our delivery system.

In order to assess the biological activity of GDNF released from the affinity-based delivery system, embryonic chick DRGs were implanted into fibrin matrices with and without the delivery system that had been previously washed 5 times over 24 h (analogous to the first day of the *in vitro* release assay). The average neurite extension was measured after 48 h and was normalized to neurite extension from DRGs grown in unmodified fibrin matrices with GDNF in the culture media at a dose of 10 ng/mL (Figure 4.4). This dose promoted the greatest level of neurite extension (optimal dose) from comparison to other doses ranging from 1 – 100 ng/mL of GDNF. Additionally, ranges of concentrations of GDNF were tested at a single peptide to heparin molar ratio (4:1) to identify an optimal dose (Figure 4.5). At this optimal dose, multiple delivery system formulations were tested by varying the peptide to heparin molar ratio (through altering the heparin concentration) in the delivery system to determine if the delivery system component ratio modulated biological activity.

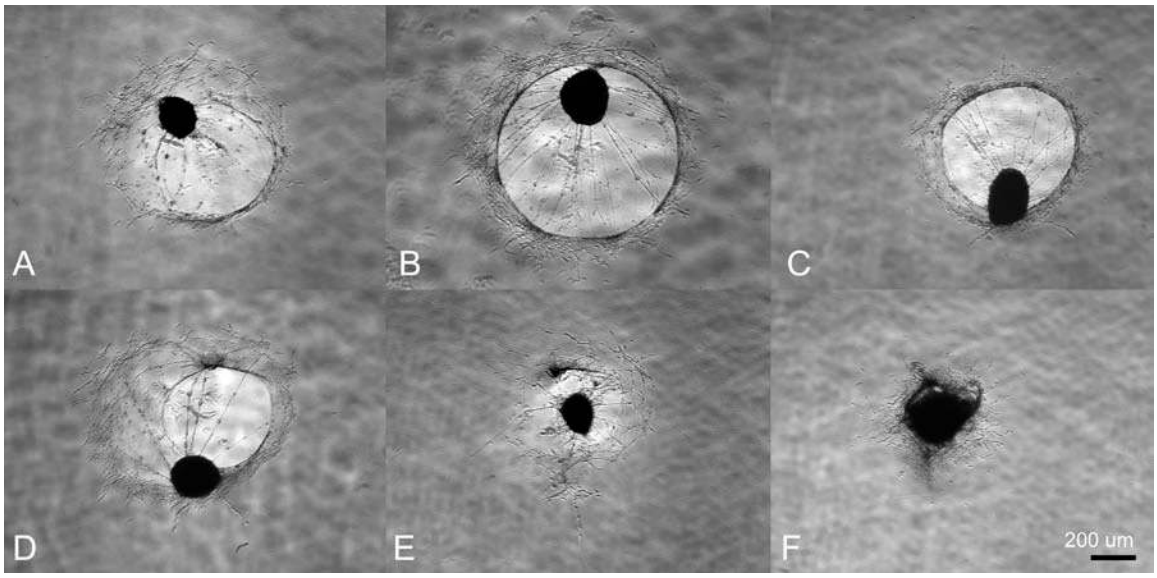

Figure 4.4. Photomicrographs of DRG cultured containing different GDNF concentrations with and without the delivery system. A) 10 ng/mL GDNF in the culture media, B) 100 ng/mL GDNF with the delivery system at a 4:1 molar ratio of peptide to heparin, C) 100 ng/mL GDNF without the delivery system, D) 250 ng/mL GDNF without the delivery system, E) 10 ng/mL GDNF with the delivery system, F) fibrin matrix with no GDNF added. These results represent sample images demonstrating DRG neurite extension resulting from the variations in the concentration of GDNF used in the assay.

In the absence of GDNF (with or without heparin and peptide) robust neurite extension was not observed, and neurite extension was significantly decreased from the positive control by ~75%. Normalized neurite extension through fibrin matrices containing 10 ng/mL of GDNF with the delivery system was similar to 10 ng/mL of GDNF in the media (positive control), while the 10 ng/mL of GDNF in fibrin matrices lacking the delivery system (GDNF and fibrin alone) resulted in decreased neurite extension versus the positive control (Figure 4.5). At other doses tested (25, 100, 250, and 500 ng/mL in fibrin matrices) neurite extension was similar to the positive control for both matrices containing the delivery system and matrices lacking the delivery system. However, at a GDNF dose of 100 ng/mL, the presence of the delivery system resulted in enhanced normalized neurite extension compared to fibrin matrices without the delivery system at this same dose.

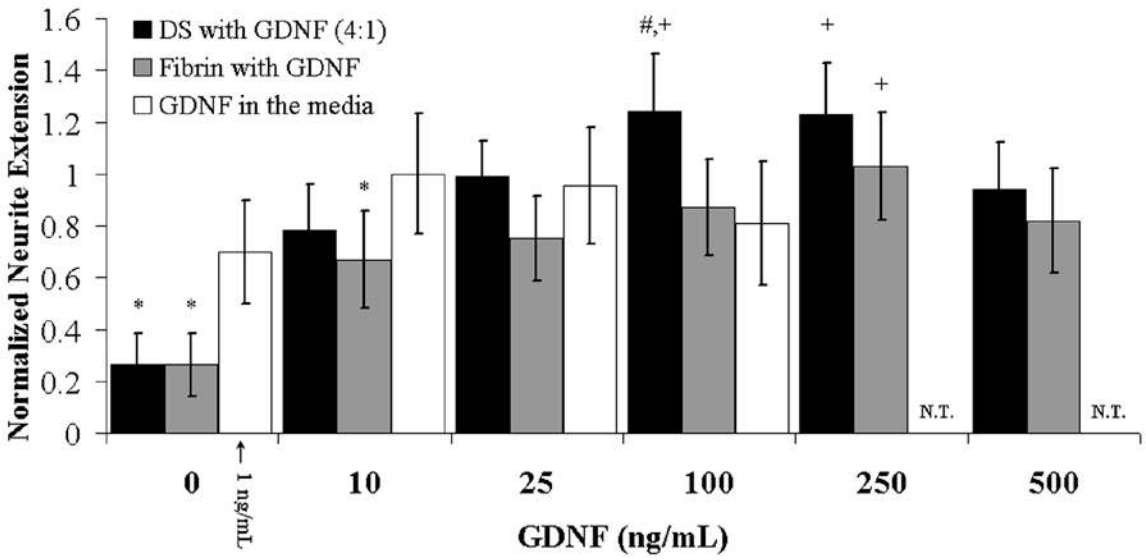

Figure 4.5. Effect of GDNF concentration on DRG neurite extension *in vitro*. Neurite extension from chick DRGs was affected by the concentration of GDNF in fibrin matrices with (DS with GDNF 4:1) or without the delivery system (GDNF alone). The delivery system at a peptide to heparin molar ratio of 4:1 at any concentration of GDNF promoted neurite extension similar to GDNF in the media at 10 ng/mL (optimal dose), while the lack of delivery system resulted in decreased neurite extension at the lowest GDNF concentration (10 ng/mL). The delivery system with 100 ng/mL of GDNF promoted enhanced neurite extension compared to GDNF at the same dose with no delivery system. Additionally, neurite extension was biphasic in response to the GDNF concentration both with and without the delivery system. All DRG neurite extension was normalized to the average neurite extension for 10 ng/mL GDNF in the media for the same experiment. Data ($n \geq 18$) represents mean ± S.D., N.T. indicates a dose of GDNF in the media that was not tested, * indicates statistical significance ($p<0.05$) compared to 10 ng/mL GDNF in the media, # indicates statistical significance ($p<0.05$) compared to GDNF alone, and + indicates statistical significance ($p<0.05$) compared to 10 ng/mL GDNF under similar conditions (with/without the delivery system).

A biphasic response was exhibited where neurite extension increased or decreased depending on the concentration of GDNF. At doses of 100 and 250 ng/mL of GDNF, neurite extension increased compared to a dose of 10 ng/mL of GDNF with the delivery system present, while 25 and 500 ng/mL of GDNF resulted in neurite extension similar to 10 ng/mL of GDNF. Also, when the delivery system was not present, all doses but 250 ng/mL of GDNF resulted in neurite extension comparable to a dose of 10 ng/mL of GDNF, where neurite extension was increased at 250 ng/mL of GDNF.

Based on these results, it was determined that at 100 ng/mL the delivery system at a peptide to heparin molar ratio of 4:1 enhanced normalized neurite extension compared to the matrices containing a similar dose of GDNF without the delivery system. Therefore, at this optimal dose the effect of the molar ratio of peptide to heparin was examined, and the effect of individual delivery system components was examined in a systematic manner to assess their contribution to the biological response. The use of the delivery system at a 40:1 peptide to heparin molar ratio resulted in enhanced normalized neurite extension compared to GDNF in the media at an optimal dose (10 ng/mL) by 32 ± 26% but was not different from the response of the delivery system at a ratio of 4:1 (Figure 4.6). The elimination of components of the delivery system resulted in similar neurite extension as was found from fibrin matrices with GDNF alone, which were again decreased versus the complete the delivery system.

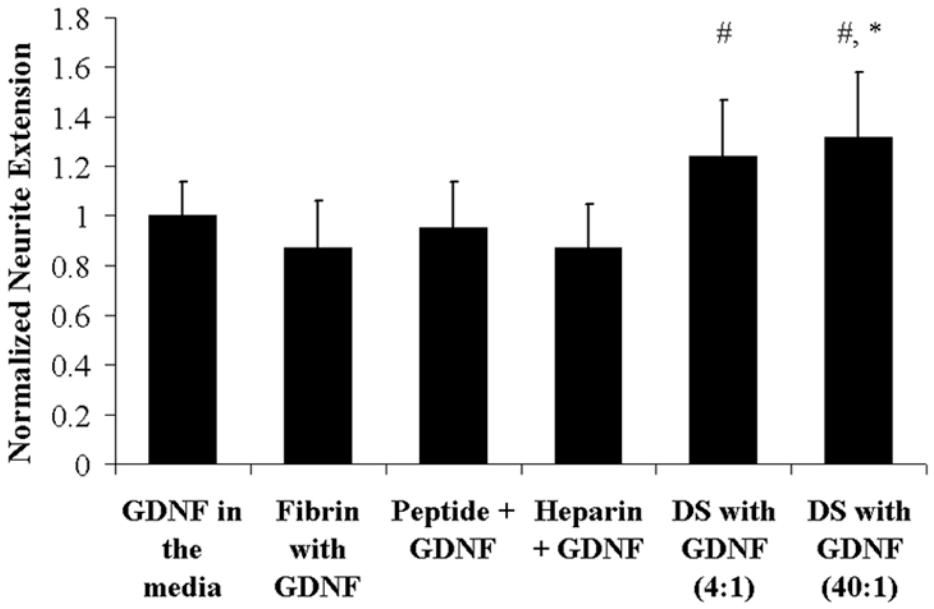

Figure 4.6. Delivery system components affect DRG neurite extension *in vitro*. Neurite extension from chick DRGs was affected by the molar ratio of peptide to heparin at 100 ng/mL of GDNF. The delivery system at a peptide to heparin molar ratio of 40:1 promoted enhanced neurite extension compared to 10 ng/mL GDNF in the media. Additionally, the entire delivery system was required to increase normalized neurite extension. All DRG neurite extension was normalized to the average neurite extension for unmodified fibrin matrices with 10 ng/mL of GDNF added to the media for the same experiment. Data ($n \geq 18$) represents mean ± S.D., # indicates statistical significance ($p<0.05$) compared to the GDNF with no delivery system or with an incomplete delivery system, and * indicates statistical significance ($p<0.05$) compared to 10 ng/mL GDNF in the media.

4.5 Discussion

This study utilized an affinity-based delivery system to sequester GDNF and control its release from fibrin matrices. Non-covalent interactions served to sequester the growth factor, limiting the fraction of GDNF in the diffusible form, thus slowing its diffusion from fibrin matrices. In cases of slow diffusion-based release, cell-mediated processes, such as neurite outgrowth induced protease activation (e.g. plasmin), could potentially serve as alternative mechanisms for regulating growth factor release from fibrin matrices. GDNF has been administered locally at the site of injury via passive diffusion (Yan, Matheson et al.

1995; Barras, Pasche et al. 2002; Fine, Decosterd et al. 2002) and systemically via subcutaneous injections (Yan, Matheson et al. 1995), both resulting in the rescue of motor neuron cell bodies from death. Furthermore, when GDNF release kinetics from a nerve guidance conduit are varied, greater nerve fiber counts but slower functional recovery result for higher rates of delivery (Piquilloud, Christen et al. 2007); therefore, it is still unclear which route is the best method for delivery. The affinity-based delivery system releases growth factor via a cell-mediated manner. Specifically, as neurites from the neuron extend their processes within the fibrin, proteases are activated by the growth cone allowing the breakdown of fibrin and other delivery system components. For example, the growth cone is known to facilitate the activation of plasminogen to plasmin, which can cleave fibrin into smaller fragments (Pittman and Buettner 1989; Pittman, Ivins et al. 1989; Herbert, Bittner et al. 1996), and cells releasing heparinase have been shown to liberate heparin-binding growth factors from extracellular matrix (ECM) (Vlodavsky, Fuks et al. 1991). In order to retain growth factor in the matrix and available for cell-mediated release, the ratio of delivery system components can be modulated to slow the passive diffusion-based release of growth factor from the matrices, as evidenced by the increased fraction of GDNF found in the matrix at 7 days with the delivery system versus without delivery system.

As the molar ratio of the peptide to heparin increased from 4:1 to 40:1, less GDNF was released over 7 days from fibrin matrices with the delivery system. Previously, when this delivery system was utilized to delivery neurotrophin-3 (NT-3), a heparin to growth factor molar ratio of 800:1 was more effective in slowing the release of growth factor versus a ratio of 8000:1 (Taylor, McDonald et al. 2004). These ratios correspond to approximate peptide to heparin ratios of 40:1 and 4:1 for the 800:1 and 8000:1 ratios, respectively (see Table 4.1). This previous result with NT-3 agrees well with release data from our present experiments

with a different growth factor, GDNF, used in the same delivery system. While the heparin to growth factor ratios differ between this previous result and the present data (chosen based on the relative affinity of the growth factors for heparin), the concentrations of heparin and peptide used in both were similar and previous mathematical modeling (Wood and Sakiyama-Elbert 2008) predicts that heparin to growth factor ratios above 100:1 would contain a vast majority of heparin-growth factor binary complexes. Therefore, the differences in the ratios of heparin to growth factor are not as important as the ratios of peptide to heparin, which were similar between both results. Additionally, mathematical modeling of this delivery system at a state of binding equilibrium between peptide, heparin, and growth factor within the fibrin matrix utilizing the growth factors NT-3 (Taylor, McDonald et al. 2004) and NGF (Wood and Sakiyama-Elbert 2008) found a range of delivery system component ratios that maximized bound growth factor within the matrices. The molar ratios tested in this study for the growth factor GDNF predict a dissociation constant (K_D) on the order of $1 – 3 \times 10^{-7}$ M (Wood and Sakiyama-Elbert 2008), which is within the range predicted by others studying GDNF's affinity for heparin ($\sim 1 \times 10^{-7}$ M (Rickard, Mummery et al. 2003; Rider 2003; Rider 2006)). This K_D would place both peptide to heparin molar ratios used in this study within the range of delivery components that would maximize bound growth factor in a ternary complex within the fibrin matrix (Wood and Sakiyama-Elbert 2008). However, a molar ratio of 4:1 is closer to the range of suboptimal equilibrium binding which could result in more release due to unbound heparin interacting with GDNF resulting in an unbound heparin-GDNF complex that would be washed away via passive diffusion.

The delivery system did not have a negative effect on the biological activity of GDNF as evidenced by similar or enhanced neurite extension to GDNF in the cell culture

media. Additionally, the delivery system's ability to retain GDNF strongly influenced GDNF biological activity. The presence of the delivery system provided the ability to retain more GDNF in the fibrin matrices after washing resulting in better neurite extension compared to GDNF loaded in the fibrin matrices without the delivery system for certain doses. Moreover, when individual components of the delivery system were eliminated at a dose of 100 ng/mL of GDNF, less GDNF was retained and neurite extension was again decreased compared to the complete delivery system. Furthermore, at this dose the delivery system was capable of enhancing the biological activity of GDNF over the positive control by modulating the number of binding sites for peptide and growth factor or concentration of delivery system components, represented by the molar ratio of peptide to heparin.

Previously, others have found that localized delivery of neurotrophic factors can enhance neurite extension more than neurotrophic factors in the media (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b; Taylor, McDonald et al. 2004). In this study, enhanced neurite extension compared to GDNF in the media was observed for a peptide to heparin molar ratio of 40:1 but not for 4:1. This increase could be due to a decrease in GDNF released from the system at a peptide to heparin molar ratio of 40:1 compared to a ratio of 4:1. Previously it was determined that the ability of this delivery system to retain mass was crucial in enhancing the biological activity of DRGs in response to the growth factor NGF (Wood and Sakiyama-Elbert 2008). Our results agree with this since at 24 h both ratios retained similar mass, but after 24 h the 40:1 ratio retained more GDNF than the 4:1 ratio.

