BIOFUNCTIONAL POLYMERS FOR THE CONTROLLED RELEASE OF GROWTH FACTORS IN THE PERIPHERAL NERVOUS SYSTEM

Thesis by

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Dedication

To Don for his constant love and support.

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Abstract

Fibrin is the natural biomaterial of nerve regeneration. Fibrin possesses the ability to promote cell adhesion and can be degraded locally by cell-regulated proteases. However, fibrin lacks sufficient neuroinductive character to promote nerve regeneration across large gaps. A previously developed method for the incorporation of peptides via the transglutaminase activity of factor XIIIa was used to immobilize cell adhesion sites and growth factors within fibrin matrices that could serve as potential therapeutics for peripheral nerve regeneration.

Heparin-binding domains from several proteins have been shown to promote neurite extension on surfaces. Four different heparin-binding domains were immobilized within fibrin matrices using factor XIIIa and all four domains were found to enhance three-dimensional neurite extension through fibrin. The ability of these domains to enhance neurite extension was found to correlate positively with their relative heparin-binding affinity.

Prolonged release of exogenous growth factors over the duration of nerve regeneration could potentially enhance regeneration. Two delivery systems were developed to provide controlled release of exogenous growth factors, where the rate of release could be regulated by cellular activity rather than by diffusion. The first system developed was designed to mimic the ability of the extracellular matrix to sequester heparin-binding growth factors based on interactions with heparin sulfate proteoglycans. It was hypothesized that by immobilizing a high excess of heparin sites within fibrin, diffusion of growth factors from the matrix could be slowed, and this would allow release to be dominated by cell-regulated matrix degradation. The ability of such systems to

immobilize growth factors and release them in an active form was assayed using neurite extension from dorsal root ganglia (DRGs).

A second method of delivery was developed consisting of recombinantly expressed nerve growth factor (NGF) fusion proteins containing an exogenous crosslinking substrate. A plasmin-degradable linker was placed between the crosslinking substrate and the NGF domain. These fusion proteins could be immobilized in fibrin using factor XIIIa and released in a native form by plasmin cleavage. Immobilized NGF fusion proteins were found to enhance neurite extension from DRGs within fibrin matrices versus similar concentrations of native NGF in the medium.

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Chapter 1 Introduction

1.1 Objective of Research

The objective of this research was to enhance the ability of fibrin-based tissue engineering scaffolds to promote peripheral nerve regeneration. Fibrin was chosen as the base material for these tissue engineering scaffolds because of its ability to promote cell adhesion and its ability to be degraded in a localized manner by cell-regulated proteases. In addition, fibrin glues are commercially available and currently approved for clinical use in both the US and Europe.

Cell adhesion proteins and peptides from adhesion proteins have previously been shown to enhance nerve regeneration. Heparin-binding domains are a class of adhesion domains that have previously been demonstrated to enhance neurite outgrowth on two-dimensional surfaces. Therefore, it was hypothesized that the immobilization of exogenous heparin-binding peptides could enhance three-dimensional neurite extension through fibrin matrices. Thus, *the first objective of this research* was to determine whether heparin-binding peptides incorporated into fibrin matrices during polymerization could serve as a potential therapeutic for nerve regeneration.

The application of exogenous growth factors has also been demonstrated to enhance peripheral nerve regeneration. It was hypothesized that prolonged release of exogenous growth factors would be more effective in promoting nerve regeneration than a one-time bolus application of growth factor. Two different drug delivery systems were developed to provide controlled release of exogenous growth factors during nerve regeneration. In both cases, the goal was to develop delivery systems in which the rate of growth factor release was regulated by cellular activity rather than by diffusion.

The first delivery system was designed mimic the extracellular matrix and its ability to sequester heparin-binding growth factors based on interactions with heparan

sulfate proteoglycans. It was hypothesized that by immobilizing an excess of heparin sites within a fibrin matrix that the diffusion of heparin-binding growth factors from the matrix could be reduced considerably. Slowing the rate of passive, diffusion-based growth factor release allows release to be regulated by the rate of matrix degradation, which can be controlled by the level of localized cellular activity, in the form of proteases. Thus, *the second objective of this research* was to develop a method for the immobilization of heparin within fibrin matrices and to test the feasibility of controlled growth factor release from such a system in an *in vitro* model of nerve regeneration.

This principle of cell-regulated release was also used to develop a second type of growth factor delivery system, consisting of recombinantly expressed growth factor fusion proteins that contained an exogenous cross-linking domain. In between the cross-linking domain and the growth factor domain, an enzymatically-degradable substrate was inserted to allow growth factor release to be regulated by cellular activity. It was hypothesized that these growth factor fusion proteins could be immobilized within fibrin matrices during polymerization and released locally as regeneration progressed through the fibrin matrix. Thus, *the third objective of this research* was to express these novel growth factor fusion proteins and determine if they had an effect on neurite extension within fibrin matrices.

This research involved the modification of fibrin-based scaffolds through the immobilization of peptides and proteins to enhance peripheral nerve regeneration. The choice of what factors to immobilize and the analysis of materials developed in this research relied on information gathered from many different disciplines. As such, an introduction to some relevant topics is presented here. The current clinical approaches to peripheral nerve repair and their shortcomings are discussed, along with the biological processes involved in nerve regeneration. The properties of fibrin and its natural role in wound healing are described. Cell adhesion domains and their potential role in nerve regeneration are discussed, with an emphasis on heparin-binding domains. Growth

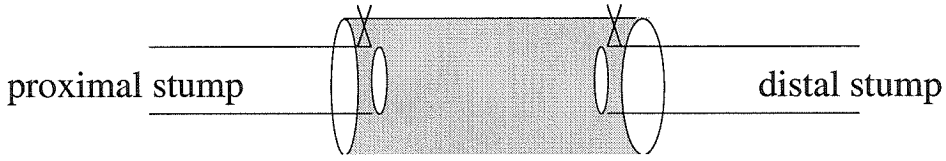
factors are also discussed both in the context of approaches for controlled release and in terms of their potential role in nerve regeneration.

1.2 Peripheral Nerve Regeneration

1.2.1 Clinical approaches

Despite recent advances in the understanding of nerve regeneration and in surgical techniques, the complete recovery of function in a damaged nerve is rare (Seckel, 1990). More than 200,000 nerve repair procedures are estimated to be performed annually in the United States alone (Archibald et al., 1991). The most common method of nerve repair is to directly suture the two severed nerve ends together; however, this is often not feasible if lacerations are present or if the gap between the two ends is too large and thus excessive tension is required to reconnect the two ends. In these cases, a nerve autograft is usually performed (Mackinnon, 1989). The autograft provides a scaffold for the regenerating nerve and guides it to its proper target. This procedure has its limitations, because of the defect created at the donor site for the autograft. Both of these procedures, direct anastamosis and nerve autograft, have poor results (Lundborg, 1990). Less than 25% of patients who received direct reanastomosis or 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 et al., 1984; Dellon and Mackinnon, 1988).

One alternative method to autografting is to use a nerve guidance tube (NGT) to connect the proximal and distal ends of the severed nerve and guide the regeneration of axons back to the appropriate target (Figure 1.1). These tubes allow the micro-environment of regeneration to be controlled by manipulating of the contents of the NGT. Numerous studies have been performed to determine some of the fundamental mechanisms of regeneration by varying the conditions within such tubes.



Nerve guidance tube

Figure 1.1 Nerve guide tube with severed nerve ends in both ends.

A comprehensive overview of the various materials used for NGT construction can be found in the review by Fields (Fields et al., 1989). Prior to 1989, silicone elastomer was the material of choice for NGT construction. More recently biodegradable NGTs have been studied, because an additional surgical procedure is not required to remove degradable NGTs. The use of poly (glycolic acid) NGTs has been studied as an alternative to autograft clinically. It was found that in gaps of up to 3 cm, degradable NGTs were at least as good as nerve autografts (Mackinnon and Dellon, 1990). The use of porous poly (L-lactic acid) NGTs have been evaluated a 10 mm gap in the rat sciatic nerve (Evans et al., 1999). Similar recovery in gait and nerve fiber density was observed for both nerve autografts and NGTs, but both treatments demonstrated incomplete recovery versus intact nerve controls. Biodegradable NGTs made of poly (lactic acid–co-ε-caprolactone) were studied in a 10 mm gap in the rat sciatic nerve, and the degradable NGTs were found to promote enhanced regeneration versus nerve autografts (den Dunnen et al., 1995). Another new family of degradable polymers that contain hydrophilic and hydrophobic block copolymers, including poly (R-3-hydroxybutyric acid-co-(R)-3-hydroxyvaleric acid)-diol (PHB), has been developed. NGTs made of PHB have been shown not to interfere with nerve regeneration across a 10 mm gap in the rat sciatic nerve (Borkenhagen et al., 1998b). In all of these studies, the researchers compare NGTs with the clinical "gold standard" for peripheral nerve repair, nerve autograft, and define success as regeneration comparable to that observed when nerve

autograft is performed, but even nerve autografts do not yield complete motor or sensory nerve regeneration.

The micro-environment of the NGT has also been studied in great detail. The effect of tube surface microgeometry on nerve regeneration has been evaluated using polyacrylonitrile-polyvinyl chloride ultra-filtration membranes (Aebischer et al., 1990). Smooth NGT surfaces resulted in the formation of longitudinally aligned fibrin matrices that promoted the formation of organized nerve fascicules. Rough tube walls led to random fibrin fiber deposition and poor regeneration. The porosity of NGTs was also found to affect nerve regeneration (Aebischer et al., 1989a). Tubular polysulfone membranes with molecular weight cutoffs of 10^5 and 10^6 Da were analyzed. It was found that regeneration was better in the 10^5 molecular weight cutoff NGTs, suggesting the high molecular weight species, such as antibodies, might be inhibitory to nerve regeneration.

Regeneration can occur through NGTs which are initially empty; however, pre-filling NGTs with saline or other adjuncts has been found in some cases to improve regeneration. These adjuncts can include cells, wound healing scaffolds including laminin or collagen, and trophic factors. The addition of saline was found to allow regeneration to occur in a larger chamber volume than possible with a NGT initially filled with air (Williams and Varon, 1985). It was also observed that when only the proximal end of the severed nerve was placed in the NGT, regeneration was not as effective as in the case when both ends of the severed nerve were placed in the NGT (Lundborg et al., 1982). The addition of Schwann cells to laminin–filled NGTs was found to increase the fasciculation of the regenerating nerve (Guenard et al., 1992).

1.2.2 Biology of wound healing

Nerve is one of the most difficult tissues in the body to regenerate; however, the peripheral nervous system has shown more potential for regeneration than the central nervous system. Peripheral nerve regeneration is a complex process because of the

requirement for both cell-cell interactions between neuronal and non-neuronal cells and interactions between cells and the surrounding extracellular matrix (ECM). Cellular processes in the form of axons must often extend distances of several centimeters or more in order to reach their proper target of innervation and reestablish the neural connections that were damaged. In order for regeneration to occur, several different types of cells must interact properly and in a time-coordinated manner.

Peripheral nerve axotomy results in distinct morphological, biochemical and physiological changes in both the cell body and axonal processes of a neuron. If the neuron survives the initial trauma, attempts at regeneration in the form of axonal sprouting may begin within 24 hours of the injury. The nerve cell body undergoes a dramatic change as it shifts from neurotransmitter production to the synthesis of proteins required for regeneration (Grafstein and McQuarrie, 1978). Cytoskeleton components, such as actin, tubulin and neurofilament, are essential for axonal elongation. The anterograde transport of these cytoskeleton proteins is relatively slow (5 to 6 mm per day) and is thought to limit the maximum rate of nerve regeneration (Lasek and Hoffman, 1976; McQuarrie, 1986). Growth-associated proteins (GAPs) are another group of proteins required for regeneration. Although the role of GAPs in regeneration is not clear, their synthesis is increased 20-100 time during nerve regeneration (Skene et al., 1986). These changes in the cell body result in neuronal dedifferentiation, and the neurons assume an embryonic state, which is more plastic and more permissive for growth (Foehring et al., 1986; Gordon, 1983; Kuno et al., 1974). These changes are postulated to occur in response to the loss of target-derived trophic factors from the periphery (Smith et al., 1988).

The proximal stump of the axon experiences "traumatic degradation" after axotomy (Ramon Y Cjal, 1928). Traumatic degradation occurs in response to ionic influxes of sodium and calcium into the cell, as well as a loss of potassium and intracellular proteins following the disruption of the cell membrane integrity (de

Medinaceli and Seaber, 1989; de Medinaceli et al., 1983; Hodgkin and Katz, 1949; Schlaepfer, 1971). The result of traumatic degradation is a zone of axonal debris that extends proximally to at least the next node of Ranvier or may even result in cell death. This zone initially prevents contact between the extending proximal nerve sprouts and the distal stump.

After traumatic degradation occurs, the distal nerve stump undergoes a process known as Wallerian degeneration. The myelin surrounding the distal nerve deteriorates and the axonal contents are lost (Dyck et al., 1984). Schwann cells in the nearby area proliferate and phagocytose the myelin debris (Aguayo et al., 1976). The basal lamina or endoneurial tubes remain after the myelin and axonal debris is removed, and can provide guidance for the regenerating nerve. The basal lamina surrounding the Schwann cells contains laminin, type IV collagen, heparan sulfate and other proteoglycans that may serve as a scaffold for nerve regeneration (Bunge and Bunge, 1983; Cornbrooks et al., 1983).

Regeneration from the proximal nerve is mediated through the elongation of growth cones from the severed axons. The growth cones extend filopodia and lamellipodia to explore the surrounding area for guidance cues, such as laminin and fibronectin in the ECM. Growth cones can also respond to soluble trophic cues, such as nerve growth factor (Gundersen and Barret, 1980). The growth cone can clear a path through the surrounding ECM by releasing proteases, such as plasmin (Krystosek and Seeds, 1981).

The cellular and molecular events of peripheral nerve regeneration through NGTs have been studied extensively. If both ends of the severed nerve are placed inside a NGT, a fibrin bridge forms between the two severed ends of the nerve (Williams et al., 1983). This fibrin bridge provides a substrate for future cell migration and can be degraded as regeneration progresses. After the formation of a fibrin bridge, fibroblasts and Schwann cells migrate along the fibrin strands. These cells secrete ECM molecules

(Bunge, 1987; Longo et al., 1984) that provide cues for axon guidance and present cell surface molecules for cell-cell recognition by extending axons (Bixby et al., 1988; Rutishauser, 1985). It is also thought that these cells might provide trophic cues to promote axon migration and neuron survival in the absence of target-derived trophic factors (Lundborg et al., 1982).

After Schwann cell and axonal migration occurs, differentiation of both neuronal and non-neuronal tissue occurs, which restores the tissue to its original functional organization. The axons then grow in diameter to re-establish their conductive properties, which is vital because axonal resistance is inversely proportional to axon diameter (Waxman, 1975). Finally, myelination occurs, which includes the formation of multilaminar sheaths around the axon by Schwann cells and the formation of the nodes of Ranvier, which are vital to conduction speed (Le Beau et al., 1988).

1.3 Fibrin

Fibrinogen is a dimeric molecule that consists of three pairs of disulfide-bonded protein sub-units, Aα, Bβ, and γ (Blomback, 1973; Blomback and Yamashina, 1958; Henschen, 1964). Fibrinogen has a molecular weight of 340,000 Da (Scheraga and Laskowski, 1957) and circulates in the blood at a concentration of about 2.5 mg/mL (Mosher, 1990). The three-dimensional structure of fibrinogen has been studied extensively. The central domain (domain E) of the fibrinogen molecule is formed by the N-termini of all six sub-unit chains, which contains a two-fold axis of symmetry through the center of the molecule. Extending from this central domain on both sides are coiled-coil regions, where each of the three α-helical protein chains wraps around one another like a rope (Doolittle et al., 1978). In the middle of these coiled-coil regions, there are small non-helical protease-sensitive domains. At the distal ends of the molecule (domain D), the Bβ and γ chains form globular regions at their C-termini (Telford et al., 1987;

Weisel et al., 1985), while the C-terminus of the Aα chains fold back and its hydrophilic residues interact with the coiled-coil domains (Medved et al., 1983).

Fibrinogen is converted from a soluble protein to an insoluble matrix during the final steps of the coagulation cascade. Upon activation of the enzyme thrombin, two small peptides, fibrinopeptides A and B are cleaved from the N-termini (E domain) of the α and β chains of fibrinogen (Blomback, 1958). Following cleavage of the fibrinopeptides, the cleaved fibrinogen molecules, referred to as fibrin monomers, non-covalently self assemble. Ligands on the exterior portion (D-domains) of the γ sub-units interact with binding-pockets on in the center (E-domain) of the fibrin monomer, which are exposed by cleavage of the fibrinopeptides (Blomback et al., 1978; Krakow et al., 1972). The resulting assembly of the center E domain of one molecule with the distal D domain of another results in the half-staggered assembly of fibrin monomers and leads to the formation of double stranded protofibrils (Ferry, 1952). These protofibrils assemble laterally via non-covalent interactions and form fibers consisting of 14-22 fibrils (Weisel et al., 1985). The fibers then aggregate to form an insoluble matrix (Hantgan and Hermans, 1979).

Covalent cross-linking of self-assembled fibrin monomers is performed by the transglutaminase factor XIIIa. Factor XIII, the zymogen of factor XIIIa, circulates in the blood at a concentration of about 10 μg/mL. Thrombin activates factor XIII by proteolytic cleavage to factor XIIIa. Factor XIIIa covalently cross-links the γ and α chains of fibrinogen via an acyl transfer reaction between the carboxyamide group on glutamine residues and the ε-amine of lysine residues (Matacic and Loewy, 1968). Cross-linking of the γ chains occurs at a single lysine and glutamine residue (Gln^{398} and Lys^{406}) resulting in γ chain dimerization that occurs early during factor XIIIa cross-linking (Chen and Doolittle, 1971; Doolittle et al., 1971). In contrast, cross-linking of the α chains can occur at several different lysine sites and results in high molecular weight polymers (McDonagh et al., 1971). Thus far, 12 lysine residues have been identified as

potential amine donor residues; however, the cross-linking of two lysine residues account of 50% of the total cross-linking observed (Lys^{556} and Lys^{580}) (Sobel and Gawinowicz, 1996).

1.3.1 Cell adhesion sites in fibrinogen

There are several cell adhesion sites present in fibrin matrices, which are found both on the fibrin itself and on other proteins that are immobilized within fibrin during coagulation. Fibrinogen contains two RGD domains on the α chain ($\alpha^{95\text{-}98}$ and $\alpha^{572\text{-}575}$) that can serve as ligands for integrins and promote adhesion of several different cell types, including endothelial cells, and fibroblasts (Smith et al., 1990b). The $\alpha^{572\text{-}575}$ RGD can interact with integrin receptors including $\alpha_v\beta_3$, which is expressed on endothelial cells and fibroblasts. Endothelial cell adhesion to fibrinogen can be almost completely inhibited by the addition of soluble GRGDSP peptide, whereas fibroblast adhesion is reduced by only 50% (Farrell and al-Mondhiry, 1997). Fibroblast adhesion is also inhibited by a peptide from the γ chain ($\gamma^{117\text{-}133}$) that is a ligand for intracellular adhesion molecule (ICAM)-1, suggesting that fibroblasts may adhere to fibrinogen by an RGD-dependent and an RGD-independent mechanism.

RGD peptides can bind to the platelet integrin receptor,$\alpha_{IIb}\beta_3$, and can inhibit the binding of $\alpha_{IIb}\beta_3$ to fibrinogen (Gartner and Bennett, 1985). However, it is unclear if RGD ligands mediate binding of $\alpha_{IIb}\beta_3$ to native fibrinogen. Some controversy has surrounded the role of RGD peptides and platelet adhesion to fibrinogen. Proteolytic fragments of fibrinogen lacking the RGD sites have exhibited decreased platelet adhesion (Savage et al., 1995). Conversely, recombinant fibrinogen, where the RGD sites were changed to RGE, has no effect on platelet aggregation or adhesion (Farrell et al., 1992). Some researchers have reported that $\alpha_{IIb}\beta_3$ may bind to the $\alpha^{95\text{-}98}$ RGD domain. However, the $\alpha^{95\text{-}98}$ RGD domain in native fibrinogen is prevented from interacting with cells due to the proximity of the coiled-coil domain (Doolittle et al., 1978). An additional platelet

adhesion site is located on the γ chain of fibrinogen with the sequence HLGGAKQAGDV that interacts with the platelet integrin receptor $\alpha_{IIb}\beta_3$ (Andrieux et al., 1989). A γ chain peptide ($\gamma^{190-202}$), WTVFQKRLDGSV, interacts with the $\alpha_M\beta_2$ integrin receptor on leukocytes and monocytes (Altieri et al., 1993).

Endothelial cell spreading on fibrinogen is mediated by a domain found at the N-terminus of the β chain after fibrinopeptide cleavage by thrombin. This peptide, β^{15-42}, is exposed by fibrinopeptide B cleavage and promotes endothelial cell spreading on fibrin. Endothelial cells can adhere in the absence of this domain, but fail to form stress fibers when only fibrinopeptide A is cleaved (Bunce et al., 1992). The cleavage of fibrinopeptide B also causes endothelial cells to release von Willebrand factor from intracellular Weibel-Palede bodies, which may contribute to cell adhesion to fibrin clots. In addition to the adhesion sequences within fibrinogen itself, fibronectin, which also contains an RGD site, is also cross-linked to fibrin during coagulation by factor XIIIa (Procyk and Blomback, 1988).

1.3.2 Degradation of fibrinogen

Fibrin matrices are degraded by the protease plasmin, and their degradation is tightly regulated through by control of plasmin activation and plasmin inhibitors. This tight regulation allows cells to migrate through fibrin by degrading the matrix in a localized manner. Therefore, both matrix density and protease inhibitors can serve to regulate the rate of cell infiltration into fibrin matrices. The fibrinolytic cascade can also allow cells to remodel modified fibrin matrices that serve as tissue engineered wound-healing scaffolds.