Additionally, the results from the biological activity assay suggested a biphasic response of DRGs to GDNF, where at a low dose of GDNF (10 ng/mL) neurite extension

was decreased from higher doses of GDNF, both with and without the delivery system. At doses higher than those doses that maximized neurite extension there was not an increase in neurite extension, but instead a decrease in neurite extension from these optimal doses resulting in neurite extension similar to a low dose of GDNF (10 ng/mL), indicative of a saturation response. Biphasic behavior has been demonstrated *in vitro* for neurotrophic growth factors administered in the media alone (Conti, Fischer et al. 1997) and when it is sequestered in a biomaterial matrix (Sakiyama-Elbert and Hubbell 2000a; Maxwell, Hicks et al. 2005; Willerth, Johnson et al. 2007), similar to our results. The response of DRGs to these doses is useful when considering potential doses for *in vivo* applications towards treatment of peripheral nerve injury, where an effective dose that encourages regeneration is desired.

The ability of the delivery system to present biologically active GDNF *in vitro* suggests it may be useful for the treatment of nerve injury *in vivo*. In peripheral nerve regeneration, when a guidance conduit is placed between the distal and proximal nerve stumps at the injury site, a fibrin bridge naturally forms between the separated nerve segments serving as a scaffold for the regeneration process (Williams, Longo et al. 1983). Therefore, a delivery system filling the lumen of a nerve guidance conduit incorporating both native ECM and beneficial neurotrophic factors could promote peripheral nerve regeneration. In addition, this delivery system allows the exploration of factors affecting local delivery of growth factors that influence regeneration. Sequestering NGF within this biomaterial matrix promoted better regeneration than free NGF in the matrix at a similar dose (Lee, Yu et al. 2003b). As mentioned above, less is known about the optimal delivery method for GDNF, but others have seen promising results when it is delivered slowly during the regeneration process. For example, transected sciatic nerves repaired with neural

conduits that slowly released GDNF supported better axonal regeneration, a greater number of myelinated axons and higher numbers of retrograde-labeled motorneurons than untreated or NGF-treated conduits (Fine, Decosterd et al. 2002). Furthermore, when the release of GDNF from a nerve guidance conduit was varied, the resulting regeneration was dependent on the rate of delivery (Piquilloud, Christen et al. 2007). Therefore, future studies will focus on the delivery of GDNF utilizing this heparin-based delivery system *in vivo* for the potential treatment of peripheral nerve injury to investigate the role of cell-mediated release in the regeneration process.

Chapter 5

Affinity-based Release of Glial-Derived Neurotrophic Factor from Fibrin Matrices Enhances Sciatic Nerve Regeneration*

5.1 Abstract

Glial-derived neurotrophic factor (GDNF) promotes both sensory and motor neuron survival. The delivery of GDNF to the peripheral nervous system has been shown to enhance regeneration following injury. In this study we evaluated the effect of affinity-based delivery of GDNF from a fibrin matrix in a nerve guidance conduit on nerve regeneration in a 13 mm rat sciatic nerve defect. Seven experimental groups were evaluated consisting of GDNF or nerve growth factor (NGF) with the delivery system within the conduit, control groups excluding one or more components of the delivery system, and nerve isografts. Nerves were harvested 6 weeks after treatment for analysis by histomorphometry and electron microscopy. The use of the delivery system (DS) with either GDNF or NGF resulted in a higher frequency of nerve regeneration versus control groups, as evidenced by a neural structure spanning the 13 mm gap. The GDNF DS and NGF DS groups were also similar to the nerve isograft group in measures of nerve fiber density, percent neural tissue, and myelinated area measurements, but not in terms of total fiber counts. In addition, both groups contained a significantly greater percentage of larger diameter fibers with GDNF DS having the largest in comparison to all groups, suggesting more mature neural content. The delivery of GDNF via the affinity-based delivery system

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can enhance peripheral nerve regeneration through a silicone conduit across a critical nerve gap and offers insight into potential future alternatives to the treatment of peripheral nerve injuries.

5.2 Introduction

Despite recent advances in the understanding of peripheral nerve injury and regeneration, functional outcomes are still suboptimal. In nerve transection injuries, the current standard of care is a primary end to end repair. In nerve gap injuries, when tension precludes a primary repair, an autograft is used to provide a scaffold for the regenerating nerve. This procedure, however, has limitations due to donor site availability and morbidity (Meek and Coert 2002; Chen, Zhang et al. 2006) . One alternative to autografting is the use of a nerve guidance conduit (NGC). NGCs facilitate bridging the gap between a proximal and distal nerve, protect regenerating axons from infiltrating scar tissue, and allow the microenvironment of the regenerating nerve to be manipulated by controlling biochemical and physical contents (Meek and Coert 2002; Battiston, Geuna et al. 2005).

A variety of materials have been investigated for use as scaffolds to fill the lumen of a NGC, including the extracellular matrix proteins collagen (Chamberlain, Yannas et al. 1998; Labrador, Buti et al. 1998), fibronectin (Chen, Hsieh et al. 2000), and laminin (Labrador, Buti et al. 1998) as well as naturally-derived matrices such as agarose (Labrador, Buti et al. 1995; Yu and Bellamkonda 2003) and alginate (Ohta, Suzuki et al. 2004; Mohanna, Terenghi et al. 2005). Fibrin also has been used as a biomaterial scaffold to support neural regeneration within a NGC (Lee, Yu et al. 2003a; Galla, Vedecnik et al. 2004; Marcol,

Kotulska et al. 2005) and may offer an advantage over other materials because it naturally forms within an empty silicone conduit connecting the damaged ends of rat sciatic nerve (Williams, Longo et al. 1983). Furthermore, fibrin contains sites for cell binding via integrin receptors (Thiagarajan, Rippon et al. 1996), including cell binding sites for Schwann cells (Chernousov and Carey 2003) that may facilitate cellular migration.

Numerous drug delivery methods have also been used with NGCs (Barras, Pasche et al. 2002; Fine, Decosterd et al. 2002; Whittlesey and Shea 2004; Piquilloud, Christen et al. 2007; Dodla and Bellamkonda 2008; Pfister, Alther et al. 2008). However, diffusion-based release of growth factors from degradable polymers is the most common delivery method (Barras, Pasche et al. 2002; Fine, Decosterd et al. 2002; Burdick, Ward et al. 2006). One shortcoming of this approach is that the release rate cannot be modulated or controlled by cells during regeneration. One alternative is to use an *affinity-based* delivery system (DS) that allows the release of growth factors to be controlled by cell-based degradation of the delivery system (Sakiyama-Elbert and Hubbell 2000a). Our lab has developed an affinity-based delivery system that sequesters heparin-binding proteins within a fibrin matrix using non-covalent interactions (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b). This system contains a bi-domain peptide containing a transglutaminase substrate domain and a heparin-binding domain. Based on the α_2-plasmin inhibitor substrate (Ichinose, Tamaki et al. 1983; Kimura, Tamaki et al. 1985), the peptide is able to crosslink into the fibrin matrix during polymerization via the transglutaminase activity of Factor XIIIa, leaving the other domain free to interact (Sakiyama, Schense et al. 1999; Schense and Hubbell 1999). This heparin-binding domain has the capability to sequester various neurotrophic factors due to their ability to bind to heparin via the sulfated domains on the heparin (Yamada 1983). This delivery system has been used with a variety of growth factors

in many potential treatment applications (Sakiyama-Elbert and Hubbell 2000a; Lee, Yu et al. 2003a; Taylor, McDonald et al. 2004; Taylor and Sakiyama-Elbert 2006; Gelberman, Thomopoulos et al. 2007; Wood and Sakiyama-Elbert 2008). Specifically, we have characterized the effect of affinity-based delivery of nerve growth factor (NGF) on peripheral nerve regeneration (Lee, Yu et al. 2003a).

Glial-derived neurotrophic factor (GDNF) has shown promise in the treatment of peripheral nerve injuries. While GDNF is has been found to promote the survival of both sensory and motor neurons, multiple studies report it to be the most potent motor neuron trophic and survival factor (Henderson, Camu et al. 1993; Li, Wu et al. 1995; Oppenheim, Houenou et al. 1995; Yan, Matheson et al. 1995; Oppenheim, Houenou et al. 2000; Hoke, Gordon et al. 2002). GDNF expression in peripheral nerves is also upregulated significantly in the distal stump of injured sciatic nerve, as well as in the corresponding muscle (Trupp, Ryden et al. 1995; Naveilhan, ElShamy et al. 1997). Given the ability of GDNF to enhance peripheral nerve regeneration (Barras, Pasche et al. 2002; Fine, Decosterd et al. 2002; Piquilloud, Christen et al. 2007), we chose to examine controlled delivery of GDNF from our affinity-based delivery system *in vitro* and found that GDNF could be retained and released from the delivery system in a biologically active form (Wood, Borschel et al. 2009).

In the present study, we evaluated the effects of controlled release of GDNF from a fibrin matrix containing our affinity-based delivery system within a NGC on nerve regeneration *in vivo* using a rat sciatic nerve injury model. We included NGF in the current study for comparison to our previous study. We hypothesized that controlled delivery of GDNF would enhance nerve regeneration and have histomorphometric equivalence to a nerve isograft.

5.3 Materials and Methods

5.3.1 Experimental animals

Adult male Lewis rats (Harlan Sprague-Dawley, Indianapolis, IN), each weighing 250-300 g were used in this study. All surgical procedures and peri-operative care measures were performed in strict accordance with the National Institutes of Health guidelines and were approved by the Washington University Animal Studies Committee. All animals were housed in a central animal facility, given a rodent diet (PicoLab Rodent Diet 20 #5053, PMI Nutrition International) and water *ad libitum*. After surgical procedures, animals recovered in a warm environment and were closely monitored for 2 hours. Animals were then returned to the animal facility and monitored for weight loss, infection, and other morbidities.

5.3.2 Experimental Design

Eighty-four animals were randomized into seven groups (n = 12) as shown in Table 5.1. An additional six animals served as sciatic nerve isograft donors. In all experimental groups, the sciatic nerve was transected and a 5 mm segment was excised just proximal to the trifurcation of the nerve. The nerve was repaired with a 15 mm silicone conduit containing fibrin matrices with or without delivery system and growth factor. One millimeter of nerve was incorporated into each end of the conduit to create a 13 mm nerve gap, exceeding the "critical gap" of spontaneous rat sciatic regeneration through silicone conduits by 3 mm (Lundborg, Dahlin et al. 1982a; Williams, Longo et al. 1983) (Figure 5.1). Group I served as the untreated control group and received an empty conduit. Group II, III, and IV were additional control groups receiving conduits containing fibrin alone, fibrin with

the delivery system (no growth factor) or fibrin with the growth factor, but no delivery system. These groups examined the isolated effects of the delivery system components. The remaining groups (V, VI) were implanted with conduits containing the fibrin matrix containing the delivery system with doses of GDNF or NGF, which were selected based upon *in vitro* DRG dose studies and preliminary data obtained from dose-response pilot studies in the sciatic nerve model (test doses included 25, 50, 100, and 250 ng/mL GDNF with the delivery system). Group VII served as a positive control receiving reversed nerve isografts from syngeneic donor animals.

Table 5.1: Experimental Design

Group	GF dose (ng/ml)	Delivery System (DS)	Fibrin Matrix	Number of rats	Number of rats with neural regeneration
I	0	No	No	12	3
II	0	No	Yes	12	5
III	0	Yes	Yes	12	3
IV	100 – GDNF	No	Yes	12	1
V	100 – GDNF	Yes	Yes	12	6
VI	50 - NGF	Yes	Yes	12	7
VII	Isograft	No	No	12	12

Abbreviations: GF, Growth Factor; GDNF, glial-derived neurotrophic factor; NGF, nerve growth factor.

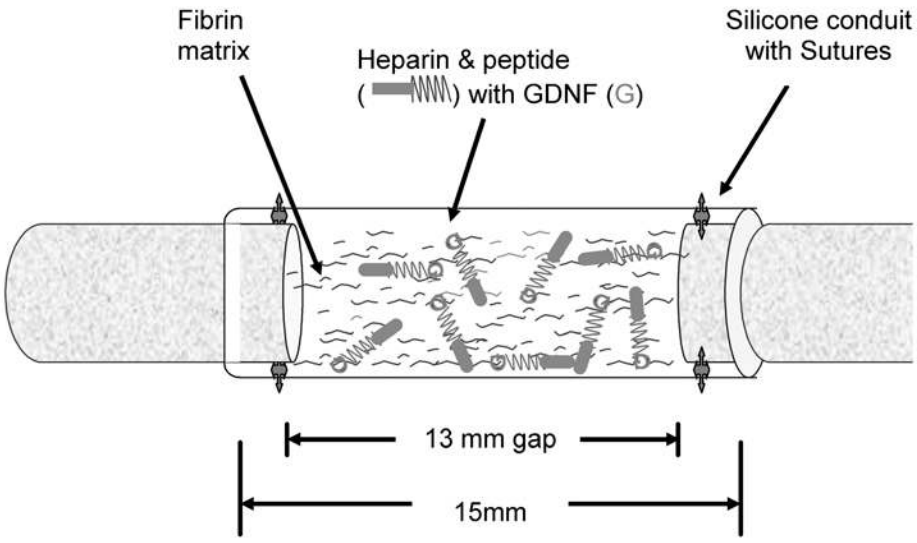

Figure 5.1. Schematic representation of surgical implantation of nerve guidance conduit containing the affinity-based delivery system. A 13 mm nerve gap was repaired with a 15 mm silicone conduit containing fibrin matrices with or without delivery system and growth factor and sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end. The delivery system consisted of a bi-domain peptide cross-linked into the fibrin matrix at one domain while the other binds heparin by electrostatic interactions. The growth factor can then bind to the bound heparin, creating a matrix-bound, non-diffusible complex, which can be retained for cell mediated degradation of the fibrin matrix.

5.3.3 Preparation of fibrin matrices

Fibrinogen solutions were prepared by dissolving human plasminogen-free fibrinogen in deionized water at 8 mg/mL for 1 h and dialyzing versus 4L of Tris-buffered saline (TBS) (33 mM Tris, 8 g/L NaCl, 0.2 g/L KCl) at pH 7.4 overnight to exchange salts present in the protein solution. The resulting solution was sterilized by filtration through 5.0 μm and 0.22 μm syringe filters, and the final fibrinogen concentration was determined by measuring absorbance at 280 nm. For the delivery system, a bi-domain peptide (ATIII) based on a modified version of the antithrombin III-heparin binding domain (*(AcG)NQEQVSPK*(βA)FAKLAAR-LYRKA, where AcG denotes N-acetyl-glycine and the tranglutaminase substrate is given in italics) (Tyler-Cross, Sobel et al. 1994; Sakiyama,

Schense et al. 1999) was synthesized as described previously (Wood, Borschel et al. 2009). Fibrin matrices were prepared as previously described. Components were mixed to obtain the following final solution concentrations: 4 mg/mL fibrinogen, 2.5 mM Ca^{++}, 2 NIH units/mL of thrombin, 0.25 mM peptide (which results in 8 moles of cross-linked peptide per mole fibrinogen (Sakiyama, Schense et al. 1999; Schense and Hubbell 1999)), 62.5 μM heparin (sodium salt), and recombinant human GDNF or NGF (at proper dose, Peprotech, Table 5.1).

Silicone tubing (SF Medical, Hudson, MA) (1.5 mm inside diameter x 0.3 mm wall thickness) was autoclaved overnight, cut into 15 mm segments, and soaked in 70% ethyl alcohol. Prior to filling, the tubes were rinsed with sterile saline solution. The fibrinogen solution was drawn into the silicone tube using a pipette and allowed to polymerize for 10 minutes prior to implantation.

5.3.4 Operative Procedure

All surgical procedures were performed using aseptic technique and microsurgical dissection and repairs. Under subcutaneous anesthesia with ketamine (75 mg/kg) and medetomidine (0.5 mg/kg), the hind leg of the rat was prepped and the sciatic nerve was exposed through a dorsolateral gluteal muscle splitting incision. A 5 mm nerve segment was excised proximal to the trifurcation of the sciatic nerve and a 15 mm silicone tube, with the fibrin matrices with or without delivery system and growth factor, was sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end as described above (Figure 5.1). Four 9-0 nylon interrupted microepineurial sutures were used to secure the conduit. In animals receiving the isograft control, a 13 mm segment of sciatic

nerve was harvested from a syngeneic donor animal and inserted into the recipient animal. Wounds were irrigated with saline, dried, and closed with a running 5-0 vicryl suture in muscle fascia, and then interrupted 4-0 nylon skin sutures.

Anesthesia in experimental animals was then reversed with a subcutaneous injection of atipamezole HCl (1mg/kg) (Pfizer Animal Health, Exton, PA), and the animals recovered in a warm environment. After recovery, the animals were returned to the housing facility.

Six weeks postoperatively, all animals were re-anesthetized and nerve harvests were performed by reopening the prior muscle splitting incision. The nerve conduit and a 5 mm portion of native nerve both proximally and distally were harvested. The specimens were marked with a proximal suture and stored in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C until histomorphometric analysis was performed. Under anesthesia, the animals were then euthanized with intracardiac injection of Euthasol (150mg/kg) (Delmarva Laboratories, Des Moines, IA).

5.3.5 Histomorphometric and Electron Microscopic Evaluation

The tissues were post-fixed with 1% osmium tetroxide, ethanol dehydrated and embedded in Araldite 502 (Polyscience Inc., Warrington, PA). Thin (1 μm) sections were made from the tissue using a LKB II Ultramicrotome (LKB-Produckter A.B., Broma, Sweden) and then stained with 1% toluidine blue for examination by light microscopy. The slides were evaluated by an observer blinded to the experimental groups for overall nerve architecture, quantity of regenerated nerve fibers, degree of myelination, and the presence of Wallerian degeneration (Hunter, Moradzadeh et al. 2007).

Proximal and distal cross sections from the host nerve, and sections through the conduit or graft were evaluated. At 1000X magnification, six representative fields per nerve were evaluated with an automated digital image-analysis system linked to morphometry software using previously described methods to measure nerve morphometry (Hunter, Moradzadeh et al. 2007). Briefly, total fascicular area and total fiber number were measured. At least 80% of the nerve area was measured to determine the fiber diameters and density. From these primary measurements the following morphometric indices were calculated: total number of nerve fibers, nerve fiber density (fiber number/mm^2), percent neural tissue (100 x neural area/intrafascicular area), and nerve fiber width. Morphometric indices from experimental neural specimens were compared to the isograft controls.

For electron microscopy, ultrathin 70 nm sections of the embedded tissues were cut by a LKB III Ultramicrotome and stained with uranyl acetate-lead citrate. These sections were examined with a Zeiss 902 electron microscope (Zeiss Instruments, Chicago, IL). Quality of myelination, relative prevalence of unmyelinated fibers, and the area of myelinated and unmyelinated fibers were evaluated.

5.3.6 Statistical analysis

All results are reported as mean ± standard error of the mean. Statistical analyses were performed using Statistica version 6 (Statsoft Inc., Tulsa, OK). All data were evaluated for differences between groups using the Kruskal-Wallis analysis of variance (ANOVA) and median test. Post hoc Mann-Whitney tests were used for determining differences between groups with significance set at $\alpha = 0.05$. Additionally, fiber width data was evaluated for

differences between groups using ANOVA, and post hoc LSD tests were used for determining which groups differed with a significance set at $\alpha = 0.05$.

5.4 Results

5.4.1 Nerve guidance conduit harvest

The effectiveness of GDNF in promoting nerve regeneration across a critical nerve gap was evaluated *in vivo* after sciatic nerve transaction and NGC implantation. After 42 days, groups with the delivery system and growth factor resulted in more successful regeneration as demonstrated by a neural structure spanning the 13 mm gap with higher frequency. Six of 12 conduits from the 100 ng/mL GDNF with delivery system (GDNF DS) and 7 of 12 conduits from 50 ng/mL NGF with DS (NGF DS) groups contained regenerated nerve cables. Five of 12 conduits from the group containing fibrin, 3 of 12 conduits from DS alone with no growth factor (DS alone (no GF)) group, 1 of 12 of the 100 ng/mL GDNF (GDNF (no DS)) group, and 3 of 12 from the empty group demonstrated nerve regeneration (Table 5.1). All 12 animals in the isograft group demonstrated regeneration. The gross appearance of the regenerated nerves in the GDNF DS group exhibited a larger, more robust nerve cable in comparison to the other experimental groups. The neural structure was centered compactly in the conduit, away from the walls, in all conduit specimens. All conduit specimens demonstrated intact connections to the proximal or distal sciatic nerve, despite variability in regeneration.

5.4.2 Histology

Qualitative examination of the midline of the conduits or grafts by light microscopy revealed differences in nerve architecture as reflected by the arrangement and description of the regenerating axons (Figure 5.2). In particular, the normal rat sciatic nerve contains myelinated fibers in a packed, semi-symmetric, uniform arrangement with fibers that are relatively similar in size and shape to one another. Overall, this arrangement can be described as "organized" architecture. The isograft, GDNF DS and NGF DS, reflect this organized appearance, while the fibrin alone group demonstrated more random spacing and swirling of fibers, as well as, less symmetric shape of the individual myelinated fibers. In addition, groups with growth factor and the delivery system appeared to have more tightly packed fibers than the isograft, which was likely due to the compact area for neural regeneration in the silicone tube. No inflammatory response or residual fibrin was appreciated.

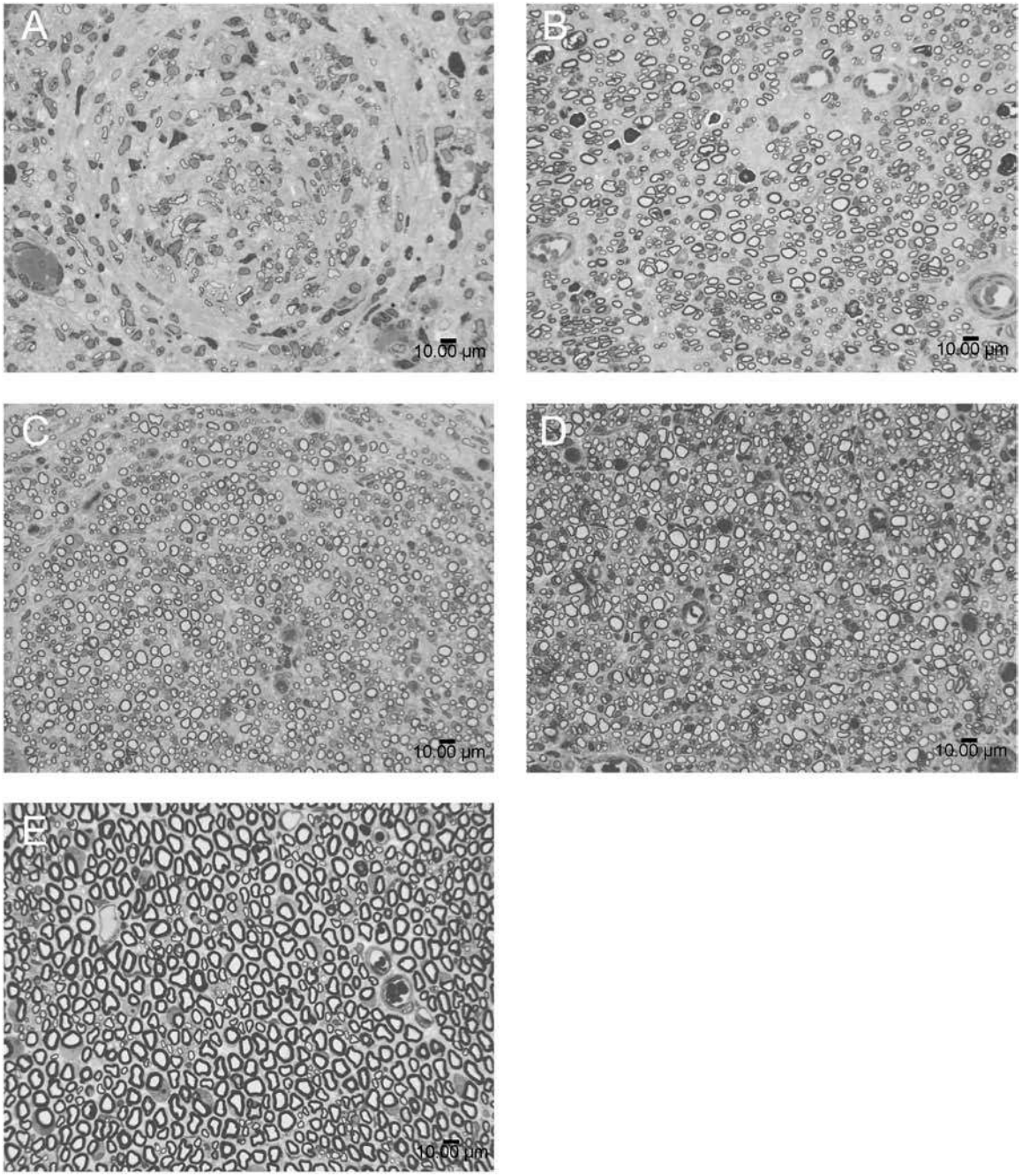

Figure 5.2. Histological sections of regenerating nerves at the midline of the conduit (or graft). (A) Fibrin alone; (B) Isograft; (C) delivery system incorporating GDNF (GDNF DS); (D) delivery system incorporating NGF (NGF DS); (E) normal uninjured nerve. Thin (1 µm) sections of sciatic nerve specimens were stained with 1% toluidine blue for qualitative examination of the midline of the conduits by light microscopy. GDNF DS and NGF DS groups demonstrated more organized neural architecture, closely approximating the isograft, in comparison to the fibrin alone group. Scale bar, 10 µm.

5.4.3 Histomorphometry

At six weeks, the average total myelinated fiber count, one measure of the effectiveness of neural regeneration, was 12,000 ± 600 fibers (n = 12) for the isograft, while the conduits with GDNF DS contained 3500 ± 1500 fibers (n = 12) (Figure 5.3A). The NGF DS group had 2000 ± 770 fibers (n = 12), and the fibrin alone group had 1700 ± 800 fibers. The GDNF alone, DS alone (no GF), and empty conduit groups had little regeneration and resulted in fiber counts less than 1700. The isograft had significantly more fibers than all other groups. The average number of fibers in a normal rat sciatic nerve is approximately 7200 ± 410 (Mackinnon, Dellon et al. 1991).

Nerve fiber density is another measure of neural regeneration. The nerve fiber density at the midline for the GDNF DS (~13,000 fibers/mm^2) and NGF DS (~15,000 fibers/mm^2) groups were not significantly different from the isograft controls (~20,000 fibers/mm^2) (Figure 5.3B). For normal sciatic nerve, the fiber density is ~12,000 fibers/mm^2 (Mackinnon, Dellon et al. 1991). Both groups incorporating the delivery system and growth factors also had similar percentages of neural tissue at the midline of the conduit (15 – 16 %) compared to the isografts (~19 %) (Figure 5.3C). This assessment provides a measure of quality of the regenerating nerve and suggests that the quality of nerve regeneration is better in groups containing both the delivery system and growth factor compared to control groups.

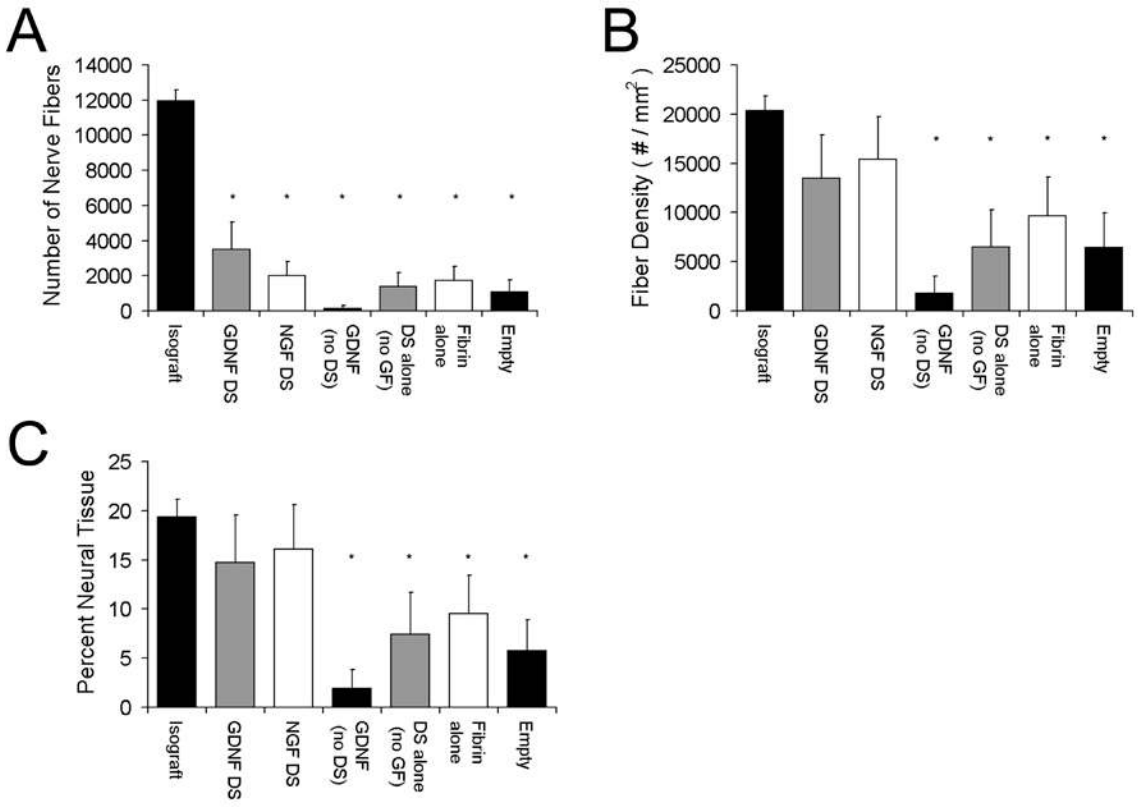

Figure 5.3. Histomorphometric analysis of nerves at the midline of the conduit (or graft). The total number of myelinated nerve fibers, density, and percent neural tissue were measured by quantitative histomorphometry. No groups were similar to the isograft group in terms of total number of nerve fibers (A), but the delivery system with GDNF (GDNF DS) or NGF (NGF DS) was similar to the isograft in terms of fiber density (B) and percent neural tissue (C). Data (n = 12) represents mean ± S.E.M. and * indicates statistical significance ($p<0.05$) compared to the isograft.

The myelinated nerve fiber width was assessed as a measure of maturity of the regenerating nerve fibers, and groups that were most effective in promoting neural regeneration were assessed for their fiber width distribution in regenerating nerves. All groups utilizing fibrin within conduits for regeneration contained fewer smaller nerve fibers (2 – 3 μm) compared to isograft controls (Figure 5.4A); furthermore, the GDNF DS group also contained fewer nerve fibers than the isograft in the 3 – 4 μm distribution. Both groups containing the delivery system and growth factor demonstrated higher percentages of larger nerve fibers (4 – 5, 5 – 6 μm) compared to the isograft, but only the GDNF DS group

promoted the higher percentages of large caliber nerve fibers (6 – 7 μm) compared to the isograft, suggesting more mature regenerating fibers. Overall, conduit groups contained larger average fiber widths compared to the isograft (Figure 5.4B). The normal median rat sciatic nerve fiber width is ~6.5 μm (Mackinnon, Dellon et al. 1991).

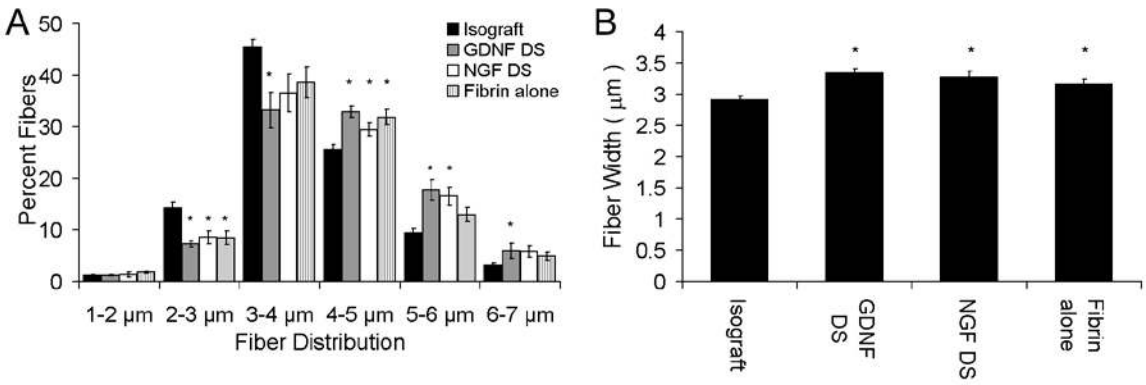

Figure 5.4. Myelinated fiber size distribution of regenerating nerves at the midline of the conduit (or graft). The nerve fiber width distributions (A) and averages (B) were measured by quantitative histomorphometry. The percentage of large regenerating nerve fibers (4-5, 5-6 μm) were larger in the GDNF DS and NGF DS groups compared to the isograft group. GDNF DS had significantly the greatest percentage of the largest fibers (6-7μm) compared to the isograft. Overall, conduit groups average fiber widths were larger than isografts. Data represents mean ± S.E.M. and * indicates statistical significance ($p<0.05$) compared to the isograft.