Plasmin is a serine protease that degrades fibrin in the final step of the fibrinolytic cascade. Plasminogen is the zymogen of plasmin, which is activated through proteolytic cleavage by plasminogen activators including tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). Plasminogen is a glycoprotein with a molecular

weight of 92 kD and circulates at a concentration of about 2 μM (Collen and de Maeyer, 1975). The three-dimensional structure of plasminogen has been studied in detail and consists of an N-terminal pre-activation peptide, five kringle domains and a protease domain (Sottrup-Jensen et al., 1978). Removal of the preactivation peptide by proteolytic cleavage gives rise to a form of plasminogen referred to as Lys-plasminogen (Wallen and Wiman, 1970; Wallen and Wiman, 1972). This form has a different confirmation than native plasminogen, which allows it to bind to fibrinogen with higher affinity and causes it to be activated more easily. The kringle domains confer lysine-binding ability to plasminogen (Lijnen et al., 1980; Wiman and Wallen, 1977).

The catalytic domain of plasminogen is activated by cleavage of the Arg^{561}-Val^{562} bond by a plasminogen activator (Robbins et al., 1967). Plasminogen activation usually occurs following plasminogen binding to fibrin, because plasmin circulating in the blood stream could result in generalized proteolysis and is therefore rapidly inactivated by plasmin inhibitor. Fibrinogen binding occurs through the kringle domains (Garman and Smith, 1982; Wu et al., 1990). tPA is a 68 kD protease found in many tissues and is synthesized mainly by endothelial cells (Saksela and Rifkin, 1988). It circulates at a concentration of about 70 nM with a half-life of about 4 minutes (Bachmann and Kruithof, 1984) and is usually found complexed with its inhibitor, plasminogen activator inhibitor 1 (PAI-1). tPA has four types of domains in within its structure: a finger domain that is responsible for binding to fibrin, an EGF domain, two kringle domains with lysine-binding affinity, and the catalytic domain (van Zonneveld et al., 1986; Verheijen et al., 1986). The rate of plasminogen activation by tPA is greatly enhanced in the presence of fibrin (Camiolo et al., 1971).

uPA is largely found in tissues where it serves to promote degradation of the ECM and enable cell migration through fibrin matrices (Saksela and Rifkin, 1988). uPA is a glycoprotein with molecular weight 55 kD that circulates in the blood at a concentration of about 0.05 nM (Husain et al., 1983; Wun et al., 1982). It contains four

different domains in its structure: an EGF domain that binds to the uPA receptor on cell, a kringle domain, and a catalytic domain (Blasi, 1988). uPA is activated by cleavage between Lys^{158} and Ile^{159} by trypsin, plasmin, kallikrein, or cathepsin-L (Ichinose et al., 1986).

Several inhibitors of the fibrinolytic cascade are present in the blood. Plasminogen activator inhibitors (PAIs) inhibit the activation of plasminogen. PAI-1 inhibits tPA and uPA. It has a molecular weight of 52 kD and is produced by endothelial cells (Schleef et al., 1990). In its active form, it binds to plasminogen activator forming a 1:1 complex with inactivated tPA (Lindahl et al., 1989). Most tPA in blood is bound to PAI-1 (Chandler et al., 1990). PAI-2 is a uPA inhibitor with a molecular weight of 47 kD in its nonglycosylated form (Kawano et al., 1970). Antiplasmin, or α_2-plasmin inhibitor (α_2-PI), is a 70 kD glycoprotein that circulates at a concentration of about 1 μM (Lijnen and Collen, 1989). α_2-PI inhibits plasmin by blocking the binding of plasminogen to fibrin thus limiting its conversion to plasmin, and it has specific binding sites for plasmin and fibrin (Mimuro et al., 1987). α_2-PI can also be cross-linked to fibrin matrices during coagulation by factor XIIIa and serves to slow the degradation of fibrin (Sakata and Aoki, 1980). Plasmin inhibitor binds to the lysine-binding site of plasmin with relatively high affinity (Wiman et al., 1979).

Another inhibitor of fibrinolysis is α_2-macroglobulin, a glycoprotein of molecular weight 725 kD that circulates at a concentration of 3 μM. It inactivates plasmin, tPA and other proteases in the fibrinolytic cascade and acts as a scavenger protease inhibitor. ω-aminocarboxylic acids, such as ε-aminocaproic acid (EACA) can inhibit plasmin by binding the lysine-binding site and blocking the binding of plasminogen to fibrin (Markus et al., 1978). Aprotinin is a small polypeptide derived from bovine lung that inhibits serine proteases through the formation of a 1:1 complex with the protease (Fritz and Wunderer, 1983).

1.3.3 Proteases in neurite growth within fibrin

Endogenous proteases play a key role in three-dimensional neurite outgrowth. They allow the advancing axons to remodel the ECM as regeneration proceeds. Dorsal root ganglia (DRGs) were found to secrete plasminogen activators using fibrin overlay methods (Krystosek and Seeds, 1984). Fibrinolysis was observed in the vicinity of the somata and cell processes, including growth cones. Two proteases were secreted from sympathetic and sensory neurons, a calcium-dependent metalloprotease and a plasminogen activator (Pittman, 1985). A strong correlation was observed between metalloprotease activity and the ability of neurites to penetrate collagen-containing gels (Pittman and Buettner, 1989). Almost 40% of plasminogen activator (PA) release was found to occur at the most distal processes and growth cones of sympathetic neurons, demonstrating that fibrinolysis is highly localized to the vicinity of the growth cone where regeneration occurs. Plasmin has been demonstrated to play an important role in neurite extension through three-dimensional fibrin matrices (Herbert et al., 1996).

Plasminogen activators released from sympathetic neurons and PC12 cells can interact with three different binding sites on the cell surface (Figure 1.2) (Pittman et al., 1989). Two of these receptors are for uPA and one is for tPA. One of the uPA receptors binds to the active site of uPA and prevents degradation of the matrix (Seeds et al., 1992). The other uPA receptor binds to a region of uPA that does not inhibit its activity and serves to localize the activation of plasminogen to plasmin (Pittman et al., 1989). This limits the region in which matrix degradation occurs to the area immediately surrounding the growth cone. PC12 cells overexpressing tPA were found to migrate more quickly than control cells in a three-dimensional Matrigel® matrix (laminin-containing gel) (Pittman and BiDenedetto, 1995).

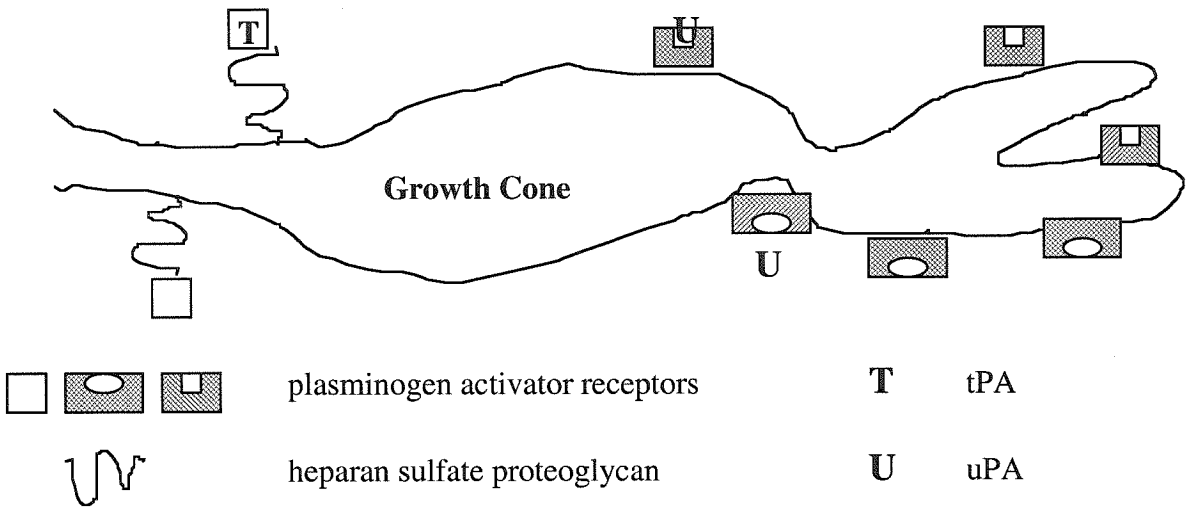


Figure 1.2 Neuronal plasminogen activator receptors on the growth cone.

1.3.4 Role of fibrin in wound healing

Fibrin is the natural biomaterial for wound healing in many tissues, including peripheral nerve and the vascular system. Fibrin matrices are formed during injury whenever the vasculature of a tissue is damaged, and they can serve as scaffolds for wound healing and tissue regeneration. Fibrin contains several characteristics that are desirable for a wound-healing scaffold. Fibrin provides signals for cell adhesion and infiltration and can be enzymatically degraded by cells as they migrate through fibrin matrices.

Fibrin serves as the natural scaffold for peripheral nerve regeneration. When the two ends of a severed nerve are placed within a NGT, an oriented fibrin bridge forms spontaneously between the two ends of the nerve during the first week of regeneration within the NGT (Williams et al., 1983). Williams and coworkers postulated that this fibrin bridge might play a role in nerve regeneration and that the formation of a fibrin bridge might be the rate-limiting step in nerve regeneration through NGTs. The addition of dialyzed plasma to the NGT resulted in the formation of an oriented fibrin matrix at an earlier time point than was observed in a saline-filled NGT (Williams, 1987; Williams et

al., 1987). The earlier formation of an oriented fibrin matrix also resulted in faster regeneration, yielding a great number of axons in the regenerating nerve than a saline-filled control NGT. However, fibrin alone does not provide sufficient neuroinductive capacity to promote nerve regeneration across long gaps in the peripheral nervous system.

Fibrin also serves as a wound-healing matrix in the vascular system following the injury of a blood vessel. Plasma that leaks from damaged vessels forms a temporary fibrin matrix. The fibrin matrix serves a haemostatic barrier to prevent further blood loss from the damaged vessel and also as a scaffold for angiogenesis. Endothelial cells can invade the fibrin matrix by activating plasmin from plasminogen, which degrades the fibrin matrix locally. This local degradation allows both cell infiltration for new vessel formation, and degradation of fibrin on the lumenal side of the newly formed microvessels (van Hinsbergh et al., 1997).

1.3.5 Incorporation of peptides into fibrin matrices

Because fibrin alone is not sufficient to promote peripheral nerve regeneration across long gaps, other researchers have sought to modify fibrin-based materials to enhance their ability to promote peripheral nerve regeneration *in vitro* and *in vivo*. Curtis Herbert sought to enhance the ability of fibrin matrices to promote nerve regeneration through the incorporation of exogenous adhesion factors by modifying fibrin matrices with poly(acrylic acid) polymers grafted with bioactive peptides (Herbert, 1996). Jason Schense continued this research and developed a new method for the incorporation of bioactive peptides using the transglutaminase activity of factor XIIIa to incorporate the bioactive peptides during fibrin polymerization (Schense and Hubbell, 1999). Schense demonstrated that nerve regeneration through fibrin matrices was enhanced by the incorporation of exogenous peptides, such as neurite promoting peptides from laminin (Schense et al., 2000). In the present work, I sought to enhance the ability of fibrin

matrices to promote neurite extension through fibrin matrices by incorporating heparin-binding cell adhesion peptides and through the controlled delivery of growth factors.