5.4.4 Electron microscopy

Electron microscopy was performed to evaluate the regenerative nerve ultrastructure. Representative sections from groups that were most effective in promoting nerve regeneration (GDNF DS, NGF DS, fibrin alone and isograft) are shown in Figure 5.5. The myelinated and unmyelinated fiber areas were determined from randomly selected specimens from each group by a researcher blinded to the groups ($n = 3$). Qualitatively, the GDNF DS group again appeared to have more organized structure and more uniform fiber shape with larger myelinated fibers than the other experimental groups including the isograft,

while fibrin alone had the most disorganized appearance and less uniform fiber shapes. The myelinated area of the GDNF DS (27 ± 2.4 μm^2) and NGF DS (23 ± 5.1 μm^2) groups were equivalent to the isograft (25 ± 5.5 μm^2), whereas fibrin alone (19 ± 1.4 μm^2) had significantly less myelinated fiber area (Figure 5.6A). There were no differences among groups for unmyelinated fiber area (Figure 5.6B).

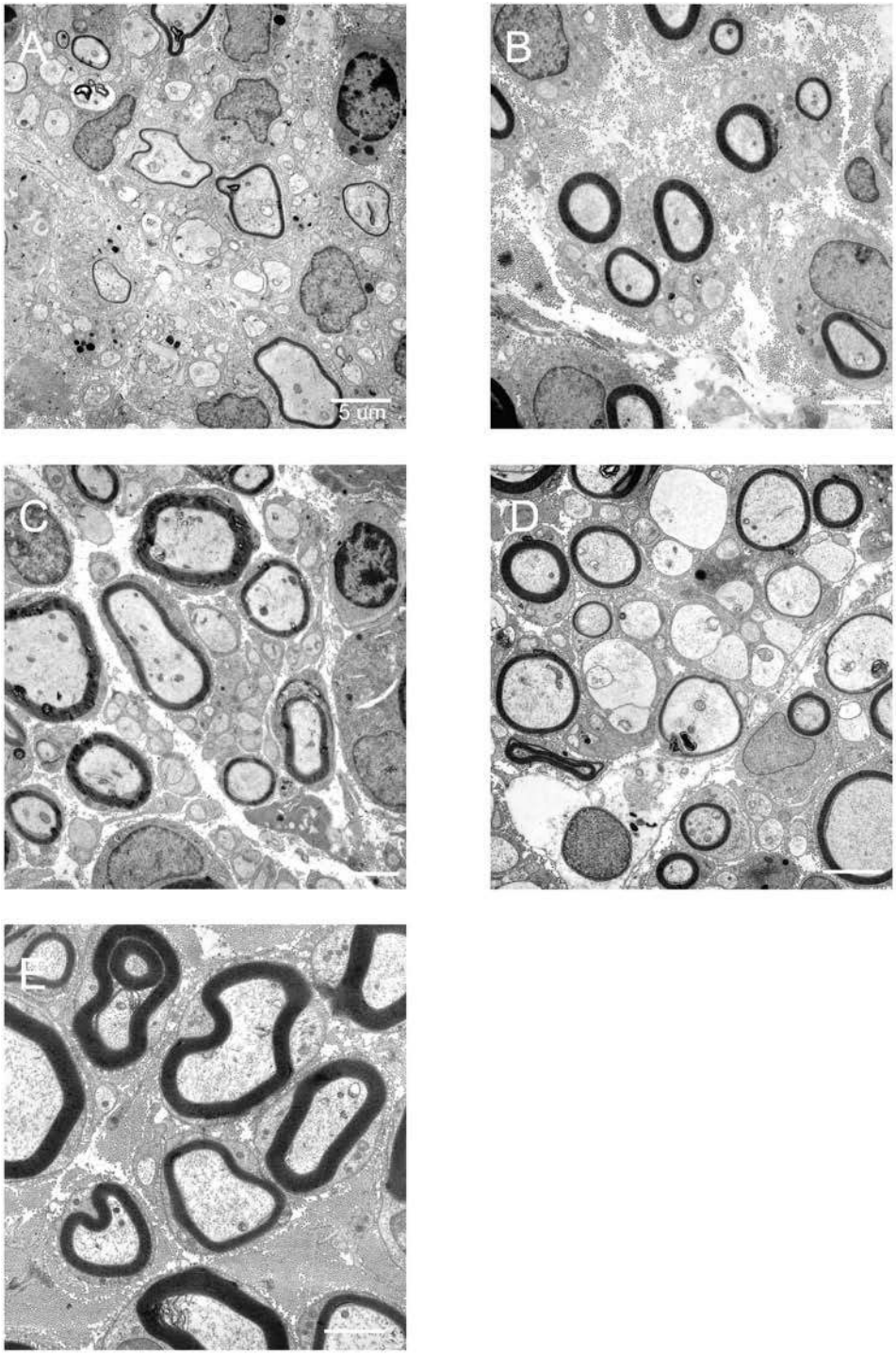

Figure 5.5. Electron micrographs of regenerating nerves at the midline of the conduit (or graft). (A) Fibrin alone; (B) Isograft; (C) delivery system incorporating GDNF (GDNF DS); (D) delivery system incorporating NGF (NGF DS); (E) normal uninjured nerve. Ultrathin 70 nm sections of the embedded tissues were cut and stained with uranyl acetate-lead citrate. Qualitatively, GDNF DS and NGF DS appeared to have more uniform fiber structures with larger myelinated fibers than fibrin alone, which had the most disorganized fiber appearance. Scale bar, 5 μm.

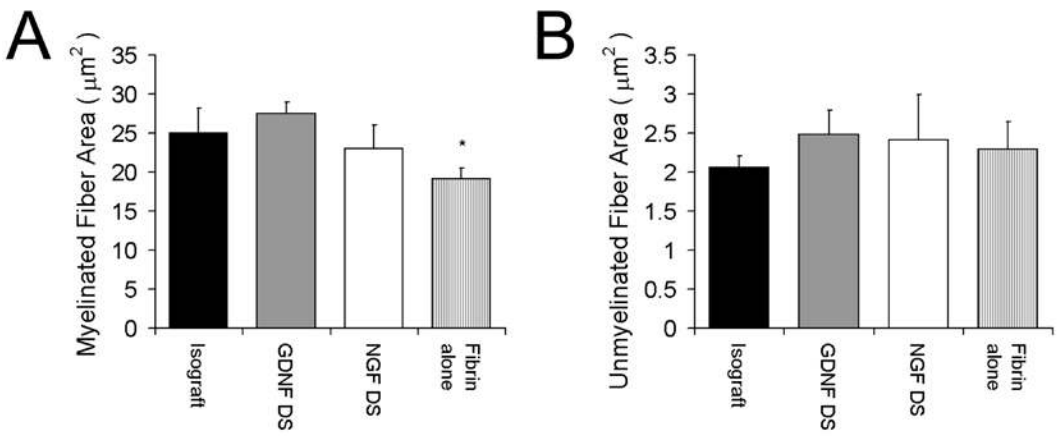

Figure 5.6. Myelinated and unmyelinated fiber areas of regenerating nerves at the midline of the conduit (or graft). The myelinated and unmyelinated fiber areas were determined from randomly selected specimens by electron microscopy from each group. The myelinated areas of for groups with the delivery system incorporating GDNF (GDNF DS) or NGF (NGF DS) were equivalent to the isograft, whereas the fibrin alone group had a lower myelinated fiber area (A). There were no differences among groups in unmyelinated fiber area (B). Data (n = 3) represents mean ± S.E.M. and * indicates statistical significance ($p < 0.05$) compared to the isograft.

5.5 Discussion

Peripheral nerve injuries are devastating and alternatives to standard repairs of nerve gaps rarely lead to complete clinical recovery. In this study we examined the effect of growth factor delivery using a NGC to bridge and enhance nerve regeneration across a critical nerve gap length. We used an affinity-based delivery system within a fibrin matrix to immobilize growth factors, slow their diffusion from the matrix, and allow release by cell-mediated degradation of the matrix, thus, controlling their delivery to the regenerative site. This "cell-mediated" matrix degradation and subsequent growth factor release may be facilitated through the processes of axonal outgrowth and cell-induced protease activation (e.g. plasmin activation on the growth cone) (Kalderon 1984; Krystosek and Seeds 1984; Alvarez-Buylla and Valinsky 1985; Pittman and Buettner 1989; Herbert, Bittner et al. 1996).

We have previously examined controlled release of NGF with this delivery system *in vivo* and found that affinity-based release of NGF increased myelinated nerve fiber sprouting and outgrowth compared to diffusion-based release from fibrin matrices alone (Lee, Yu et al. 2003a). In the present study, we focused on the controlled delivery of another neurotrophic factor, GDNF, to investigate its effect on peripheral nerve regeneration in a sciatic nerve injury model. We found that controlled delivery of GDNF was superior to controls in aspects of nerve regeneration including neural fiber size and organized nerve architecture, suggesting more mature neural content. The use of our affinity-based delivery system directly affected regeneration across a 13 mm gap and the inclusion of the delivery system sequestering GDNF had a greater effect in eliciting neural regeneration than providing GDNF in an unbound form to the regenerative site. This effect may be explained by the delivery's system's ability to sequester GDNF and avoid an initial burst of drug release, as found *in vitro* (Wood, Borschel et al. 2009). Others have cited initial bursts of GDNF release to be detrimental to nerve regeneration (Barras, Pasche et al. 2002).

We anticipated the ability of an exogenous fibrin matrix, with or without an affinity-based delivery system, to facilitate bridging a critical nerve defect because a fibrin matrix naturally forms within an empty silicone conduit connecting the damaged ends of rat sciatic nerve over a one week period (Williams, Longo et al. 1983). However, we were surprised to see three out of twelve *empty* conduits had regeneration across this critical defect, although the neural content was histologically poorly organized. Nerve regeneration has been shown to be inconsistent beyond a 10 mm defect without the addition of other components, such as extracellular matrix molecules, Schwann cells, plasma, or neurotrophic factors (Lundborg, Dahlin et al. 1982a; Williams, Longo et al. 1983). The regeneration in our empty conduits may be a result of superior neural regeneration for rats, as it has been shown that the rat is

able to spontaneously regenerate into an unfilled 4.5 cm nerve gap to a distance of 2.4 cm after 5 months (Mackinnon, Hudson et al. 1985). Despite the regeneration in some of the controls, the inclusion of our affinity-based delivery system incorporating GDNF or NGF more closely approximated neural regeneration of an isograft across a critical gap defect.

The efficacy of affinity-based delivery of GDNF or NGF to the regenerative site was also observed in the histomorphometry and electron microscopy results. Both groups containing the delivery system and neurotrophic factors were not statistically different from the isograft controls with regards to fiber density and percent neural tissue, both measures of nerve quality. Beyond enhancing the quality of regeneration, we found that the delivery of GDNF and NGF from the affinity-based delivery system improved the maturity of the regenerating fibers. It is well known that axon size and myelin thickness are measures of maturity (Aitken, Sharman et al. 1947; Young 1949; Williams and Wendell-Smith 1971; Fraher and Dockery 1998). Both growth factors increased fiber maturity, as seen in histomorphometric fiber distributions as well as myelinated fiber area from electron microscopy. Our findings suggest that although not equivalent in fiber number, the delivery of GDNF by our delivery system produces larger, mature nerve fibers. Of particular importance, the GDNF group had more 6-7 μm diameter fibers than all other groups including the isograft. In the normal, uninjured sciatic rat nerve, the average myelinated fiber width is 6-7 μm. Furthermore, studies have shown that larger axon diameter and myelination results in larger conduction velocity and can be correlated to greater function as compared to smaller, less myelinated fibers (Williams and Wendell-Smith 1971; Fraher and Dockery 1998). Although we did not look at functional outcomes in this study, parallel studies are underway to explore if these larger diameter fibers result in greater return of function.

Our study corroborates previous findings in the literature on the effect of GDNF on peripheral nerve regeneration. Barras *et al.* evaluated the effects of GDNF delivery from an ethylene vinyl acetate polymer rod across an 8 mm rat facial nerve defect. Similar to our results, they found that GDNF delivery increased myelination and fiber size. In this pure motor model, they also found that GDNF promoted increased motor neuron labeling in comparison to neurotrophin-3, suggesting that GDNF has a significant role in motor nerve regeneration (Barras, Pasche et al. 2002). Given the strong evidence of GDNF in motor neuron survival and enhancement of peripheral nerve regeneration, further studies looking at the affinity-based delivery of GDNF to specific motor and sensory nerves will be performed to elucidate this neurotrophic factor's impact on modality specific regeneration.

In addition, Fine *et al.* evaluated axonal regeneration across a 15 mm long gap in the rat sciatic nerve in the presence of GDNF or NGF provided by synthetic nerve guidance channels continuously releasing the neurotrophic factors. They found that the average number of myelinated axons at the midpoint of the regenerated nerves was greater in the presence of GDNF than NGF. The GDNF group also had significantly greater numbers of retrograde labeled motoneurons. Thus, these authors report GDNF to have greater efficacy than NGF in overall regeneration (Fine, Decosterd et al. 2002). In contrast, we did not find significant differences between GDNF and NGF, except in fiber size distribution. The difference in our findings may be attributed to our method of delivery. In particular, Fine *et al.* used diffusion-based delivery of GDNF or NGF from a biodegradable conduit where the dose delivered to the regenerating nerve was constant for a period of time. Our delivery method relies on cell-mediated release of sequestered growth factor from a luminal matrix, which allows the release rate to vary based on the presence of cells and position within the NGC. This result suggests that this method of growth factor delivery may play a role in

regeneration. The effect of delivery rate has been noted by others, such as Piquilloud *et al.* who used a resorbable conduit that released GDNF at three different delivery rates and found differences in neural regeneration due to release rate (Piquilloud, Christen et al. 2007).

We have examined our delivery system with a silicone NGC because of the biocompatibility of the product, its mechanical stability, and well known critical defect length in our surgical model. However, clinically, silicone conduits are not ideal and have associated morbidities. Silicone conduits have been reported to cause chronic nerve compression, irritation at the implantation site requiring removal, and inflammatory and fibrotic reactions impacting nerve regeneration (Merle, Dellon et al. 1989; Dellon 1994; Battiston, Geuna et al. 2005). Thus, the combination of our drug delivery system with a biodegradable conduit would be more desirable for clinical peripheral nerve injury repairs. Future studies directed toward this goal would be of certain benefit in translating our delivery system into clinical practice.

Lastly, previous studies have demonstrated that both NGF and GDNF can enhance peripheral nerve regeneration (Fine, Decosterd et al. 2002; Boyd and Gordon 2003). However, NGF has been shown to primarily promote sensory nerve regeneration in the peripheral nervous system (Terenghi 1999; Boyd and Gordon 2003) as motor neurons do not express NGF or its receptors (Boyd and Gordon 2003). Our study further confirms the assertion that the delivery of these neurotrophic factors enhances peripheral nerve regeneration in a sciatic nerve model, a model that contains both sensory and motor fibers. Given that that GDNF and NGF affect different cell populations (Boyd and Gordon 2003) and *in vitro* work demonstrates increased neurite outgrowth with the combination of the two factors more than either alone (Deister and Schmidt 2006), an interesting investigation

would be to evaluate the possible synergistic effects of delivering a combination of both neurotrophic factors on peripheral nerve regeneration.

5.6 Conclusions

In summary, the goal of this study was to evaluate and elucidate the efficacy of affinity-based delivery of GDNF to the regenerative site in a critical size defect model. We examined histological outcomes of this neurotrophic factor and compared it to controls as well as NGF. Given the increased maturity and organized architecture of the regenerating nerve under the influence GDNF, we believe affinity-based delivery of GDNF offers insight into potential future alternatives for the treatment of peripheral nerve injuries.

Chapter 6

Fibrin Matrices with Affinity-based Delivery Systems and Neurotrophic Factors Promote Functional Nerve Regeneration

6.1 Abstract

Glial-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) have both been shown to enhance peripheral nerve regeneration following injury and target different neuronal populations. Therefore, the delivery of either growth factor may result in differences in motor and sensory nerve regeneration and functional recovery. In this study we evaluated the effect of affinity-based delivery of GDNF or NGF from a fibrin matrix in a nerve guidance conduit (NGC) on modality specific nerve regeneration and functional recovery in a 13 mm rat sciatic nerve defect. Seven experimental groups were evaluated consisting of GDNF or NGF and the delivery system (DS) within the conduit, control groups excluding the DS and/or growth factor, and nerve isografts. The DS with either GDNF or NGF resulted in a higher frequency of nerve regeneration versus control groups with a nerve cable spanning the 13 mm gap within the conduit. All groups with a fibrin matrix and growth factor in the conduit performed similar to the isograft in behavioral measures and had similar relative muscle mass at 12 weeks, while the GDNF DS group had better behavioral outcomes than the isograft. While no differences were observed in nerve compound nerve action potentials, groups with GDNF had greater extensor digitorum longus twitch and tetanic specific muscle force. Modality specific regeneration assessed by

retrograde labeling revealed the number of labeled ventral horn neurons in the GDNF and NGF DS groups were similar to the isograft; however, these counts were greater than those of groups without growth factor. Only the empty conduit group had a lower number of labeled DRG neurons and all other groups showed no differences. Overall, the ability of the GDNF DS group demonstrated better functional recovery and equivalent motor nerve regeneration compared to the isograft, suggesting it has potential as a treatment for motor nerve injury.