1.4 Adhesion domains relevant for nerve regeneration

Cell adhesion can occur through many different types of interactions. These interactions typically occur between cell-surface receptors and ligands in the ECM or on the surface of other cells. The most extensively studied cell adhesion receptors are the integrins, which mediate cell adhesion to ECM proteins such as fibronectin and vitronectin. Although integrins are the most common type of cell adhesion receptors, there are also non-integrin receptors for ECM proteins, which mediate cell adhesion to the ECM and bind to proteins, such as laminin. Cell-cell adhesion can occur through homophilic interactions between cell adhesion molecules on the surfaces of adjacent cells that are mediated through proteins, such as neural cell adhesion molecule (NCAM) and N-cadherin. Heparin-binding domains are another type of cell adhesion domain, which possess the ability to bind heparin and other sulfated glycosaminoglycans, which are typically found on cell-surface and ECM proteoglycans.

1.4.1 Cell adhesion via integrin receptors

The integrin receptors play an important role in cell adhesion for many different types of cells. Integrins are transmembrane cell adhesion receptors that serve to link the ECM to the cytoskeleton. In addition to transducing physical forces from the outside of the cell to the inside of the cell, integrins also serve as biochemical signal transducers. Integrins consist of two protein subunits, an α and a β subunit, which are non-covalently associated in 1:1 stochiometry to form heterodimers. Each different combination of subunits has a different ligand specificity, and much work has been done to elucidate subtle differences in ligand affinity for difference integrins (Koivunen et al., 1993; Koivunen et al., 1995; Pasqualini et al., 1995; Pasqualini and Ruoslahti, 1996).

The most well know integrin ligand is the cell-binding domain from fibronectin, the tripeptide arg-gly-asp (RGD) (Pierschbacher and Ruoslahti, 1984). The RGD sequence is found in many cell adhesion proteins in the extracellular matrix and in blood. The amino acid residues surrounding the RGD sequence serve to enhance the affinity of different RGD substrates for different integrins. Although most integrin research has focused on non-neuronal cell types, integrins also play an important role in neural cell adhesion during development and nerve regeneration.

Fibronectin is an ECM protein that serves as an integrin ligand and has been shown to promote neurite outgrowth in the peripheral nervous system. DRG neurite outgrowth on fibronectin can be selectively inhibited by antibodies to the integrin subunit β_1 (Haugen et al., 1992; Rogers et al., 1985). Pheochromocytoma (PC12) cells adhere to fibronectin-coated substrates and extend neurites in the presence of NGF. This adhesion and neurite extension can be inhibited completely by the addition of soluble RGD (Akeson and Warren, 1986). Cell adhesion to fibronectin can occur through a variety of combinations of integrin subunits, including $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (Hautanen et al., 1989; Pytela et al., 1985). The $\alpha_5\beta_1$ receptor is expressed following injury to the peripheral nervous system, suggesting a possible role in regeneration (Lefcort et al., 1992). Recently, $\alpha_5\beta_1$ expression has been observed on the growth cones of PC12 cells, DRGs and regenerating nerves, and was found to co-localize with actin in the growth cone (Yanagida et al., 1999).

Laminin has been extensively studied for its ability to promote neurite outgrowth *in vivo* and *in vitro*. Although the classical neurite promoting domains from laminin are recognized by non-integrin receptors, laminin can also interact with integrins as well. Neural crest cells express $\alpha_1\beta_1$, a laminin-binding integrin. The integrins $\alpha_1\beta_1$ and $\alpha_3\beta_1$ function as laminin receptors on DRG neurons and PC12 cells (Tomaselli et al., 1993) and $\alpha_6\beta_1$ serves as a functional laminin receptor on retinal ganglia and ciliary ganglia (McKerracher et al., 1996). The LRGDN sequence of the laminin α1 chain has been

shown to promote neuronal adhesion (Aumailley et al., 1990; Ignatius et al., 1990a; Kirchhofer et al., 1990). Deletion of the $\alpha_7\beta_1$ integrin, which binds to laminins, has been shown to impair peripheral nerve regeneration of motor neurons (Werner et al., 2000).

Collagen is another ECM protein that can serve as a ligand for neural integrins. Collagen has been shown to interact with $\alpha_2\beta_1$ integrins on the surface of developing retinal cells (Bradshaw et al., 1995). Embryonic sympathetic neuronal outgrowth on type IV collagen occurs via the $\alpha_1\beta_1$ integrin (Lein et al., 1991). A short peptide ligand for $\alpha_2\beta_1$ has been identified as DGEA (Zutter and Santaro, 1990).

1.4.2 Non-integrin adhesion ligands

Laminins are members of a family of large glycoproteins made of three subunits, α, β, and γ. Their role in promoting neurite outgrowth has been extensively studied to better understand neuronal adhesion and migration on laminins during development and regeneration. Laminin has several isoforms that contain different combinations of the protein subunits. Laminin-2 is a component of the basal lamina in peripheral nerve and is produced by Schwann cells (Yurchenco and O'Rear, 1994). It is likely that laminin-2 plays a role in the ability of peripheral nerve basal lamina to promote regeneration (Toyota et al., 1990).

There are several domains in laminin that are known to promote neurite outgrowth and neuronal adhesion that do not interact with integrins (Yamada and Kleinman, 1992). The YIGSR sequence of the laminin β1 chain has been shown to promote neurite outgrowth (Graf et al., 1987a; Graf et al., 1987b; Kleinman et al., 1989; Massia et al., 1993) and binds a 67 kD non-integrin cell surface receptor (Kleinman et al., 1993). The SIKVAV sequence on the α1 chain has also been shown to promote neurite outgrowth (Tashiro et al., 1989) and interacts with the 110 kD laminin receptor (Kleinman et al., 1993). The sequence RNIAEIIKDI of the γ1 chain has also be reported to promote neurite extension at low concentrations (Liesi et al., 1989).

1.4.3 Cell-cell adhesion proteins

Cell-cell adhesion proteins are also believed to play an important role during neural development and regeneration. Cell-cell contact between neurons and Schwann cells is important for nerve regeneration, and Schwann cells have been shown to be an effective substrate for promoting neurite outgrowth from DRGs and ciliary ganglia (Bixby et al., 1988; Letourneau et al., 1990). One of receptors on the Schwann cell that has been shown to regulate neurite outgrowth is the cell-cell adhesion protein N-cadherin. The LRAHAVS sequence from N-cadherin has been identified as a substrate for neurite outgrowth (Blaschuk et al., 1990). NCAM and L1 are related members of the cell adhesion molecule (CAM) protein family. They are involved in homophilic cell surface receptor interactions, similar to N-cadherin, and are found on the surface of neuron and Schwann cells in the peripheral nerve.

1.4.4 Heparin-binding domains

Heparin-binding domains are found in a variety of proteins of different types, including ECM proteins, cell-cell adhesion proteins, growth factors, and proteins found in the blood. In nature heparin-binding domains serve a variety of roles including promoting cell adhesion, binding heparin in blood, and immobilizing proteins to the ECM. Heparin-binding domains can interact with cell-surface proteoglycans to serve as cell adhesion domains and, in the case of neurons, they also to promote neurite extension. Heparin-binding domains have been found to enhance neurite extension from both peripheral and central neurons, when studied as proteolytic cleavage fragments or peptides adsorbed to surfaces (Perris et al., 1989; Rogers et al., 1985; Sephel et al., 1989).

ECM proteins that contain heparin-binding domains include laminin, fibronectin and vitronectin. The heparin-binding domain of fibronectin has been extensively studied. The ability of proteolytic digest fragments from fibronectin to promote neurite outgrowth has been studied. It has been observed that the 31 kD fragment containing the

heparin-binding domain promotes the same level of neurite outgrowth from retinal explants as native fibronectin, while the 105 kD fragment containing the RGD domain did not promote neurite outgrowth (Carri et al., 1988). It has also been observed that CNS neurons prefer the heparin-binding domain of fibronectin versus the RGD domain. DRG neurons prefer the RGD domain, but neurite outgrowth from DRGs on native fibronectin is sensitive to inhibition by heparin, suggesting that the heparin-binding domain of fibronectin may also play a role in DRG neurite outgrowth on fibronectin (Haugen et al., 1992; Rogers et al., 1985). Researchers often used platelet factor 4 (PF4) as a control heparin-binding protein for the fibronectin studies because it contains a heparin-binding domain but has no RGD site. PF4 was observed to promote neurite outgrowth in a manner similar to the heparin-binding domain of fibronectin.

Laminin is another ECM protein that promotes neurite outgrowth and has been shown to contain heparin-binding domains. Heparin-binding domains have been identified in the E8 region of laminin, and antibodies to this domain inhibit neurite outgrowth from sympathetic neurons on laminin (Edgar et al., 1984). Other heparin-binding domains have been identified in the cross-region of the protein (Skubitz et al., 1988). Neural crest cell migration on laminin is reduced by the heparin, suggesting a role for the heparin-binding domain of laminin in neural development (Perris et al., 1989).

CAMs are responsible for cell-cell adhesion and are another class of proteins that often contain heparin-binding domains. Although NCAMs are best know for homophilic interactions, they also possess a heparin-binding domain (Cole et al., 1986). The kinetics of NCAM binding to heparin-sepharose have been quantified and a dissociation constant of 5.2×10^{-8} M has been measured (Nybroe et al., 1989). The heparin-binding domain from NCAM has been shown to mediate cell-substrate adhesion of embryonic retinal cells (Cole and Glaser, 1986). Previous studies of the NCAM heparin-binding domain suggest that cell-surface proteoglycans, rather than soluble proteoglycans, mediate adhesion of neurons to heparin-binding domains (Kallapur and Akeson, 1992).

Heparin-binding growth associated molecule (HB-GAM) is a cell surface and ECM-associate protein that promotes neurite outgrowth *in vitro* and lines regions of axonal outgrowth during neural development (Rauvala, 1989). N-synedecan is a heparan sulfate proteoglycan that is expressed in the brain and has been identified as the receptor for HB-GAM, further suggesting the role of cell-surface proteoglycans as a general class of receptors for heparin-binding domains (Raolo et al., 1994). Neurite outgrowth on HB-GAM is inhibited by the heparin or heparan sulfate proteoglycans (Kinnunen et al., 1996).

Midkine is a heparin-binding protein of molecular weight 13 kD that shares a similar primary structure to HB-GAM. It acts as both a diffusible factor and a substrate molecule for neuronal adhesion. It is expressed in the brain during development, and its expression is highly regulated. Midkine serves as a substrate that can promote neurite outgrowth and the survival of embryonic brain cells when immobilized on a surface (Muramatsu and Muramatsu, 1991). It also serves as a survival factor for CNS and PNS neurons in the soluble form (Kikuchi et al., 1993).

Many proteins involved in the coagulation and fibrinolysis cascades also possess heparin-binding domains. Fibrinogen has a heparin-binding domain near the N-terminus of the β chain, and the affinity of this heparin-binding domain increases upon cleavage of fibrinogen by thrombin (Odrljin et al., 1996). von Willebrand factor (vWF) also contains two heparin-binding domains, one of which constrains the factor VIII binding site on vWF (Mohri et al., 1989). tPA and plasminogen also possess heparin-binding domains.