6.2 Introduction

Despite significant advances in the treatment of peripheral nerve injury, complete clinical recovery is rare suggesting that an alternative to the current standard of care (nerve autograft) is needed. Critical nerve defects treated by nerve autograft suffer from donor site morbidity and remain deficient in positive functional outcomes (Beazley, Milek et al. 1984; Dellon and Mackinnon 1988). An alternative repair strategy involves a nerve guidance conduit (NGC), which can be filled with a biomaterial matrix and growth factors, to bridge the defect and enhance axonal regeneration (Schmidt and Leach 2003; Bellamkonda 2006). NGCs have demonstrated similarities to autografts by histological measures, but data supporting their role in motor nerve regeneration and functional recovery is limited. Therefore, alternative strategies such as NGCs should focus not only on promoting nerve regeneration but also improving functional recovery.

Functional recovery following peripheral nerve injury has been difficult to achieve because the biology of modality specific nerve regeneration (sensory versus motor nerve

regeneration) and the requirements for stimulating proper muscle reinnervation are not well understood. One hypothesis is that trophic support acts as a cue to promote the specificity of nerve regeneration (Uschold, Robinson et al. 2007). Through a series of experiments, Madison and colleagues demonstrated that motor axons regenerated to their correct parent branch due to trophic support derived from their end-organ targets (Robinson and Madison 2004; Madison, Robinson et al. 2007; Uschold, Robinson et al. 2007). Alternatively, others have suggested that the nerve branches may be a source of modality specific trophic support. For example, the use of ventral root (motor) nerve segments in silicone conduits stimulated better muscle reinnervation compared to dorsal root (sensory) nerve segments in a sciatic nerve defect, possibly due to cues for motor nerve regeneration contained in the ventral root nerve segments (Lago, Rodriguez et al. 2007). Recent work by Hoke *et al.* demonstrated that Schwann cells (SCs) within the nerve branches may be the source of the modality specific trophic support. They observed differences in growth factor expression levels before and after injury when comparing motor and sensory nerve branches (Hoke, Redett et al. 2006). Additionally, motor and sensory neurons express varying levels of growth factor receptors (Boyd and Gordon 2003). For example, motor neurons and their axons express receptors for glial-derived neurotrophic factor (GDNF (GDNFRα1 and RET) but not NGF (TrkA) (Boyd and Gordon 2003). Therefore, the inclusion of growth factors that target motor neurons in a conduit may better stimulate motor nerve regeneration and in turn better promote functional recovery.

Differences in nerve regeneration modalities have been observed with growth factor delivery from NGCs that target different neuronal populations. Fine *et al.* found that the controlled delivery of GDNF from synthetic conduits stimulated more motor neurons to regenerate versus NGF as assessed by retrograde labeling of the regenerating ventral horn

axons (Fine, Decosterd et al. 2002). Additionally, Barras *et al.* found that a greater number of axons regenerated with controlled delivery of GDNF from synthetic conduits compared to neurotrophin-3 (NT-3) in a rat facial nerve injury model, which is a primarily motor nerve (Barras, Pasche et al. 2002). Both studies utilized diffusion-based release of growth factors from the NGC. Based on these studies we hypothesized that an alternative approach, *affinity-based* delivery, could potentially stimulate modality specific nerve regeneration due to growth factor release.

In contrast to diffusion-based release, an *affinity-based* DS allows the release of growth factors to be controlled by cell-based degradation of the DS (Sakiyama-Elbert and Hubbell 2000a). Our lab has developed an affinity-based DS that sequesters the drug of interest into a fibrin matrix using non-covalent interactions (Sakiyama-Elbert and Hubbell 2000a; Sakiyama-Elbert and Hubbell 2000b). This system consists of a bi-domain heparin-binding peptide, where one domain is a transglutaminase substrate, based on α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983; Kimura, Tamaki et al. 1985), allowing the peptide to be crosslinked into fibrin matrices during polymerization by the transglutaminase Factor XIIIa. The other domain consists of a modified version of the heparin-binding domain from antithrombin III (Tyler-Cross, Sobel et al. 1994; Tyler-Cross, Sobel et al. 1996; Sakiyama, Schense et al. 1999), that allows non-covalent immobilization of heparin to peptide crosslinked within a fibrin matrix. The heparin-binding domain has the ability to sequester various growth factors based on their interaction with heparin via the sulfate groups (Yamada 1983).

Delivery of NGF and GDNF from our affinity-based DS has previously been found to promote nerve regeneration in short-term studies (Lee, Yu et al. 2003a; Wood, Hunter et

al. 2009; Wood, Moore et al. 2009). In the present study, we evaluated the effects of the controlled release of GDNF and NGF from a fibrin matrix containing our affinity-based DS within a NGC on modality specific nerve regeneration and functional recovery *in vivo* using a rat sciatic nerve injury model.

6.3 Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

6.3.1 Experimental animals

Adult male Lewis rats (Harlan Sprague-Dawley, Indianapolis, IN), each weighing 250-300 g were used in this study. All surgical procedures and peri-operative care measures were performed in strict accordance with the National Institutes of Health guidelines and were approved by the Washington University Animal Studies Committee. All animals were housed in a central animal facility, given a rodent diet (PicoLab Rodent Diet 20 #5053, PMI Nutrition International) and water *ad libitum*. After surgical procedures, animals recovered in a warm environment and were closely monitored for 2 hours. Animals were then returned to the animal facility and monitored for weight loss, infection, and other morbidities.

6.3.2 Experimental Design

One hundred twelve animals were randomized into seven groups (n = 16) as shown in Table 6.1. Fifty-six of those animals were distributed equally by group to the evoked motor response portion of the study, while the remaining animals were distributed to the retrograde labeling portion of the study. An additional eight animals served as sciatic nerve isograft donors. Empty conduits served as the untreated control group. Additional control groups received conduits containing fibrin with the DS (no growth factor) or fibrin with growth factor, but no DS. These groups examined the effects of the incomplete DS. The remaining groups were implanted with conduits containing fibrin with the DS and GDNF or NGF. Doses of GDNF and NGF were selected based upon previous *in vivo* studies (Wood, Moore et al. 2009). Reversed nerve isografts from syngeneic donor animals served as a positive isograft control.

Table 6.1: Experimental Design

		Motor Response Study		**Retrograde Study**	
Group Name	**Group Description**	**Number of Rats**	**Number with Regeneration**	**Number of Rats**	**Number with Regeneration**
Isograft	Isograft	8	8	8	8
GDNF DS	Fibrin + DS + GDNF	8	5	8	5
GDNF (no DS)	Fibrin + GDNF	8	2	8	0
NGF DS	Fibrin + DS + NGF	8	5	8	6
NGF (no DS)	Fibrin + NGF	8	4	8	1

DS (no GF)	Fibrin + DS (no GF)	8	3	8	3
Empty	Empty	8	3	8	2

Abbreviations: DS, Delivery System; GF, Growth Factor; GDNF, glial-derived neurotrophic factor (100 ng/mL); NGF, nerve growth factor (50 ng/mL).

6.3.3 Preparation of fibrin matrices

Fibrinogen solutions were prepared by dissolving human plasminogen-free fibrinogen in deionized water at 8 mg/mL for 1 h and dialyzing versus 4L of Tris-buffered saline (TBS, 33 mM Tris, 8 g/L NaCl, 0.2 g/L KCl; Fisher Scientific, Pittsburgh, PA) at pH 7.4 overnight to exchange salts present in the protein solution. The resulting solution was sterilized by filtration through 5.0 μm and 0.22 μm syringe filters, and the final fibrinogen concentration was determined by measuring absorbance at 280 nm. For the DS, a bi-domain peptide (ATIII) based on a modified version of the antithrombin III-heparin binding domain (*(AcG)NQEQVSPK*(βA)FAKLAARLYRKA, where AcG denotes N-acetyl-glycine and the transglutaminase substrate is given in italics) (Tyler-Cross, Sobel et al. 1994; Sakiyama, Schense et al. 1999) was synthesized as described previously (Wood, Borschel et al. 2009). Fibrin matrices were prepared as previously described (Sakiyama-Elbert and Hubbell 2000a). Components were mixed to obtain the following final solution concentrations: 4 mg/mL fibrinogen, 2.5 mM Ca^{++}, 2 NIH units/mL of thrombin, 0.25 mM peptide (which results in 8 moles of cross-linked peptide per mole fibrinogen (Sakiyama, Schense et al. 1999; Schense and Hubbell 1999)), 62.5 μM heparin (sodium salt), and recombinant human GDNF or β-NGF (100 ng/mL and 50 ng/mL, respectively; Peprotech Inc., Rocky Hill, NJ).

Silicone tubing (SF Medical, Hudson, MA) (1.5 mm inside diameter x 0.3 mm wall thickness) was autoclaved overnight, cut into 15 mm segments, and soaked in 70% ethyl alcohol. Prior to filling, the tubes were rinsed with sterile saline solution. The fibrinogen solution was drawn into the silicone tube using a pipette and allowed to polymerize for 10 minutes prior to implantation.

6.3.4 Operative Procedure

All surgical procedures were performed using aseptic technique and microsurgical dissection and repairs. Four percent isoflurane gas (Vedco Inc., St Josephs, MO) anesthesia was used for animal induction followed by 2% isoflurane gas to maintain anesthesia. The hind leg of the rat was prepped and the sciatic nerve was exposed through a dorsolateral-gluteal muscle splitting incision. A 5 mm nerve segment was excised proximal to the trifurcation of the sciatic nerve and a 15 mm silicone tube, containing fibrin with or without DS and growth factor, was sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end (resulting in a 13 mm gap). Four 9-0 nylon interrupted microepineurial sutures were used to secure the conduit. In animals receiving the isograft control, a 13 mm segment of sciatic nerve was harvested from a syngeneic donor animal and inserted into the recipient animal in reverse orientation. Wounds were irrigated with saline, dried, and closed with a running 5-0 vicryl suture in muscle fascia, and then interrupted 4-0 nylon skin sutures. Experimental animals were recovered in a warm environment, and after recovery the animals were returned to the housing facility.

6.3.5 Behavioral Analysis

Two behavioral tests were performed to assess functional recovery in experimental groups before any surgical procedures and every 4 weeks before the experimental end point at 12 weeks. Walking track analysis, as developed by de Medinaceli *et al.*, was utilized to assess the recovery of hindlimb function (de Medinaceli, Freed et al. 1982). The rats' hind feet were coated with nontoxic finger paint and the rats walked down a closed, narrow path on construction paper. The footprints were assessed for toe spread, intermediate toe spread, and print length as according to de Medinaceli *et al.* (de Medinaceli, Freed et al. 1982) and scored using the sciatic functional index (SFI) previously developed by Bain *et. al* (Bain, Mackinnon et al. 1989).

Additionally, a grid-grip test modified from Johnson *et al.* was utilized to measure functional recovery in the hindlimb (Johnson, Parker et al. 2009). For this test, rats were observed walking on a fixed grid of bars spaced 1.5 inches apart for 3 min. Successful grips of the grid, defined as 2 or more toes of the injured foot griping the bar and successful movement to another bar without slipping, and the total number of steps with the injured foot were counted during the allotted time period. The percentage of successful grid-grips was recorded and improvement in the percentage of successful grid-grips was indicative of functional recovery.

6.3.6 Measurement of compound neural action potentials (CNAPs) and evoked motor responses

Twelve weeks postoperatively, sciatic nerve function was assessed by examining CNAPs of the peroneal branch of the sciatic nerve and the motor response in reinnervated EDL muscle upon stimulation of the sciatic nerve. All animals were re-anesthetized and following isolation of the nerve branches and immersion of tissue in a mineral oil bath, cathodic, biphasic electrical impulses (duration = 50 μsec, variable amplitudes) were generated by a single-channel isolated pulse stimulator (Model 2100, A-M Systems Inc., Carlsborg, WA) and delivered to the sciatic nerve proximal to the regenerated nerve segment via bipolar silver wire hook electrodes (7 mil, California Fine Wire, Grover Beach, CA). Using similar wire hook electrodes, CNAPs were recorded and the resulting signal was amplified (gain = 1000X) using an instrumentation amplifier (AD620, Analog Devices Inc., Norwood, MA) powered by a constant voltage source before being recorded on a desktop PC (Dell Computer Corp., Austin, TX) equipped with a data acquisition board (DT3003/PGL, Data Translations, Marlboro, MA) and custom Matlab software (The MathWorks Inc., Natick, MA). Stimulation with a 1 msec delay and recording were synchronized through custom software such that electrical stimulation coincided with the initiation of a 500 msec recording period, wherein data was sampled at 40 kHz for an average of 25 trials per current amplitude tested. Current amplitudes were varied to determine the maximum CNAP and the threshold at which a CNAP and foot twitch (motor response) were observed.

Following measurements of CNAPs, the distal portion of the EDL muscle was separated from the leg by severing the distal tendons on the dorsum of the foot and

fastening the tendons to a stainless steel S-hook at the musculotendinous junction using 5-0 nylon suture. Animals were subsequently placed in a custom-designed force measurement jig where the leg was immobilized by anchoring the femoral condyles. The stainless steel S-hook attached to the EDL muscle was connected to a 5 N thin film load cell (S100, Strain Measurement Devices Inc., Meriden, CT) supported on an adjustable mount. Cathodic, monophasic electrical impulses (duration = 200 μsec, frequency = single-200 Hz, amplitude = 0-3 V) were generated and delivered to the sciatic nerve proximal to the regenerated nerve segment via bipolar silver wire electrodes. Resulting force output at the EDL muscle tendon was transduced via the load cell and the resulting signal was amplified (gain = 1000X) before being recorded as before on a desktop PC with custom Matlab software. This software calculated the passive force and active force for each recorded force trace.

Twitch contractions measured using the custom force recording system were utilized to determine the optimal stimulus amplitude (V_o) and optimal muscle length (L_o) for isometric force production in the EDL muscle. Stimulus amplitude was incrementally increased while muscle length was held constant to determine the largest active force (V_o). Muscle length was then increased in 1 mm increments from a relaxed state while the stimulation amplitude was fixed at V_o until the largest active force produced was determined (L_o). L_o was directly measured as the length of the EDL muscle from proximal to distal musculotendinous junction. All subsequent isometric force measurements were made at V_o and L_o. Single twitch contractions were recorded, and peak twitch force (F_t) was calculated. Tetanic contractions were recorded by delivering 300 μsec bursts of increasing frequency (5-200 Hz) to the sciatic nerve, while allowing two minute periods between stimuli for muscle recovery. Maximum isometric tetanic force (F_o) was calculated from the active force plateau.

Physiological cross-sectional area (PCSA) of the EDL muscle was calculated according to the following (Urbanchek, Chung et al. 1999):

$$PCSA = \frac{M \times \cos\theta}{(\rho)(Lo)(0.44)}, \quad (6.1)$$

where PCSA = physiological muscle cross-sectional area (cm^2), M = EDL muscle mass (g), cos θ = angle of pinnation for EDL muscle (0^o), ρ = density of mammalian skeletal muscle (1.06 g/cm^3), L_o = optimal muscle length (cm), 0.44 = L_f/L_m ratio for EDL muscle fiber length to the muscle belly length. Maximum specific isometric force was calculated as the maximum isometric force normalized to muscle PCSA. Healthy, uninjured sciatic nerves and EDL muscles were similarly tested and evaluated. The muscle mass of both the injured and uninjured EDL muscles were harvested after testing and weighed. The injured muscle mass was normalized to the uninjured muscle mass to determine the relative muscle mass and level of muscle atrophy following injury.

6.3.7 Retrograde labeling of spinal cord and DRG neurons

Retrograde labeling distinguishes and allows quantification of regenerating sensory and motor neurons. Twelve weeks postoperatively, the surgical site was reopened under general anesthesia. The trifurcation of the sciatic nerve 5 mm distal to the conduit or graft was transected, and the proximal portion was immediately placed in a silicone well containing 4% Fluorogold solution (Sigma-Aldrich, St. Louis, MO). The nerve was allowed 1 h to bath in the solution. The silicone well and solution were removed and the wound irrigated with saline, dried, and closed with a running 5-0 vicryl suture in muscle fascia, and

then interrupted 4-0 nylon skin sutures. The contralateral side was labeled in the same manner immediately following the experimental side as a control for the retrograde tracing. Animals were recovered in a warm environment, and after recovery the animals were returned to the housing facility.

Ten days following the procedure animals were euthanized and perfused in 4% paraformaldehyde, and the spinal cord (Lumbar regions L3 – L6) and DRG (L4 & L5) harvested. Twenty μm axial sections of the DRG and lumbar spinal cord were obtained on a cryostat (Leica Microsystems). The number of labeled cell bodies on the control and experimental side of each ventral horn spinal cord and DRG section were counted using an optical dissector technique facilitated by MicroBrite Field stereology software (MBF Bioscience StereoInvestigator version 7.0, Williston, Vermont), where count estimates were accepted if the Gundersen coefficient of error was less than 0.08. Values were reported as the percentage of cell bodies labeled relative to the contralateral, uninjured side.

6.3.8 Statistical analysis

All results are reported for animals with nerve regeneration (nerve cable present in conduit/isograft) as mean ± standard error of the mean. Statistical analyses were performed using Statistica version 6 (Statsoft Inc., Tulsa, OK). All data were evaluated for differences between groups using the Analysis of Variance (ANOVA) with post hoc LSD tests with Bonferroni correction used for determining differences between groups with significance set at $\alpha = 0.05$ ($p<0.05$).

6.4 Results

6.4.1 Nerve guidance conduit harvest

The effectiveness of NGF or GDNF in promoting nerve regeneration across a critical nerve gap was evaluated *in vivo* after sciatic nerve transection and NGC implantation. After 12 weeks, groups with the DS and growth factor resulted in higher nerve regeneration effectiveness (a neural structure spanning the 13 mm gap, Table 6.1). Combining animals used in both portions of the study (evoked motor response and retrograde labeling), 10 of 16 conduits from the GDNF with DS (GDNF DS) and 11 of 16 conduits from NGF DS groups contained regenerated nerve cables. Only 2 of 16 conduits from the GDNF (no DS) group, 5 of 16 conduits from the NGF (no DS) group, 6 of 16 conduits from the DS alone with no growth factor (DS (no GF)) group, and 5 of 16 conduits from the empty group demonstrated nerve regeneration, representing a frequency of less than half the animals regenerating any neural tissue. All 16 animals in the isograft group had regeneration. The regenerated nerves in the NGF DS and GDNF DS groups exhibited a larger, more robust nerve cable in comparison to the other experimental conduit groups by gross observation. The neural structure was centered compactly in the conduit, away from the walls, in all conduit specimens and had a smaller cross-sectional area than the isograft group. All conduit specimens demonstrated intact connections to the proximal or distal sciatic nerve, despite variability in regeneration.

6.4.2 Behavioral testing

All animals regardless of nerve regeneration were assessed for functional recovery using noninvasive behavioral testing over the course of 12 weeks. Only animals with nerve regeneration, as determined at nerve harvest, were included in the behavioral analysis, which include animals pooled from the entire study, both the evoked motor response and retrograde labeling of SC and DRG neurons. The average preoperative SFI score was -7.3 ± 1.1 for experimental groups with no differences between groups, demonstrating normal function before injury. No experimental animals demonstrated improved functional recovery 4, 8 or 12 weeks after injury as SFI scores did not differ between weeks (Figure 6.1). Toe and ankle contractures (abnormal distortions in the toes and ankles) were observed at 8 and 12 weeks after injury, which disqualified these animals for use in walking track analysis testing since clear toe spreads and/or print lengths were unobtainable. However, animals without contractures did not exhibit differences in SFI scores between groups at 4, 8, or 12 weeks after injury.

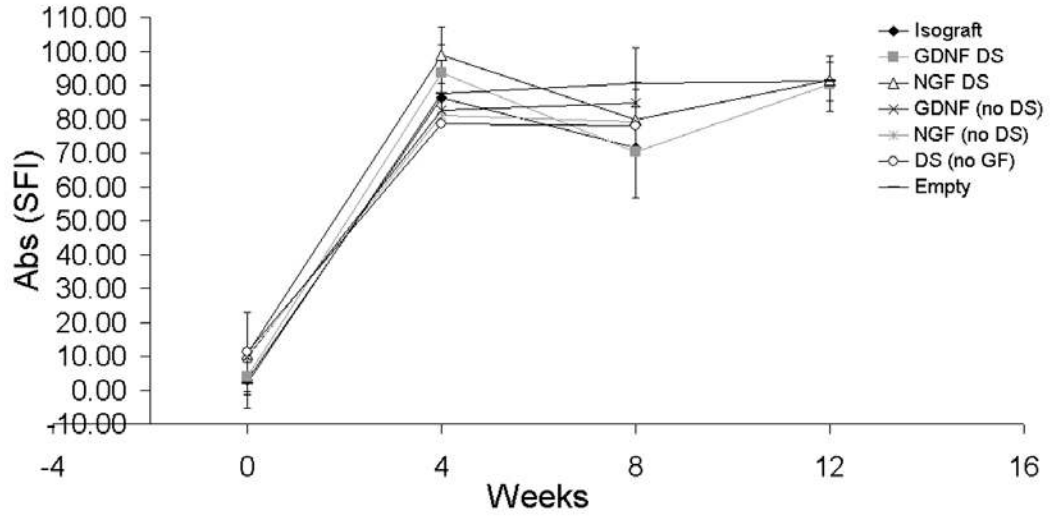

Figure 6.1. Walking track analysis for groups with nerve regeneration 12 weeks after injury. Animal hind limb foot tracks were recorded and scored for the sciatic functional index (SFI). No experimental groups demonstrated improved behavioral recovery at any time point and no differences were found between groups at any time point. Data represents mean ± S.E.M.

Animals were also placed on a wire grid every 4 weeks, and the number of times the animal successful used its toes to grip the wire mesh without slipping and with visible toe curling were recorded to measure return to normal, uninjured behavior. All groups preoperatively averaged high successful grid-grips percentages (79 ± 13%) with no differences between experimental groups. Four weeks after injury, all experimental groups demonstrated poor grid-gripping ability with successful grid-grip percentages at nearly zero for all groups, and no differences were found between groups (Figure 6.2). At 8 weeks, the isograft had improved functional recovery compared to all other groups with an average successful grid-grip of 6.8 ± 1.4%, indicating possible earlier neural reinnervation of muscles. However, at 12 weeks the GDNF DS group surpassed the isograft in functional recovery with a higher successful grid-grip (13 ± 2%) compared to the isograft (5.9 ± 1%). All other experimental groups except the empty conduit group (0.4 ± 0.4%) were similar to the

isograft at 12 weeks in successful grid-grip percentages. Additionally, all experimental groups except GDNF (no DS) had improved functional recovery compared to the empty conduit group.

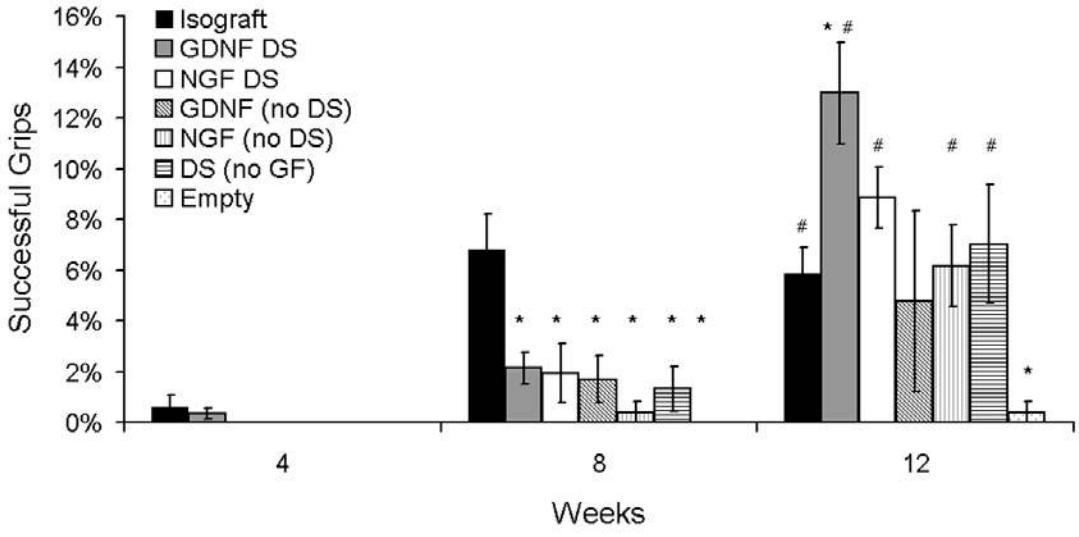

Figure 6.2. Successful grid-grip percentages for groups with nerve regeneration 12 weeks after injury. Animals were placed on a wire mesh to assess the number of times the animal successful placed its toes to grip the wire mesh without slipping and the total number of steps taken with the injured foot. The isograft had improved recovery compared to all groups at 8 weeks; however, GDNF with the delivery system (GDNF DS) had more successful grid-grips compared to the isograft at 12 weeks. All other experimental groups performed equal to the isograft except the empty conduit group at 12 weeks. Data represents mean ± S.E.M., * indicates statistical significance ($p<0.05$) compared to the isograft, and # indicates statistical significance ($p<0.05$) compared to empty.

6.4.3 Evoked Motor Response

The sciatic nerve was stimulated proximal to the injury site to determine functional recovery in the peroneal branch by measuring CNAPs for animals with nerve regeneration across the conduit. Normal, uninjured peroneal nerve produced maximum CNAPs of 12 ± 1.3 mV and first elicited a motor response (foot twitch) with CNAP at 42 ± 4 μA. The number of animals with regeneration and tested for this portion of the study is summarized

in Table 6.1. The DS (no GF) and empty groups contained inferior regeneration that complicated measurements, where neither group had more than one animal with nerve regeneration with recordable CNAPs; therefore, these groups were omitted from the analysis as statistics could not be performed for these groups. Groups with growth factor (with or without the DS) and isografts demonstrated similar CNAPs (1.5 – 0.4 mV) and similar elicited motor response thresholds (400 – 180 μA), which were different than normal nerve (Figure 6.3A & B). Data tended to have high variance due to variability in neural regeneration in combination with tissue scarring and bleeding, which complicated measurements.

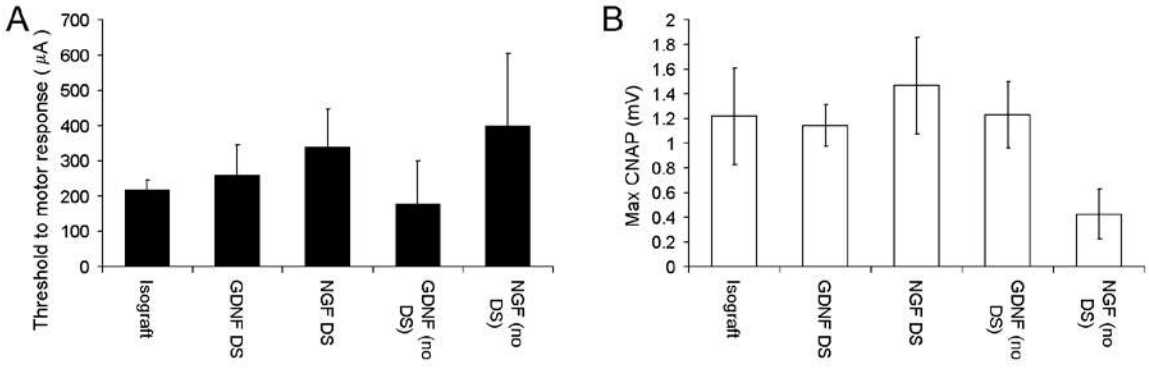

Figure 6.3. Maximum CNAPs and current thresholds to elicit motor responses for groups with nerve regeneration 12 weeks after injury. The peroneal nerve was stimulated proximal to the graft or conduit at variable current amplitudes to measure CNAPs (A) and to elicit motor responses (B). The maximum CNAP and current threshold amplitudes to elicit motor responses were not different between experimental groups. Data represents mean ± S.E.M.

The sciatic nerve was stimulated proximal to the graft or conduit to measure whole force production in the EDL. These data were normalized to the cross-sectional area of the muscle to obtain the specific force, which measures deficits in force capacity and is independent of muscle mass. Normal, uninjured nerve produced twitch and tetanic specific forces of 5.0 ± 0.48 N/cm^2 and 16 ± 1.6 N/cm^2, respectively. No experimental group

matched the normal nerve in either measure. All groups except GDNF groups produced similar twitch and tetanic specific forces compared to the isograft, including groups without growth factor, the empty and DS (no GF) conduits (Figure 6.4); however, groups without growth factor tended to have high variance due to low number of animals with nerve regeneration, which diminished power for statistical comparisons. Both twitch and tetanic specific forces increased for the GDNF DS (3.4 ± 0.36 N/cm^2 and 8.9 ± 1.2 N/cm^2, respectively) and GDNF (no DS) (3.6 ± 0.25 N/cm^2 and 11 ± 0.14 N/cm^2, respectively) groups compared to the isograft (1.4 ± 0.4 N/cm^2 and 4.0 ± 0.8 N/cm^2, respectively). These groups were also increased compared to empty (1.1 ± 1.1 N/cm^2 and 2.3 ± 2.3 N/cm^2, respectively) and DS (no GF) (0.98 ± 0.74 N/cm^2 and 3.2 ± 2.0 N/cm^2, respectively) groups. Overall, the GDNF delivery results possibly indicate superior muscle reinnervation.

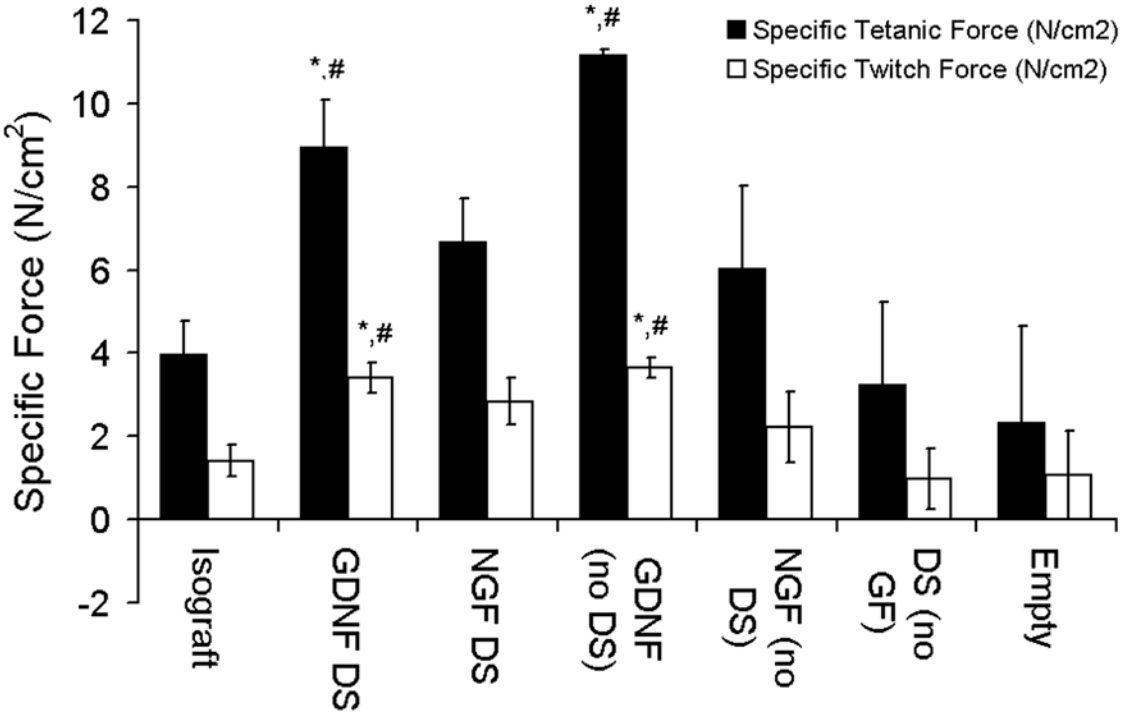

Figure 6.4. Specific force measurements of EDL for groups with nerve regeneration 12 weeks after injury. EDL muscles were stimulated proximal to the graft or conduit to the produce maximal twitch and tetanic muscle force, which were normalized to the muscle cross sectional area. All groups, except GDNF groups, produced similar specific forces compared to the isograft. Specific twitch and tetanic forces increased for GDNF groups, compared to empty and DS (no GF) groups and the isograft. Data represents mean ± S.E.M., * indicates statistical significance ($p<0.05$) compared to the isograft, and # indicates statistical significance ($p<0.05$) compared to empty or DS (no GF) groups.

EDL muscles (experimental and contralateral sides) were harvested and weighed after evoked motor response testing and normalized to the contralateral (uninjured side) muscle mass to assess the level of muscle atrophy due to loss of nerve innervation. Groups with growth factor (53 – 63%) were similar to the isograft (~ 62%), while groups without growth factor, empty (~ 30%) and DS (no GF) (~ 41%) groups, had decreased relative muscle mass compared to the isograft (Figure 6.5). Additionally, groups with growth factor, except NGF (no DS), had increased relative muscle mass compared to the empty conduit group.