Antithrombin III is a protease inhibitor found in blood that plays a vital role in preventing coagulation by inhibiting the activity of thrombin. Antithrombin III is not a very potent inhibitor of thrombin in the absence of heparin. However, when heparin is bound to antithrombin III, the affinity of antithrombin III for thrombin is greatly enhanced (Rosenberg and Damus, 1973). When antithrombin III binds heparin, it undergoes a confirmation change that renders its reactive site more readily available for

interaction with thrombin or other coagulation enzymes (Beeler et al., 1979). The reactive site of antithrombin III is found near the C-terminus of the protein at Arg^{393} and Ser^{394} (Bjork et al., 1982). The serine active center of thrombin binds to the reactive site of antithrombin, resulting in a 1:1 complex of inactivated thrombin and antithrombin. The heparin-binding domain of antithrombin III has been extensively studied because of the use of heparin as an anticoagulant. There are two domains in antithrombin III responsible for its heparin-binding affinity, residues 40-50 (Bjork et al., 1989; Olson et al., 1981) and residues 107-145 (Smith et al., 1990a). Although these regions are not continuous in the primary structure, the basic residues responsible for binding heparin are found in close proximity in the three-dimensional structure.

Growth factors are another type of proteins that contain heparin-binding domains. Heparin-binding growth factors are found in many different growth factor families, including fibroblast growth factor (FGF) family, vascular endothelial cell growth factor (VEGF) family, transforming growth factor beta (TGF-β) family, and glial-derived neurotrophic factor (GDNF) family (Klagsbrun, 1990; Lin et al., 1994; Lyon et al., 1997; Tessler et al., 1994). These growth factors affect many different types of cells, including neurons, endothelial cells, fibroblasts, epidermal cells, and glial cells. Heparin-binding growth factors can serve to modulate cell proliferation, migration, and differentiation during development, tissue regeneration and cancer.

The above list does not encompass all heparin-binding proteins by any means, but it does demonstrate that heparin-binding domains are present in wide variety of proteins that have many different functions and are present in many different tissues. Although the function of these proteins clearly varies widely, in many cases heparin-binding domains have been found to play a role in modulating the activity of these heparin-binding proteins.

While many different types of proteins contain heparin-binding domains, most heparin-binding domains have fairly similar characteristics. Cardin and Weintraub

studied the sequences of 21 heparin-binding proteins and found two consensus sequences for heparin-binding domains (Cardin and Weintraub, 1989). These two sequences are XBBXBX and XBBBXXBX, where X is a hydrophobic residue and B is a basic residue. They observed lysine to be the most commonly occurring basic residue and arginine to be the second most frequently occurring basic residue. While not all heparin-binding domains follow this exact pattern, most follow the general pattern of basic residues flanked by hydrophobic residues.

Heparin-binding domains from many different proteins have been shown to promote cell adhesion and neurite outgrowth. Proteolytic digests of fibronectin have been shown to promote neural cell migration. The heparin-binding domain and cell-binding domain (RGD-containing domain) of fibronectin promote neural crest cell migration suggesting that both domains might play a role in neural development and possibly nerve regeneration (Perris et al., 1989). A peptide mimic of the ATIII heparin-binding domain has been shown to promote neurite outgrowth, when immobilized on surfaces (Borrajo et al., 1997). The heparin-binding protein PF4 has also been shown to promote neurite outgrowth when immobilized on two-dimensional surfaces (Carri et al., 1988).

1.4.5 Therapeutic uses of adhesion domain for nerve regeneration

Cell adhesion proteins and peptides derived from adhesion proteins have been studied as potential therapies to enhance peripheral nerve regeneration through NGTs. Initial research in this area focused on the use of ECM proteins that promote neurite outgrowth *in vitro*. The effect of laminin-containing gels (e.g., Matrigel) on nerve regeneration across short sciatic nerve gaps (4mm) was studied in both nondegradable and degradable NGTs. Laminin was found to enhance cell migration into NGTs at short time points (2 weeks), but at latter time points (4 and 6 weeks), laminin gels appeared to have an inhibitory effect on regeneration (Madison et al., 1987). Matrigel or collagen

gels were also shown to enhance cell migration into silicone tubes in a 20 mm gap in the rat sciatic nerve (Madison et al., 1988). Magnetically-aligned collagen filled NGTs have been found to enhance nerve regeneration across 6 mm gaps in the mouse sciatic nerve versus randomly-oriented collagen gels, suggesting a role for orientation cues in addition to adhesion factors in nerve regeneration (Ceballos et al., 1999).

Other research has focused on the use of peptides from neurite-promoting proteins coupled to some type of three-dimensional scaffold that can serve as a filler for NGTs and can potentially enhance nerve regeneration. YIGSR peptides coupled to agarose gels were observed to enhance dorsal root regeneration *in vivo* versus unmodified agarose and saline filled tubes (Borkenhagen et al., 1998a). Combinations of neurite-promoting peptides derived from laminin have also been shown to enhance dorsal root regeneration when the peptides were enzymatically cross-linked to fibrin gels (Schense et al., 2000).

The use of fibrin matrices containing covalently immobilized heparin-binding peptides to enhance neurite extension is described in Chapter 2. Thus far, all work studying the effects of heparin-binding domains has focused on two-dimensional neurite outgrowth. However, *in vivo* neurite outgrowth occurs in three dimensions, in which numerous phenomena compete and sometimes counteract. This work utilized the method of peptide incorporation into fibrin matrices developed by Schense (Schense and Hubbell, 1999) and demonstrated that it can been used to immobilize a new class of neurite-promoting peptides. By immobilizing exogenous heparin-binding domains within fibrin matrices, the effects of bioactive factors, such as heparin-binding peptides, can be studied in three-dimensional models of neurite extension that are more realistic for nerve regeneration *in vivo*. The ability of heparin-binding peptides to promote neurite extension was found to correlate with their heparin-binding affinity. This work also shows that the ability of matrices to promote neurite extension can be tailored by using peptides of varying heparin-binding affinity.

1.5 Protein Drug Delivery

Drug delivery systems that control the release rate of drugs over prolonged times are the subject of a great deal of research. Controlled release devices offer many advantages including the constant delivery of drug at desired therapeutic levels, reduced side effects due to localized drug delivery, a potential decrease in the amount of drug required, improved patient compliance, and facilitation of the delivery of drugs with short half-lives *in vivo* (including proteins). Recent advances in drug delivery systems have impacted many fields of medicine, and the annual sales of advanced drug-delivery systems in the US alone exceeded $10 billion in 1996 (Langer, 1998). Implantable and injectable drug delivery systems accounted for about 8% of this market.

1.5.1 Review of previous approaches to protein drug delivery

There are several common mechanisms for drug delivery from polymer or lipid based systems. The most common mechanism of drug release from polymeric delivery devices is diffusion of the drug from the system. Another common mechanism of release is degradation of the delivery system via a chemical or enzymatic reaction, such as hydrolysis or proteolysis of a labile bond. Diffusion-based release often occurs in combination with the degradation of polymeric systems. A third mechanism of release is solvent activation, where drug release is initiated via osmosis or swelling

Drug delivery research initially focused on the use of polymer systems that consisted of drug entrapped inside solid non-degradable polymers. These polymers were effective for the delivery of low molecular weight drugs, such as estrogen, and are currently used clinically for products such as Norplant (Folkman and Long, 1964). However, the diffusion-based release of high molecular weight drugs, including proteins, from solid non-degradable polymer systems is too slow to be useful in most situations.

To facilitate the release of proteins and other high molecular weight drugs, porous delivery systems were developed consisting of networks of interconnected pores within matrices made of non-degradable polymers. In these delivery systems, the drug is evenly

distributed throughout the pores of the delivery system, thus as the drug near the surface is released by diffusion, a porous network is left behind that enables the release of drug from deeper within the device (Langer and Folkman, 1976).

The advent of degradable polymer delivery systems revolutionized the field of drug delivery. Poly (lactic-co-glycolic acid) copolymer systems were developed that consisted of porous matrices, similar to those described above for non-degradable polymers. Diffusion of the drug through the pores as well as polymer degradation contributed to the overall rate of drug release, from these delivery systems. This type of system is used clinically to deliver luteinizing hormone, a polypeptide hormone for the treatment of prostate cancer (Ogawa et al., 1988). Polyesters such as poly (lactic acid) and poly (glycolic acid) degrade via bulk hydrolysis, which results in non-zeroth order release kinetics.

To attain zeroth order release kinetics for high molecular weight drugs, new hydrolytically degradable polymers were developed. These polymers, polyanhydrides, are more hydrophobic and degrade via surface erosion of the polymer, allowing a more constant rate of release. Porous polyanhydride matrices have been used clinically to deliver chemotherapeutic drugs including carmustine in the treatment of glioblastoma (Brem et al., 1995).

In addition to hydrolytically degradable delivery systems, enzymatically degradable polymers have been developed to allow drug delivery that is regulated by enzymatic activity (which is presumable associated with cellular activity). Kopecek and coworkers have developed delivery systems in which the drugs are covalently linked to the system via an enzymatically degradable linker (Kopecek, 1984). They identified short oligopeptide sequences that are sensitive to lysosomal enzyme cleavage by enzymes such as cathepsin H and cathepsin B. Kopecek also developed a delivery system that incorporates both solvent activation and enzymatic degradation as mechanisms for drug release. Hydrogels based on N,N-dimethylacrlamide, N-t-butylacrylamide and acrylic

acid were cross-linked with azoaromatic compounds (Brondsted and Kopecek, 1992). These materials were protonated at low pH in the stomach, resulting in a low degree of swelling, but when the pH increased further along the gastrointestinal tract, the polymers are deprotonated and the hydrogels swell. The azo bonds are then vulnerable to cleavage by microbial enzymes in the colon, allowing colon-specific drug delivery.

1.5.2 Problems and requirement for protein delivery systems

While many low molecular weight drugs have been successfully delivered from degradable and non-degradable polymeric delivery systems in an active form, larger molecular weight proteins have proven to be more difficult to deliver. Part of the difficulty in protein delivery arises from the fact that the diffusion of proteins is much slower than the diffusion of small molecules. However, many of the problems in protein delivery arise from the fact that protein-polymer interactions are not easily generalized and studies performed with model proteins, such as bovine serum albumin (BSA), can not be readily extrapolated to other proteins. Furthermore, some interactions between polymer delivery systems and the protein drug of interest can result in irreversible adsorption of the protein to the polymer matrix, protein denaturation or aggregation, all of which reduce the activity of the protein.

Another challenge in protein delivery is that the processing conditions by which polymeric delivery systems are often prepared can result in extreme stresses on the protein. These stresses can result in protein denaturation, aggregation or degradation that lead to a decrease in protein activity. Procedures that can result in reduced protein activity include heating, exposure to shear forces, exposure to extreme pH, exposure to organic solvents, freezing and drying. In addition, protein drugs are often incorporated at relative low concentrations because of their cost and efficacy at a relatively low dose. The handling of proteins at low concentration can result in excessive loss of protein due

to adsorption of protein to plastic or glass surfaces during processing in the absence of an excess of a relatively inert carrier protein, such as BSA.

One alternative that can reduce protein adsorption to the polymer delivery system is the use of more hydrophilic polymers, such as poly (ethylene glycol). However, it is often difficult to control the pore size of highly hydrated polymer matrices because they swell in an aqueous environment. If excessive swelling of the hydrogel occurs, than the pores in the device will probably be too large, and the rate of release will probably be higher than the rate desired for the application of interest.

1.5.3 Previous approaches with affinity-based delivery systems

While the majority of growth factor delivery systems are based on diffusion of growth factors from degradable polymers, other researchers have also studied affinity-based delivery systems that immobilize and release growth factors based on non-covalent interactions. One example of an affinity-based delivery system is a heparin-based delivery system for heparin-binding growth factors, such as basic fibroblast growth factor (bFGF), developed by Edelman and coworkers (Edelman et al., 1991). This delivery system consists of heparin-conjugated Sepharose beads that are encapsulated in alginate. Heparin-binding growth factors are immobilized within this delivery system based on electrostatic interactions between basic heparin-binding domains on the growth factors and sulfated groups on heparin. The growth factors are protected from degradation by heparin and are released slowly over time. This delivery system has been tested in a number of animal models and has recently completed Phase I clinical trials successfully (Laham et al., 1999). Heparin-alginate microcapsules containing bFGF were implanted in ischemic, ungraftable myocardial territories in patients undergoing coronary bypass surgery. In the patients with the higher dose of bFGF (100 μg) after 3 months, there was significant improvement in the defect size observed by nuclear magnetic resonance

imaging; however, in the placebo group there was a trend toward worsening of the defect size.

Other affinity-based delivery systems have been developed for growth factor delivery by expressing recombinant fusion proteins of growth factors that contain non-covalent immobilization domains. Nimni and coworkers have developed a TGF-β1 fusion protein that contains an exogenous collagen-binding domain from von Willebrand factor (Tuan et al., 1996). This TGF-β1 fusion protein has been shown to bind collagen with much higher affinity than recombinant TGF-β1 that lacks the exogenous collagen-binding domain. It was also shown to promote higher levels of migration, growth and differentiation of bone marrow mesenchymal cells in collagen gels versus native TGF-β1, BMP-2 and bFGF (Andrades et al., 1999).

1.6 Growth Factors

Growth factors are powerful regulators of biological function. They serve to modulate many cellular functions including migration, proliferation, differentiation and survival. Many different classes of growth factors have been identified from different tissues. Some growth factors affect only one type of tissue, while others can affect several different tissues systemically. Generally, growth factors often serve as the initiating signal for a cascade. They act via cell-surface receptors with high affinity for a specific growth factor or family of growth factors. These receptors, which are often receptor tyrosine kinases, usually dimerize upon growth factor binding and then their cytoplasmic domains are phosphorylated. This phosphorylation initiates a signaling cascade that can result in altered DNA transcription and protein expression.

1.6.1 Fibroblast growth factor family

The fibroblast growth factor (FGF) family contains ten members and is one of the best know families of "heparin-binding growth factors." FGFs are known for pleiotropic

effects in many different cell and organ systems. bFGF, also known as FGF-2, is a potent stimulator of angiogenesis, promotes smooth muscle cell growth, wound healing and regeneration in many tissues (Basilico and Moscatelli, 1992). It may also play a role in differentiation and regeneration of the nervous system. It is expressed in neural tissue during development including the spinal chord, cortex, striatum, and in glial cells, such as astrocytes (Kushima et al., 1992; Unsicker et al., 1992; Unsicker and Westermann, 1992; Weise et al., 1993). *In vitro*, bFGF promotes the survival and neurite outgrowth of hippocampal neurons, cholinergic neurons, ciliary ganglia, and PC12 cells (Miyagawa et al., 1993).

Members of the FGF family signal through a family of high affinity receptor tyrosine kinases called FGFRTKs. These receptors share common features including two or three immunoglobulin (Ig)-like loops in their extracellular domain, a transmembrane domain and a tyrosine kinase domain. Between Ig-like loop 1 and 2, there is an acidic domain, called the "acidic box," that is believed to interact with CAMs and may be responsible for signaling of CAMs including L1 and NCAM (Williams et al., 1994). The second Ig-like loop contains the heparin-binding domain of the FGFRTKs and has a minimal binding site for all FGFs (Wang et al., 1995). The intracellular domain contains an ATP binding site and three phosphorylation sites that are important for functional signaling by FGFRTKs (Hou et al., 1993). The binding of FGFs to the FGFRTKs leads to receptor dimerization, autophosphorylation and signaling via the phospholipase Cγ pathway (Peters et al., 1992).

Heparan sulfate proteoglycans form a second class of cell surface receptors for members of the FGF family. Acidic fibroblast growth factor (aFGF) and bFGF display heparin-binding affinity, with K_D values of 10^{-8}- 10^{-9} M (Moscatelli, 1987). Although there is no direct signaling mechanism for HSPGs, they can serve to immobilize FGF to the ECM and the surface of cells. In addition, many researchers have proposed that heparin and HSPG are involved in the binding of FGFs to the high affinity cell surface

receptor, and that a trimeric complex is formed during binding. The minimum heparin sequence required to bind bFGF has been studied in great detail, and a dodecasaccharide was found to be the shortest sequence isolated that can bind both bFGF and the cell surface receptor (Guimond et al., 1993).

The kinetics of bFGF binding to heparan sulfate were studied in detail (Nugent and Edelman, 1992). The binding and dissociation rate constants for bFGF and heparan sulfate were reported to be 0.90 X 10^8 M^{-1} min^{-1} and 0.68 min^{-1}, respectively. The kinetics of bFGF binding to the cell surface receptor in the presence and absence of heparan sulfate were also determined. The dissociation rate in the absence of heparan sulfate was found to be 16 times faster, suggesting that a trimeric complex of bFGF, heparin and receptor is responsible for high affinity binding of bFGF to the cell surface receptor.

Growth factors play an important role in tissue regeneration, both naturally and therapeutically. Numerous polypeptide growth factors bind heparin with high affinity, and this binding activity is important in sequestering growth factors in the ECM, serving to localize growth factor activity, to prevent growth factor degradation, and in some cases enhancing binding to cell surface receptors (Roghani et al., 1994). Heparinase has been shown to release heparin-binding growth factors from ECM (Rifkin and Moscatelli, 1989; Vlodavsky et al., 1991). Two examples of heparin-binding growth factors that are released by heparinase are aFGF and bFGF. Both function rather broadly, inducing mitosis and modulating function in a wide range of cell types, including fibroblasts, smooth muscle cells, chondrocytes, endothelial cells, astrocytes, and Schwann cells (Baird and Walicke, 1989).

1.6.2 Neurotrophins

The neurotrophins are a family of growth factors that were isolated based on their effect on the nervous system, including their abilities to promote neuronal survival.

Nerve growth factor (NGF) was identified in the 1949 by Rita Levi-Montalcini as the molecule responsible for promoting survival and growth of sympathetic ganglion neurons. The discovery of NGF further supported the theory that neurons require target-derived molecules in order to survive and suggested that NGF was one of these survival factors. Much later, a second member of the neurotrophin family, brain derived neurotrophic factor (BDNF), was purified from the brain based on its ability to promote survival of sensory neurons (Barde, 1982). The cloning of the BDNF gene allowed the discovery of other family members including neurotrophin-3 (NT-3) and NT-4, and later NT-6 and NT-7 (whose mammalian homologues have not yet been found).

There are two types of receptors for the neurotrophin family. One type of neurotrophin receptors is the tyrosine kinase receptor family known as Trks. The Trks are high affinity neurotrophin receptors, and each Trk has a specific affinity for one or two members of the neurotrophin family. Trk A binds to NGF, Trk B binds BDNF and NT-4 and Trk C binds to NT-3. The type of Trk receptor expressed on neurons helps to determine the phenotype of the cell. There is some overlap between Trk expressing populations in the DRG, and most neurons in the DRG express at least one of the Trk receptors. The Trk receptors can activate a variety of signaling molecules and pathways, including the Ras GTPase/mitogen-actived protein (MAP) kinase pathways and the phosphatidylinositol-3 (PI-3) kinase pathways (Basu et al., 1994; Obermeier et al., 1994).

The second type of neurotrophin receptor is the p75 receptor. It has a relatively low affinity for neurotrophins, but can bind all members of the neurotrophin family. p75 has been shown to belong to the tumor necrosis factor (TNF) receptor family, which has been found to play a role in apoptosis. The p75 receptor signals via c-Jun N-terminal kinase (JNK) pathway and the nuclear factor κB pathway (Carter et al., 1996; Yoon et al., 1998). It contains an intracellular death domain, typical of the TNF receptor family (Liepinsh et al., 1997), and it has been observed that cells expressing the p75 receptor but

not any of the Trk family neurotrophin receptors may actually undergo apoptosis when exposed to NGF.

1.6.3 Therapeutic used of growth factors in nerve regeneration

The addition of trophic factors to NGTs has been evaluated in numerous studies. The ability of aFGF to enhance regeneration was evaluated using collagen-filled polyethylene NGTs, and it was reported to increase in the number of motor and sensory axons in the aFGF-treated NGTs (Cordeiro et al., 1989). Filling silicone tubes with bFGF in Dulbecco's modified Eagles media (DMEM) also protected lesioned nerves from cell loss compared with tubes filled only with DMEM (Otto et al., 1987). Placing bFGF-soaked nitrocellulose in a silicone chamber was found to enhance nerve regeneration compared with untreated nitrocellulose (Danielsen et al., 1988). However, bFGF release could only be detected by *in vitro* assay (mitogenic stimulation of 3T3 fibroblasts) for 5 days and by ^{3}H-thymidine incorporation for 10 days. Poly(ethylene-co-vinyl actetate) NGTs containing bovine serum albumin (BSA) and bFGF were reported to stimulate regeneration across a gap where no regeneration was seen in the presence of only BSA (Aebischer et al., 1989b). However, even in the presence of bFGF only 20% of the normal number of axons regenerated, perhaps due to the need for the release kinetics of bFGF to be optimized. aFGF has also been shown to promote regeneration of the spinal cord in combination with peripheral nerve grafts (Cheng et al., 1996).

Neurotrophins have also been shown to enhance peripheral nerve regeneration. NGF was reported to protect neurons from injury-induced death in lesioned sciatic nerves (Otto et al., 1987). The effect of filling silicone tubes with NGF in saline was evaluated and an increase in the number of myelinated axons in the regenerating nerve was observed (Rich et al., 1989). NGF has also been shown to facilitate regeneration of hippocampal neurons across a peripheral nerve bridge (Varon and Conner, 1994). NT-3 was found to increase the number of myelinated axons in a regenerating dorsal root

through NGTs with NT-3 releasing rods in the wall (Borkenhagen, 1997). Several researchers have shown the potential of NT-3 to promote spinal cord regeneration (Schnell et al., 1994).

DRGs consist of a heterogeneous population of motor and sensory neurons. The neurotrophins NGF, BDNF, and NT-3 have been shown to promote the survival and differentiation of distinct and overlapping populations of sensory and motor neurons in DRG (DiStefano and Curtis, 1994). Lindsay reported that each of these neurotrophins binds to a different cell surface receptor, and that these receptors serve to identify different populations of neurons within the DRG (Lindsay, 1994). Furthermore, knockouts of BDNF, NGF and NT-3 all lack the neuronal populations that require the knocked-out factor for survival (Eriksson et al., 1994). Supplying one or more of these neurotrophins during regeneration could promote neuron survival during the regeneration process, when these target-derived growth factors are no longer accessible to the severed nerve.

Based on these studies, it appears that trophic factors from both the neurotrophin and fibroblast growth factor families have the potential to enhance peripheral nerve regeneration. However, in many of these studies, the authors point out difficulties in maintaining growth factor release over the rather long duration of nerve regeneration. It seems likely that a delivery system in which active regeneration-mediated release, rather than passive release, would be more effective at delivering growth factor over the duration of wound healing. Such a paradigm would mimic the natural situation, in which FGFs exist in the sequestered state and are released, to become bioavailable, by active, cell-mediated processes.