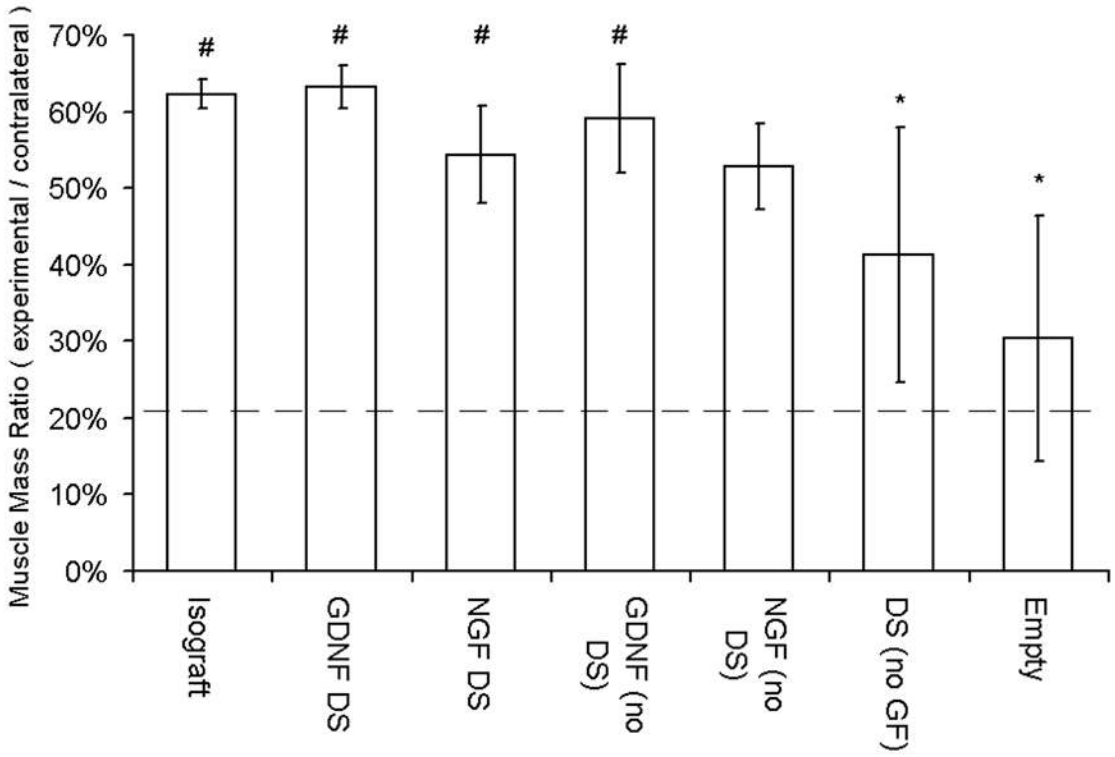

Figure 6.5. Relative muscle mass of EDL for groups with nerve regeneration 12 weeks after injury. EDL muscles (experimental and contralateral sides) were harvested and weighed, and the experimental muscle mass normalized to the contralateral mass. Groups with growth factor were similar to the isograft in relative muscle mass. Data represents mean ± S.E.M., * indicates statistical significance ($p<0.05$) compared to the isograft, and # indicates statistical significance ($p<0.05$) compared to empty.

6.4.4 Retrograde labeling

Experimental and contralateral (for normalization) sciatic nerves were labeled with Fluorogold solution to stain ventral horn and DRG neurons for animals with nerve regeneration. Cell counts of labeled axons in both regions allow quantification of regenerating motor and sensory neurons, respectively. The number of animals with regeneration and tested for this portion of the study is summarized in Table 1. Neither NGF (no DS) nor GDNF (no DS) had more than one animal with nerve regeneration for this portion of the study; therefore, these groups were omitted from the analysis as statistics

could not be performed for these groups. Differences in cell sizes, such as diameter, or locations within the spinal cord or DRG between experimental groups could indicate differences in neuronal populations and fiber types regenerating. However, no qualitative differences in labeled neurons were observed between GDNF DS, NGF DS, and isograft groups (Figure 6.6).

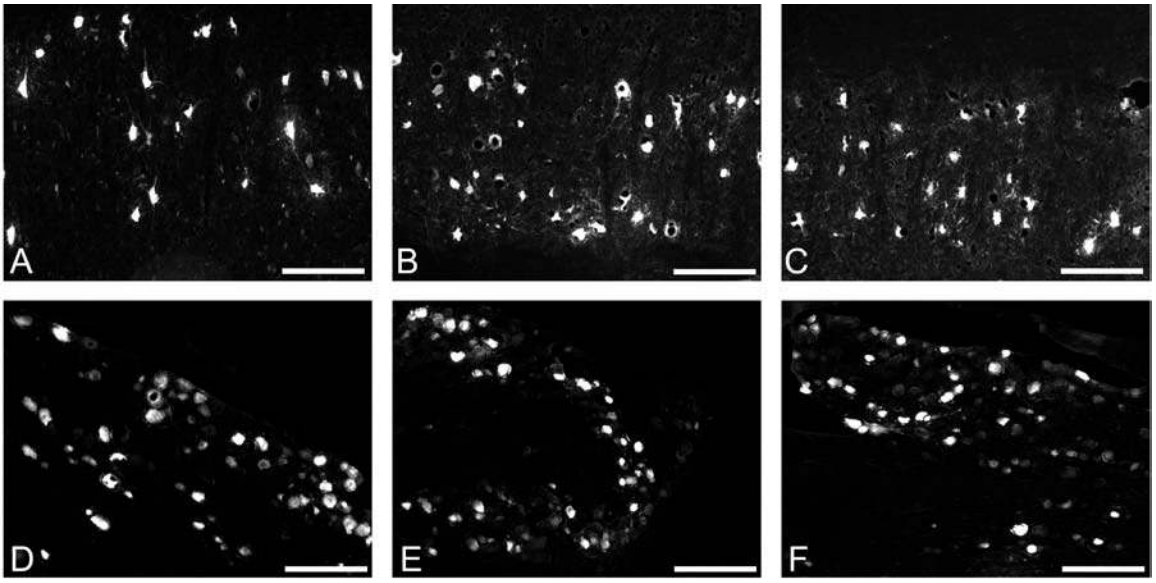

Figure 6.6. Representative sections of retrograde labeled ventral horn SC and DRG neurons 12 weeks after injury. The experimental and contralateral sciatic nerves were labeled with 4% fluorogold to stain sensory (DRG) and motor (ventral horn SC) neurons. No qualitative differences in cell sizes within the SC (A – C) or DRG (D – F) between experimental groups were observed between isograft (A, D), GDNF DS (B, E), or NGF DS (C, F) groups. Scale bar represents 200 μm.

Normal, uninjured sciatic nerve had 3200 ± 180 ventral horn neurons and 11,000 ± 560 total L4 & L5 DRG neurons labeled. All experimental group ventral horn and DRG neuron counts were normalized to contralateral (uninjured side) nerve counts in their respective region to yield a percentage of regenerating neurons. GDNF DS (62 ± 11%) and NGF DS (62 ± 7.1%) groups were comparable to the isograft (57 ± 4.5%) in the percentage of normalized ventral horn neurons (Figure 6.7A). These three groups all had greater

percentages of normalized ventral horn neurons compared to empty (1.4 ± 1.4%) and DS (no GF) (23 ± 7.5%) groups. The percentage of normalized DRG neurons were similar for GDNF DS (44 ± 4.7%), NGF DS (41 ± 7.2%), and DS (no GF) (44 ± 6.9%) groups compared to the isograft (45 ± 2.9%). Additionally, these groups had increased percentages of normalized DRG neurons compared to the empty conduit (14 ± 14%) group (Figure 6.7B). No differences between GDNF DS or NGF DS groups were found in either percentage of normalized ventral horn or DRG neurons.

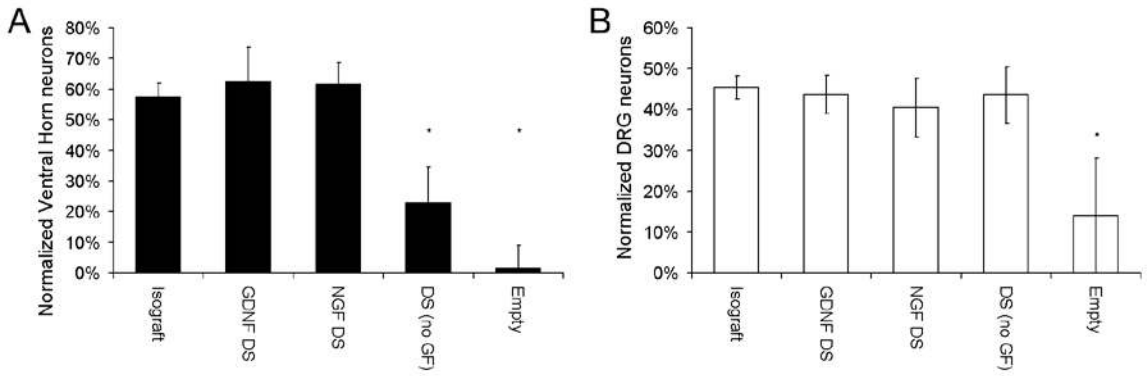

Figure 6.7. Percentage of normalized ventral horn SC and DRG neurons retrograde labeled 12 weeks after injury. The experimental and contralateral sciatic nerves were labeled distal to the conduit or graft with 4% fluorogold to stain regenerating motor (ventral horn SC; A) and sensory (DRG; B) neurons. DRG (L4 & L5) and spinal cords (ventral horn region) were harvested 10 days after labeling, and 20 μm sections were evaluated for total cell numbers using stereology techniques. GDNF DS and NGF DS were comparable to the isograft in percentages of normalized ventral horn and DRG neurons. Additionally, these three groups contained more labeled ventral horn and DRG neurons compared to empty conduit groups. Data represents mean ± S.E.M. where all cell counts were normalized to the contralateral side, and * indicates statistical significance ($p<0.05$) compared to the isograft.

6.5 Discussion

This study investigated whether the delivery of growth factors using affinity-based delivery can affect modality specific nerve regeneration and functional recovery. Diffusion-based delivery systems have utilized growth factor delivery to promote nerve regeneration

and modality specific nerve regeneration (Fine, Decosterd et al. 2002). We considered an affinity-based DS, which uses non-covalent interactions to sequester and slow the release of growth factor leaving it available for cell-mediated release. Cell-mediated processes, such as neurite outgrowth induced protease activation (e.g. plasminogen to plasmin), cleave fibrin into smaller fragments, which can regulate growth factor release from fibrin matrices (Kalderon 1984; Krystosek and Seeds 1984; Alvarez-Buylla and Valinsky 1985; Pittman and Buettner 1989; Pittman, Ivins et al. 1989; Herbert, Bittner et al. 1996). Therefore, this study investigated whether a different delivery method, our affinity-based DS, could stimulate modality specific nerve regeneration.

Regardless of growth factor used, the effectiveness of nerve regeneration or frequency of neural regeneration across the 13 mm nerve gap, in the form of a nerve cable spanning the conduit, was increased with the presence of the DS (see Table 6.1). Nerve regeneration effectiveness decreased in groups without growth factor or DS establishing that both are essential to improve effectiveness. Others have observed similar increases in nerve regeneration effectiveness with controlled growth factor delivery but decreased effectiveness without controlled growth factor delivery (Barras, Pasche et al. 2002; Fine, Decosterd et al. 2002; Dodla and Bellamkonda 2008). Previously, an initial burst was observed *in vitro* from fibrin matrices with free growth factor (either NGF (Wood and Sakiyama-Elbert 2008) or GDNF (Wood, Borschel et al. 2009)), while sequestered growth factor improved neurite outgrowth compared to free growth factor with no DS (Sakiyama-Elbert and Hubbell 2000a; Wood and Sakiyama-Elbert 2008; Wood, Borschel et al. 2009). A possible reason that fibrin matrices with free growth factor (no DS) did not improve regeneration effectiveness could be due to an initial burst of growth factor from the conduit, which has been cited by others as detrimental to nerve regeneration (Barras, Pasche et al. 2002). An initial burst would also

leave less growth factor remaining to promote regeneration for migrating cells and axons growing into the conduit. A need for sustained growth factor release could also explain the low regeneration effectiveness in groups without growth factor.

Two behavioral analyses were performed to detect differences in functional recovery between groups. No differences in behavioral recovery were observed between groups as measured by SFI scores. The lack of differences in SFI scores determined by walking track analysis was likely due to frequent foot contractures observed in animals, which decreases the number of animals that could be assessed using this method and has been cited by others (Hare, Evans et al. 1992; Hare, Evans et al. 1993), Conversely, in the second analysis, grid-grip, contracture formation analysis does not prevent measurements and is therefore beneficial compared to walking track analysis in this regard.

In grid-grip analysis, groups with fibrin were similar to the isograft in behavioral recovery. The overall improvement in successful grid-grip in groups with a fibrin matrix could be due to the inclusion of a substrate for cellular adhesion, which is lacking in empty conduits. Initial experiments in the rat sciatic nerve revealed that at one week a fibrin matrix spans a silicone conduit used to contain nerve stumps, which is later followed by nerve regeneration (Williams, Longo et al. 1983). Furthermore, fibrin has been used as a biomaterial to support nerve regeneration within a NGC (Lee, Yu et al. 2003a; Galla, Vedecnik et al. 2004; Marcol, Kotulska et al. 2005) and can promote cell adhesion because it contains binding sites for integrins (Thiagarajan, Rippon et al. 1996) and Schwann cells (Chernousov and Carey 2003), Therefore, a fibrin matrix can promote neural regeneration.

We observed faster behavioral recovery for the isograft group compared to conduit groups, as the isograft had a higher successful grid-grip percentage at 8 weeks. However, the

GDNF DS group had improved behavioral recovery compared to the isograft at 12 weeks. The improved recovery for the isograft group at 8 weeks may be due to a previous observed increase in nerve fibers compared to other groups at 6 weeks (Wood, Moore et al. 2009), which correlates with improved function (Aydin, Mackinnon et al. 2004; Lien, Cederna et al. 2008). However, in the same studies, GDNF DS had a greater number of larger (5 – 7 μm) mature fibers than the isograft (Wood, Moore et al. 2009), which can correlate with greater return in function compared to smaller fibers (Williams and Wendell-Smith 1971; Fraher and Dockery 1998). Additionally, increases in EDL twitch and tetanic specific forces for the GDNF DS group correlated with an increase in grid-gripping ability.

There were no differences between experimental groups in measured CNAPs or current amplitude thresholds to elicit motor responses. The largest contributing factor to this outcome was high variance in recording, likely due to tissue scarring near the nerve and blood from the surrounding tissue interfering with neural electrode contact. However, CNAP recordings do not clearly indicate return of function because the peroneal nerve contains a mixture of sensory and motor fibers. Therefore, measured CNAPs are the summation of all nerve fiber types generating action potentials and recording muscle force production can better indicate functional recovery.

EDL specific force production measurements demonstrated that treatment with growth factor produced muscle force that was comparable to the isograft. Most groups with growth factor (except NGF (no DS)) also had decreased muscle atrophy compared to groups without growth factor. Taken together, these results indicate that muscle reinnervation may generally improve due to growth factor release. While the empty and DS (no GF) conduit groups produced similar specific forces as the isograft, this result is likely

due to high variation in nerve regeneration between animals. Rats have superior neural regeneration compared to humans, as the rat is able to spontaneously regenerate into an unfilled 4.5 cm nerve gap to a distance of 2.4 cm after 5 months (Mackinnon, Hudson et al. 1985). Therefore, negative controls in this injury model tend to have high biological variance, which compounded with low regeneration effectiveness in these groups, results in poor statistical comparisons.

A significant outcome in the study was increased twitch and tetanic specific EDL forces for GDNF, with or without the DS, compared to groups without growth factor or the isograft. These results suggest superior muscle reinnervation in groups that received GDNF. GDNF is upregulated in Schwann cells in the distal nerve stump and skeletal muscle after injury (Nagano and Suzuki 2003; Zhao, Veltri et al. 2004) and regulates presynaptic differentiation and neuromuscular junction connections (Nagano and Suzuki 2003; Yang and Nelson 2004). Therefore, in our study, exogenous delivery of GDNF at the site of injury could have amplified the existing endogenous mechanism for motor nerve regeneration and resulted in improved functional reinnervation of the muscle. This hypothesis may explain why groups with GDNF regardless of controlled delivery performed better than the isograft in EDL twitch and tetanic specific force production. Furthermore, normalized motor neuron counts for the GDNF DS were comparable to the isograft, which would indicate that the motor neurons available are establishing more meaningful functional connections with muscles.