In Chapter 3, the development of fibrin-based heparin-containing delivery systems for heparin-binding growth factors is described. The delivery system developed contains electrostatically-immobilized heparin sites that can sequester heparin-binding growth factors in a manner similar to the way they are sequestered in the ECM.

Mathematical modeling was performed to test the feasibility of prolonged release from such delivery systems and to determine the conditions required for slow diffusion-based release of growth factor. These conditions were then tested in a model of *in vitro* nerve regeneration to determine the biological activity of growth factors delivered from these novel delivery systems.

The use of heparin-containing delivery systems to deliver growth factors that have relatively low heparin-binding affinity, such as NGF, is described in Chapter 4. Although the release of low heparin-binding affinity growth factors is faster than that of heparin-binding growth factor, the release of growth factors, such as NGF, from the delivery system is still greatly prolonged compared to the case of simple diffusion. The results presented here also demonstrate the biological activity of neurotrophins delivered from fibrin-based heparin-containing delivery systems.

In Chapter 5, novel recombinantly expressed NGF fusion proteins are described, which contain a substrate for factor XIIIa immobilization within fibrin matrices. These NGF fusion proteins also have a substrate for plasmin cleavage that allows for release of NGF in a relatively native form. The bioactivity of these NGF fusion proteins was determined using two *in vitro* activity assays. The ability of the immobilized fusion protein to promote neurite extension from DRGs was also compared with that of native NGF in the medium.

Chapter 2 Incorporation of Heparin-Binding Peptides into Fibrin Gels Enhances Neurite Extension *

2.1 Abstract

The goal of this work was to improve the potential of fibrin to promote nerve regeneration by enzymatically incorporating exogenous neurite-promoting heparin-binding peptides. The effects on neurite extension of four different heparin-binding peptides, derived from the heparin-binding domains of antithrombin III, neural cell adhesion molecule and platelet factor 4, were determined. These exogenous peptides were synthesized as bi-domain peptide chimeras, with the second domain being a substrate for factor XIIIa. This coagulation transglutaminase covalently bound the peptides within the fibrin gel during polymerization. The heparin-binding peptides enhanced the degree of neurite extension from embryonic chick dorsal root ganglia through three-dimensional fibrin gels, and the extent of enhancement was found to correlate positively with the heparin-binding affinity of the individual domains. The enhancement could be inhibited by competition with soluble heparin, by degradation of cell-surface proteoglycans, and by inhibition of the covalent immobilization of the peptide. These results demonstrate an important potential role for proteoglycan-binding components of the extracellular matrix in neurite extension, and they suggest that fibrin gels modified with covalently-bound heparin-binding peptides could serve as a therapeutic agent to enhance peripheral nerve regeneration through nerve guide tubes. More generally, the results demonstrate that the biological responses to fibrin, the body's natural wound healing matrix, can be dramatically improved by the addition of exogenous bioactive peptides in a manner such that they become immobilized during polymerization.

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2.2 Introduction

Despite recent advances in the understanding of nerve regeneration and in surgical techniques, the complete recovery of function in a damaged nerve is rare. The most common method of nerve repair is to directly suture the two severed nerve ends together; however, this is often not feasible if lacerations are present or if the gap between the two ends is too large and thus requires excessive tension to reconnect the two ends. In these cases, a nerve autograft is generally performed. The autograft provides a scaffold for the regenerating nerve and guides it to its proper target. This procedure has its limitations, due to the defects created at the donor site for the autograft. Both of these procedures, direct anastamosis and nerve autografting, have poor results (Lundborg, 1990).

One alternative method to autografting is to use nerve guide tubes to connect the proximal and distal ends of the severed nerve and guide the regeneration of axons back to the appropriate target. These tubes allow the micro-environment of regeneration to be controlled by manipulation of the contents of the nerve guide tube. Numerous studies have been performed to determine some of the fundamental mechanisms of regeneration by varying the conditions within such tubes. Williams et al. have observed the spontaneous formation of oriented fibrin matrices during the first week of regeneration within nerve guide tubes and postulated that this fibrin bridge might play a role in nerve regeneration (Williams, 1987; Williams et al., 1987). They found that the addition of dialyzed plasma led to the formation of an oriented fibrin matrix within 24 hr and that the formation of a fibrin matrix earlier allowed regeneration to occur more quickly. Aebischer et al. have studied the effects of tube surface microgeometry on nerve regeneration and have found that the inner surface of tubes must be smooth for a longitudinally oriented fibrin matrix to form (Aebischer et al., 1990). In this study, we sought to modify fibrin, the natural biomaterial of nerve regeneration, by covalently incorporating exogenous adhesion peptides to enhance its ability to promote nerve

regeneration. By such means we would hope to supply signals found in the extracellular matrix and on cell surfaces within the nerve bundle, to endow the fibrin matrix with some of the characteristics of the natural micro-environment of the nerve.

Cell adhesion is mediated by many different types of interactions between cell-surface receptors and ligands in the extracellular matrix or on the surface of other cells. Neuronal cell attachment to substrates can occur via cell-surface receptor binding extracellular matrix proteins, including to receptor-binding sequences found in proteins such as laminin and fibronectin. For example, several domains in laminin have been shown to support neuron adhesion: YIGSR of the laminin β1 chain (Graf et al., 1987a; Graf et al., 1987b; Kleinman et al., 1989; Massia et al., 1993), LRGDN of the laminin α chain (Aumailley et al., 1990; Ignatius et al., 1990b; Kirchhofer et al., 1990), SIKVAV of the laminin α chain (Tashiro et al., 1989), and RNIAEIIKDI of the laminin γ chain (Liesi et al., 1989). Neuronal adhesion can also occur through homophilic interactions between cell-cell adhesion molecules on the surfaces of adjacent cells, mediated through proteins such as neural cell adhesion molecule (NCAM) and N-cadherin. For example, the LRAHAVS sequence from N-cadherin has been shown to potentate neurite outgrowth by binding to N-cadherin (Blaschuk et al., 1990).

Another class of adhesion domains are the heparin-binding domains, which possess the ability to bind heparin and other sulfated glycosaminoglycans, typically components of cell-surface and extracellular matrix proteoglycans. It is with the role of heparin-binding domain peptides in promoting neurite extension, and possibly nerve regeneration, that this paper is concerned. Heparin-binding domains are found in a variety of proteins of different types, including laminin, fibronectin, NCAM, midkine, heparin-binding growth associated molecule (HB-GAM), and fibrinogen (Cole and Glaser, 1986; Edgar et al., 1984; Kaneda et al., 1996; Maccarana and Lindahl, 1993; Rauvala et al., 1994; Rogers et al., 1985; Smith and Knauer, 1987). These domains can interact with cell-surface proteoglycans to promote cell adhesion and, in the case of

neurons, to promote neurite extension. Heparin-binding domains have been found to enhance neurite extension from both peripheral and central neurons, when studied as proteolytic cleavage fragments or peptides adsorbed to surfaces (Edgar et al., 1984; Perris et al., 1989; Rogers et al., 1985; Sephel et al., 1989). Previous work with the NCAM heparin-binding domain has suggested that membrane-bound proteoglycans, rather than soluble proteoglycans, mediate adhesion to heparin-binding domains (Kallapur and Akeson, 1992). The ubiquitous presence of heparin-binding domains in a variety of extracellular matrix and cell attachment proteins, as well as their general bioactivity in promoting neurite extension, suggests a potential role in both neuronal development and regeneration (Perris et al., 1989).

Thus far, all work studying the effects of heparin-binding domains has focused on two-dimensional neurite extension. However, in vivo neurite extension occurs in three-dimensions, in which numerous phenomena compete and sometimes counteract. This distinction can be quite important. For example, inhibition of neurite-associated protease activity can have opposite effects in three- versus two-dimensional cultures, decreasing neurite extension in three-dimensional culture but increasing it in two-dimensional culture (Herbert et al., 1996). In the present study, three-dimensional culture was performed within fibrin gels. A novel method for covalently attaching exogenous peptides to fibrin gels using the coagulation transglutaminase factor XIIIa has recently been developed (Schense and Hubbell, 1999). In this method, a bi-domain peptide chimera is enzymatically coupled into a fibrin gel during polymerization, the amino terminal domain of this peptide chimera being a factor XIIIa substrate and the carboxyl terminal domain being a candidate peptide domain of study. This method allows the effects of bioactive factors, such as heparin-binding peptides, to be studied in three-dimensional models of neurite extension that are realistic for nerve regeneration.

The experiments described below demonstrate the potent influence of heparin-binding domains on three-dimensional neurite extension. Furthermore, the influence of

heparin-binding affinity is demonstrated, stronger binding peptides leading to more extensive neurite extension. Finally, the materials investigated in this study may be therapeutically useful in promoting peripheral nerve regeneration following transection due to trauma or surgery, providing within the fibrin matrix a biomimetic micro-environment employing adhesion and signaling domains from non-fibrin proteins.

2.3 Materials and Methods

All materials were obtained from Fluka (Buchs, Switzerland) unless otherwise specified.

2.3.1 Synthesis of peptides

Five bi-domain peptide chimeras were synthesized containing a heparin-binding domain at the carboxyl terminus and the factor XIIIa substrate domain from α_2-plasmin inhibitor at the amino terminus. The sequence of the factor XIIIa domain employed was NQEQVSP (Ichinose et al., 1983). The sequences of five bi-domain peptide chimeras are shown in Table 2.1. For clarity, the heparin-binding domains are shown in italics herein. In the first peptide, denoted $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$, the peptide contains the functional factor XIIIa substrate, NQEQVSP, in its amino terminal domain. The carboxyl-terminal domain contains a modified sequence from the heparin-binding domain of antithrombin III (ATIII) (Tyler-Cross et al., 1994), as shown in Table 2.1. A dansylated leucine residue at the amino-terminus was employed as a label to determine the extent of incorporation of the peptide chimera into the fibrin gel during polymerization (see below). The second peptide, denoted $\alpha_2PI_{1\text{-}7,\,Q^2\rightarrow G}$-$ATIII_{121\text{-}134}$, is similar, but contains a non-functional factor XIIIa substrate obtained by substitution of glycine for glutamine in the transglutaminase cross-linking site. The third peptide, denoted $\alpha_2PI_{1\text{-}7}$-$NCAM_{133\text{-}146}$, contains a functional factor XIIIa substrate and the heparin-binding domain from NCAM (Kallapur and Akeson, 1992). The fourth peptide, denoted as $\alpha_2PI_{1\text{-}7}$-$PF4_{60\text{-}67}$, contains a functional factor XIIIa substrate and the heparin-binding domain from platelet factor 4

(PF4) (Zucker and Katz, 1991). The fifth peptide, denoted as α_2PI_{1-7} -$ATIII_{121-134, K121, 125, 133 \rightarrow R}$ contains a functional factor XIIIa substrate and a modified heparin-binding domain from ATIII, where all the lysine residues in the Tyler-Cross domain have been replaced with arginine residues.

Table 2.1: Sequences and notation for bi-domain peptide chimeras containing heparin-binding domains.