It was not anticipated that the NGF DS would have been similar to the isograft in functional recovery as well as motor neuron counts because NGF cannot target motor neurons due to a lack of receptors. Previous studies using NGCs with growth factors that

targeted motor neurons or axons, such as GDNF, have increased motor axon counts (Barras, Pasche et al. 2002) or retrograde labeled motor neuron counts (Fine, Decosterd et al. 2002) compared to other growth factors such as NGF and NT-3. We observed no differences in retrograde labeled motor neuron counts between GDNF or NGF delivered with the DS but did observe functional differences, as GDNF performed better than the isograft in functional recovery measures. The differences in our study results compared to the previous studies could be the inclusion of a material for cellular migration and axonal growth within the NGC and the delivery of growth factors from the material, as opposed to diffusion-based delivery from the NGC. We hypothesize that the inclusion of the DS with growth factors within the material facilitates both modality specific nerve regeneration and Schwann cell migration into the conduit, promoting neural regeneration. The Mackinnon lab has demonstrated that isografts facilitate better nerve regeneration compared to decellularized nerve allografts based on measures of nerve regeneration effectiveness and histomorphometry (Whitlock, Tuffaha et al. 2009). They attributed the enhanced nerve regeneration in part to Schwann cells retained in isografts promoting nerve regeneration. Additionally, Schwann cells contain receptors for both NGF (Taniuchi, Clark et al. 1988; Anton, Weskamp et al. 1994) and GDNF (Iwase, Jung et al. 2005), which can facilitate cell signaling leading to increased cell proliferation and migration (Anton, Weskamp et al. 1994). Therefore, our DS may target motor axons with GDNF, explaining the improvement in functional recovery compared to the isograft, while also encouraging Schwann cell migration, explaining similarities in functional recovery and motor neuron counts between NGF DS and the isograft.

Silicone conduits serve as a useful model to study nerve regeneration, but clinically have associated morbidities. Silicone conduits have been reported to cause chronic nerve

compression, irritation at the implantation site requiring removal, and inflammatory reactions impacting nerve regeneration (Merle, Dellon et al. 1989; Dellon 1994; Battiston, Geuna et al. 2005). We examined our DS within a silicone NGC because of the biocompatibility of the product, the mechanical stability of the conduit wall thickness chosen, and well characterized critical defect length in our surgical model. The combination of our DS with a biodegradable conduit would be more desirable for clinical peripheral nerve injury repairs. Based on the positive functional outcomes observed in our study, work directed toward this goal would be beneficial in translating our DS into clinical practice.

6.6 Conclusions

In summary, the goal of this study was to determine if sensory or motor nerve fibers regenerated and the functionality of regeneration due to growth factor delivery with an affinity-based DS to the regenerative site in a rat sciatic nerve critical defect. We examined behavioral outcomes and electrophysiological responses including evoked motor responses and compared the measures to controls. We found similar functional outcomes as the isograft with the delivery of NGF, but superior functional outcomes with the delivery of GDNF, both supported by histological counts of regenerating motor and sensory neurons. Due to the observed improved functional measures, we believe that affinity-based delivery of growth factors offers insight into potential future alternatives for the treatment of peripheral nerve injuries.

Chapter 7

Summary and Future Directions

7.1 Summary of Findings

The results of this thesis work indicated that a fibrin affinity-based delivery system (ABDS) delivering growth factors can promote peripheral nerve regeneration. These results were determined through five studies. These objectives of the studies were: to assess the role of peptide binding affinity for heparin in nerve regeneration through the delivery of nerve growth factor (NGF); to assess the ability of a different growth factor, glial-derived neurotrophic factor (GDNF), to promote nerve regeneration when delivered from the ABDS; and to determine the effect of growth factor delivery from the ABDS in promoting functional recovery following injury.

The first and second study of this thesis work addressed the role of peptide binding affinity for heparin in promoting nerve regeneration. The first study determined if peptide binding affinity for heparin and the molar ratio of peptide to heparin affected the release rate and biological activity of NGF. Mathematically modeling of the delivery system and *in vitro* experiments confirmed that release rates could be controlled by both peptide binding affinity for heparin and the molar ratio of peptide to heparin. The ABDS was also found to present biologically active NGF as assayed by chick embryo dorsal root ganglia (DRG) neurite extension, regardless of peptide binding affinity for heparin. Thus release rate appeared to be the main mechanism controlling the biological activity of released NGF.

The second study extended the first study to evaluate the efficacy of the ABDS *in vivo* to promote nerve regeneration in a rat sciatic nerve critical defect and determine if peptide binding affinity for heparin affected nerve regeneration. Histological outcomes demonstrated that the ABDS with any affinity peptide and NGF was similar to the isograft in aspects of nerve regeneration including: nerve fiber density, the quality of nerve regeneration, nerve fiber maturity, and the neural fiber organization of the regenerating nerve 6 weeks after treatment. Additionally, no differences in nerve regeneration due to heparin-binding affinity were observed, but general trends indicated that stronger peptide binding affinity for heparin promoted superior nerve regeneration.

Based on the results of the first and second studies, the third and fourth study addressed the role of GDNF in the context of the ABDS. These studies focused on the delivery of GDNF from the ABDS incorporating the strongest heparin-binding peptide, ATIII. The ABDS effectively sequestered and slowed the release of GDNF, and the ratio of peptide to heparin was found to modulate the rate of GDNF release *in vitro*. The ABDS and GDNF were found to promote neurite extension comparable to GDNF and fibrin matrices alone at specific concentrations.

The fourth study evaluated the efficacy of affinity-based delivery of GDNF in a rat sciatic nerve critical defect. Histological outcomes of nerve fiber density, the quality of nerve regeneration, nerve fiber maturity, myelination, and the neural fiber organization of the regenerating nerve demonstrated that the ABDS and GDNF were similar to the isograft 6 weeks after treatment. Therefore, the ABDS can promote peripheral nerve regeneration following injury.

Based on the outcome in short-term (6 week) *in vivo* studies, the objective of the fifth study was to assess functional recovery and modality specific nerve regeneration with the ABDS and growth factor in a rat sciatic nerve critical defect over 12 weeks following treatment. Behavioral outcomes and electrophysiological responses including evoked motor responses were similar to functional outcomes in the isograft with the delivery of NGF, but superior to the isograft with the delivery of GDNF. Additionally, both GDNF and NGF delivery supported the regeneration of motor and sensory neurons equivalent to the isograft, as assessed by retrograde labeling. Overall, this work indicates that affinity-based growth factor delivery from fibrin matrices enhances nerve regeneration.

7.2 Recommendations for Future Direction

This thesis work evaluated the role of peptide affinity and growth factor delivery with an ABDS for peripheral nerve injury. Based on the observed histological outcomes and functional measures, affinity-based delivery of growth factors offers potential alternatives for the treatment of peripheral nerve injuries. During the evaluation in the short-term animal studies (6 weeks), all experimental groups with a conduit for treatment of nerve injury demonstrated decreased regenerating nerve fiber counts compared to the isograft. This may be due to the silicone conduit limiting the area of nerve regeneration. This limitation could be addressed with a new conduit material (or structure) to contain the luminal fibrin matrix and ABDS. Additionally in the 6 week animal studies, the ABDS with GDNF or NGF were similar to the negative control (empty conduits) in regenerating nerve fiber counts. This result could again be due to the above mentioned silicone issue, which could limit the ability to demonstrate differences in fiber counts within the conduit. Alternatively, the fibrin

matrix and ABDS could be modulated to provide additional guidance cues for axons. As observed in the final study, GDNF promoted better functional recovery than even the isograft while NGF promoted functional recovery similar to the isograft. This result with NGF delivery was not anticipated due to limitations of NGF in stimulating and targeting motor neurons, which do not express the major receptor for NGF (TrkA). Overall, this result may indicate that growth factor delivery in general has a synergistic effect on nerve regeneration. Therefore, incorporating other known therapies for peripheral nerve injury, such as the incorporation of cells into a fibrin scaffold with the ABDS, may further effect nerve regeneration.

7.2.1 Advanced material and delivery strategies for nerve regeneration

In this thesis work, both GDNF and NGF promoted nerve regeneration. Although it is not clear from this work, either growth factor may have promoted nerve regeneration to an extent due to targeting and stimulating axonal growth in different neuronal populations, as sensory and motor neurons express different receptors. An interesting extension of this work would be to simultaneously present these growth factors to target the neuronal populations. Deister *et al.* demonstrated that presenting multiple growth factors to DRG can produce synergistic effects on neurite extension *in vitro* (Deister and Schmidt 2006). This ABDS is capable of binding multiple growth factors simultaneously (Willerth, Rader et al. 2008), which could allow for delivery of both NGF and GDNF from the same scaffold or other growth factor combinations.

This ABDS could alternatively include additional cell adhesion molecules or peptides that interact with heparin. For example, the peptide sequence IKVAV is the neurite binding

sequence contained on laminin (Tashiro, Sephel et al. 1989), and as a synthetic peptide could be chemically modified to include a heparin-binding domain. The incorporation of both cellular adhesive molecules and growth factors could have a synergistic or additive effect on nerve regeneration. For example, adding laminin to agarose gels improved nerve regeneration compared to agarose gels alone; however, combining laminin and NGF to agarose gels improved nerve regeneration more than laminin alone in agarose gels (Yu and Bellamkonda 2003). One consideration when incorporating additional growth factors or proteins that interact with heparin would be the availability of binding sites for these proteins, which limits the concentration of bound proteins. Based on the mathematical modeling done in this work, the use of multiple growth factors would be reasonable. The ratio of heparin to growth factor in the ABDS was on the order of thousands for the sustained and controlled release of a single growth factor. Cellular adhesion molecules and proteins are typically used at greater concentrations than growth factors; therefore, their use would be limited to lower concentrations with this delivery system.

The fibrin matrices used in this study form via an enzymatic reaction that covalently links randomly oriented fibrin monomers. Consequently, during polymerization fibrinogen fibers within a fibrin matrix possess the ability to be aligned by a magnetic field. Parallel, longitudinal arrangements of fibers in a material can promote nerve regeneration better than random oriented fibers. Work from the Bellamkonda laboratory recently demonstrated that synthetic polymer fibers oriented in a magnetic field to run parallel to regenerating nerve fibers enhanced nerve regeneration compared to randomly-oriented, synthetic polymer fibers (Kim, Haftel et al. 2008). Additionally, *in vitro* studies have demonstrated that similar alignment can increase neurite outgrowth two-fold over randomly oriented fibers in a fibrin matrix (Dubey, Letourneau et al. 2001). Incorporating the ABDS into a fibrin matrix with

aligned, parallel fibers could positively affect nerve regeneration. While it is unclear how the delivery of growth factor would be affected by the alignment, the presentation of growth factors from aligned fibers within the fibrin matrix would better mimic native nerve, which contains parallel, longitudinally-oriented basal lamina tubes.

Alternatively, the ABDS could be used to create a concentration gradient of growth factors or proteins that interact with heparin. *In vivo* concentration gradients of neurotrophic factors promote enhanced nerve regeneration compared to isotropic presentation of the same factors (Dodla and Bellamkonda 2008). Commonly for a concentration gradient of growth factors to exist in an implantable material, the growth factors are immobilized within the material. The ABDS sequesters growth factors due to a fast binding constant (k_f), which prevents long-term diffusion. Therefore, this ABDS could potentially sequester a gradient of a number of growth factors to construct an implantable material to promote nerve regeneration.

7.2.2 Alternative structure to contain the material for nerve regeneration

Although the ABDS with either GDNF or NGF promoted nerve regeneration equivalent to the isograft by many histological measures, this result could be improved. The silicone conduit used to contain the system proved detrimental in nerve regeneration as measured by a decrease in the total number of regenerating nerve fibers for any experimental groups with a conduit. Other researches have noticed this effect on nerve regeneration as well (Lundborg, Dahlin et al. 1982b). Additionally, the implantation of a permanent conduit could result in adverse tissue reactions, as noted by others (Dellon 1994), which could

require a second surgery to remove it. Therefore, a different structure to house the material would be useful to eliminate the need to permanently implant a device and potentially improve nerve regeneration due to an increase in area available for nerve regeneration.

Loading the scaffold into a biodegradable nerve guidance conduit (NGC) would eliminate the safety concerns of permanently implanting a medical device. It also capitalizes on the natural degradation of the fibrin scaffold to construct a device that completely degrades over time leaving behind the regenerated nervous tissue. While this ABDS is capable of delivering multiple growth factors, as previously discussed, a biodegradable NGC offers the potential to deliver growth factors as well. Growth factor delivery from the NGC and the luminal scaffold would permit greater degrees of freedom in modulating release rates, which can affect nerve regeneration. Previously Piquilloud *et al.* designed collagen NGCs with layers of poly(lactide-coglycolide) that varied in thickness to produce conduits that can deliver GDNF at variable rates based on layer thickness. The NGCs with highest release rate of GDNF resulted in improved histological outcomes compared to delivery at slower release rates (Piquilloud, Christen et al. 2007). Therefore, an additional release vehicle could allow release rates of growth factors to be modulated to best promote nerve regeneration. Additionally, the ABDS used in this work is limited in its ability to deliver growth factors over long periods by degradation of the fibrin matrix, which completely degrades by 2-4 weeks (unpublished data from the Sakiyama-Elbert lab). Delivery from a NGC could provide growth factor delivery for periods beyond the fibrin degradation limit. Overall, biodegradable NGCs have benefits compared to non-resorbable NGCs; however, biodegradable NGCs still demonstrate problematic issues similar to silicone, where the available area for nerve regeneration is limited in the NGC before the NGC degrades

(Francel, Smith et al. 2003), and the NGC can cause compression on the nerve as the conduit degrades (Borkenhagen, Stoll et al. 1998).

Materials, such as fibrin, are not limited to a NGC, and due to its ability to polymerize to a stable hydrogel, it could be incorporated into other structures, such as biological grafts. In particular, this scaffold could be utilized in decellularized biological grafts to possibly improve their outcome. For example, decellularized muscle grafts do not produce nerve regeneration similar to autografts, but can match autografts in some histomorphometric measures of nerve regeneration when beneficial cues for nerve regeneration, such as Schwann cells (SCs), are loaded into the muscle graft (Fansa and Keilhoff 2004). Therefore, the use of both an inductive material, a fibrin matrix with an ABDS as demonstrated in this work, and decellularized biological graft could produce additive or synergistic effects on nerve regeneration. Perhaps the ideal structure would be a decellularized nerve allograft, which would combine the alignment and natural structure of nerve with a vehicle to deliver growth factors.

7.2.3 Cell transplantation for nerve regeneration

The migration of SCs into the NGC was not studied in this thesis work, but may have played a role in promoting nerve regeneration as they are known to secrete trophic support and can adhere to a fibrin scaffold. This ABDS has been used for many other applications including a scaffold for cellular growth, specifically with embryonic stem cell-derived neural progenitor cells (Willerth, Rader et al. 2008). Furthermore, the fibrin scaffold with the ABDS was capable of promoting these cells to differentiate due to controlled growth factor release from the ABDS. Therefore, SCs cultured within the scaffolds with

controlled growth factor delivery may enhance their proliferation, migration, and myelination capabilities. The transplantation of such a scaffold could enhance peripheral nerve regeneration.

Previous work with the transplantation of SCs has shown mixed success. For example, SCs loaded into biological grafts have proven beneficial (Fansa and Keilhoff 2004), but others have not observed beneficial aspects. Specifically, Evans *et al.* loaded SCs into a collagen scaffold in a degradable NGC to treat rat sciatic nerve injury. However, the combination approach did not perform better in nerve regeneration measures than collagen alone (Evans, Brandt et al. 2002); therefore, care must be taken to optimize SC growth parameters with that of axonal regeneration in order to promote additive or synergistic effects on nerve regeneration due to their transplantation.

Another aspect of SC transplantation that would be of interest to study would be the effect of modality specific SC transplantation. Hoke *et al.* determined that SCs from primarily sensory or motor specific nerve branches express different levels of growth factors, indicating that SCs have specific phenotypes (Hoke, Redett et al. 2006). This result could be further extrapolated to explore modality specific regeneration. For example, motor branches may contain SCs that guide motor axons to the correct parent target (Brushart 1988). Therefore, the transplantation of phenotypic specific SCs within a conduit or graft could affect nerve regeneration modalities.

Although the proposed future work focuses on the use of the ABDS for treatment of peripheral nerve injury, there could be potential for treatment of other diseases and injuries. As already mentioned, this ABDS can be employed using a variety of growth factors that interact non-covalently with heparin and to culture cells. It has been used for a

variety of potential treatment applications, which include flexor tendon repair (Gelberman, Thomopoulos et al. 2007) and spinal cord injury (Taylor, McDonald et al. 2004; Taylor and Sakiyama-Elbert 2006). Due to the positive outcomes demonstrated in this thesis work, future work with this biomaterial for local drug delivery and cell culture would be beneficial.

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