Denotation	Sequence	Source of factor XIIIa substrate	Source of heparin-binding domain
α_2PI_{1-7}-$ATIII_{121-134}$	dLNQEQVSP*K(βA)FAK LAARLYRKA*-NH_2	α_2-plasmin inhibitor, functional	antithrombin III
$\alpha_2PI_{1-7, Q2\rightarrow G}$-$ATIII_{121-134}$	LNGEQVSP*K(βA)FAK LAARLYRKA*-NH_2	α_2-plasmin inhibitor, nonfunctional	antithrombin III
α_2PI_{1-7} -$ATIII_{121-134, K121, 125, 133 \rightarrow R}$	LNQEQVSP*R(βA)FAR LAARLYRRA*-NH_2	α_2-plasmin inhibitor, functional	antithrombin III, modified Lys→Arg
α_2PI_{1-7}-$NCAM_{133-146}$	LNQEQVSP*KHKGRD VILKKDVR*-NH_2	α_2-plasmin inhibitor, functional	neural cell adhesion molecule
α_2PI_{1-7}-$PF4_{60-67}$	LNQEQVSP*YKKII KKL*- NH_2	α_2-plasmin inhibitor, functional	platelet factor 4

Peptides were synthesized on solid amide resin (NovaSyn TGR, Novabiochem, Laüfelfingen, Switzerland) using an automated peptide synthesizer (Pioneer peptide synthesizer, PerSeptive Biosystems, Framingham, MA) with standard 9-fluorenyl-methyloxycarbonyl chemistry (Fields and Noble, 1990). Peptides used in cross-linking quantification were labeled with a fluorescent probe by placing an α-dansyl leucine at the amino terminus of the sequence. All solvents for peptide synthesis were obtained from Paul Bucher Company (Basel, Switzerland). All other reagents for peptide synthesis were obtained from Novabiochem, unless noted. Peptides were cleaved using 88% trifluoroacetic acid (Paul Bucher Company), 0.5% phenol, 0.5% water, and 0.2% triisopropylsilane for 2-3 hr. Following cleavage, the peptides were precipitated into 10 volumes of cold ethyl ether, and the precipitate was recovered by filtration and washed twice with ethyl ether to remove hydrophobic protecting groups and scavengers. The peptides were dried for 4 hr under vacuum and dissolved into 20 mL of deionized water.

They were dialyzed against 4 L of deionized water for 24 hr and lyophilized. Peptides were dissolved in Tris-buffered saline (TBS) (33 mM Tris, 8 g/L NaCl, 0.2 g/L KCl, pH 7.4) at a concentration of 0.01 M and syringe filtered (0.22 μm) prior to use.

2.3.2 Quantification of peptide incorporation

The amount of peptide cross-linked into fibrin gels through the action of factor XIIIa was determined by size exclusion chromatography, employing methods exactly as previously reported (Schense and Hubbell, 1999). Briefly, the peptide to be incorporated was added to the polymerization mixture and cross-linked to the fibrin gel during polymerization. Unincorporated peptide was washed from the gels with TBS and the gels were degraded with a minimal amount of plasmin. The degraded gel fragments were analyzed by size exclusion chromatography to determine whether the peptide was cross-linked to fibrinogen fragments or was present as free peptide. A decrease in the elution time of the fluorescence signal, indicative of an increase in molecular weight, would demonstrate coupling of the peptide to the fibrin gel. As controls, the peptide was analyzed by size exclusion chromatography both pure and as added to the plasmin-degraded fibrin, to demonstrate that this apparent increase in molecular weight could only be explained by covalent binding of the chimeric peptide to the fibrin gel. The effect of heparin on peptide incorporation was analyzed by including heparin and peptide in the polymerization mixture, degrading the gels with plasmin and running the degradation products on HPLC, as described.

2.3.3 Preparation of fibrin gels

Fibrinogen solutions were prepared exactly as described previously, using plasminogen-free fibrinogen from pooled human plasma (Schense and Hubbell, 1999). Dorsal root ganglia (DRGs) were dissected from day 8 White Leghorn chicken embryos (Varon, 1972) and placed in Hanks-buffered salt solution (HBSS) (Gibco, Basel, Switzerland). The DRGs were pipetted into the bottom of flat 24-well tissue culture

plates (1 per well) and fibrin gels were polymerized around the ganglia such that the ganglia were three-dimensionally embedded within the gel. Fibrin gels (400 μL per well) were made by mixing the components to obtain the following final solution concentrations: 3.5 mg/mL fibrinogen, 2.5 mM Ca^{++}, 2 NIH units/mL of thrombin, 0.25 mM peptide or 0.125 mM peptide (to obtain 8 moles of cross-linked peptide per mole fibrinogen or 4 moles of cross-linked peptide per mole fibrinogen, respectively), and 0.125 mM heparin (sodium salt from porcine intestinal mucosa, 176 USP U/mg, when used). The polymerization mixture was incubated for 60 min at 37 °C, 95% relative humidity, and 5% CO_2.

2.3.4 DRG culture and analysis

After polymerization to form the fibrin gel, 1 mL of modified neural basal medium was added to each well, consisting of insulin (5 μg/mL), transferrin (100 μg/mL), progesterone (6.4 ng/mL), putrescine (16.11 μg/mL), selenite (5.2 ng/mL) (all from Gibco), 5 μg/mL fibronectin, 0.1% bovine serum albumin, 20 ng/mL mouse nerve growth factor, 0.5 mM L-glutamine, 25 μM L-glutamate (all from Fluka), and 1% antibiotic-anti-mycotic solution (Gibco) added to neural basal medium (Gibco). The medium was changed at 3, 6, 9, and 24 hr to wash out uncross-linked peptide. DRGs were cultured within fibrin gels without peptide, and all measurements of neurite extension were normalized with respect to this level. Normalized neurite extension of 1 corresponded to 0.74 mm at 48 hr culture time. Neurite growth from dorsal root ganglia was normalized to growth in unmodified fibrin gels at 48 hr culture time. Experiments with the $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ peptide and $\alpha_2PI_{1\text{-}7,\,Q^2\rightarrow G}$-$ATIII_{121\text{-}134}$ peptide at 8 moles per mole fibrinogen were performed in quadruplicate, and experiments with the $\alpha_2PI_{1\text{-}7}$ - $ATIII_{121\text{-}134,\,K121,\,125,\,133\,\rightarrow R}$, $\alpha_2PI_{1\text{-}7}$-$NCAM_{133\text{-}146}$, $\alpha_2PI_{1\text{-}7}$-$PF4_{60\text{-}67}$ peptides at 8 moles per mole fibrinogen, the medium containing heparin, and $\alpha_2PI_{1\text{-}7,\,Q^2\rightarrow G}$-$ATIII_{121\text{-}134}$ peptide at 4

moles per mole fibrinogen were performed in duplicate, each with 6 ganglia per replication.

Enzymatic removal of glycosaminoglycans from cell surfaces was performed using heparitinase or chondroitinase ABC. Upon dissection, DRGs were incubated in HBSS containing 0.1 NIH units/mL of either heparitinase or chondroitinase ABC (Fluka). Culture medium for these ganglia contained 0.1 NIH units/mL of either heparitinase or chondroitinase ABC in the modified neural basal medium described above. Experiments with heparitinase and chondroitinase ABC added to the culture medium were performed in duplicate with 6 ganglia per replication.

Bright field images of the ganglia were taken at 24 and 48 hr with 4.0x Achroplan (24 hr) and 2.5x Plan (48 hr) objective (Zeiss, Zurich, Switzerland). The images were digitized with a Shimatsu chilled color 3-chip CCD camera, Matrox Meteor PCI frame grabber (Matrox Electronic Systems, Dorval, Quebec, Canada), and Leica Qwin software (both from Leica, Zurich, Switzerland). These images were then analyzed to determine the average length of neurite extension, which was calculated as the radius of an annulus between the DRG body and the outer halo of extending neurites, exactly as described previously (Herbert et al., 1996). Neurite length for each experiment was normalized by the average neurite extension through unmodified fibrin gels from the same experiment at the same time point.

Confocal scanning laser microscopy of DRGs was performed at 48 hr with a 10x/0.30 Plan Neofluor objective (Zeiss) using a MRC 600 confocal system (Biorad, Glattbrugg, Switzerland). 200 μL fibrin gels containing DRG were polymerized in the center of a 35 mm petri dish to allow imaging of the sample from above. DRGs were cultured as usual except that 2 mL of media per sample was used in order to cover the gel completely. Samples were stained at 48 hr, prior to imaging, by adding 4 μL of a 5 mg/mL stock solution of fluorescein diacetate in acetone to 2 mL of TBS. Samples were placed in the incubator for 5 min and then washed with TBS prior to imaging.

Approximately 50 to 100 images were taken at 7-10 μm intervals and a composite image was assembled using Imaris (Bitplane, Zurich, Switzerland) image processing software on an Indigo2 extreme Silicon Graphics Workstation (Silicon Graphics, Mountain View, CA).

2.3.5 Heparin-affinity chromatography

The relative affinity for heparin of the heparin-binding peptides used in these studies was determined by heparin-affinity chromatography, using a TSK-GEL Heparin-5PW (7.5 cm x 7.5 mm ID) column (TosoHass, Stuttgart, Germany). Samples of the bi-domain peptides were injected in 20 mM Tris, pH 7.4, 0.05 M NaCl. Elution was accomplished by running a gradient of NaCl from 0.05 M to 2.0 M over 40 min, and the NaCl concentration at which elution was observed was taken as a measure of the heparin-binding affinity of the peptide.

2.3.6 Statistics

Statistical analysis was performed using Statview® 4.5 (Abacus Concepts, Berkeley, CA). Comparative analyses were completed using the Scheffe's F post-hoc test by Analysis of Variance (ANOVA) at a 95% confidence level. Mean values and standard error of the mean are reported.

2.4 Results

2.4.1 Quantification of peptide incorporation

Size exclusion chromatography was used to determine the amount of peptide cross-linked into fibrin gels using the previously developed incorporation method (Fig. 2.1). A bi-domain peptide ($\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$) containing the factor XIIIa substrate from α_2-plasmin inhibitor, the heparin-binding domain from ATIII, and a fluorescent label was incorporated into fibrin gels during polymerization. The free peptide was washed from the gels (as determined by measuring fluorescence in the wash fluids), and the fibrin gel

was degraded with plasmin. The degradation products were analyzed by size exclusion HPLC to determine the amount of peptide (by fluorescence from the dansyl label) incorporated per mole of fibrinogen (by UV absorbance). The fluorescence signal from peptide-modified gels appeared at an earlier elution time (24 - 36 min) than did the signal from free peptide added to the degraded fibrin or chromatographed alone (45 min), indicating that all peptide present in the modified gels was covalently attached to protein in the degraded fibrin and as such had been present cross-linked to the fibrin gel (Fig. 2.1). When additional peptide was added free to degraded fibrin gel that also contained enzymatically incorporated peptide, the region of the peak corresponding to free peptide was observed to split, indicating the possibility of physiochemical interaction between the free peptide and the fibrin degradation products. This eluted at a much later time than did the covalently incorporated peptide, demonstrating unequivocally that the peptide eluting at times earlier than 40 min in the samples with enzymatic incorporation was indeed covalently coupled to the fibrin gel. Quantification based on standards of known concentration for both peptide and fibrin gels degraded with plasmin showed incorporation of 8.7 ± 0.2 moles of peptide per mole of fibrinogen (n=10), which is in close agreement with previously published results for a peptide containing the same factor XIIIa substrate domain but a vastly different carboxyl terminal sequence (Schense and Hubbell, 1999).

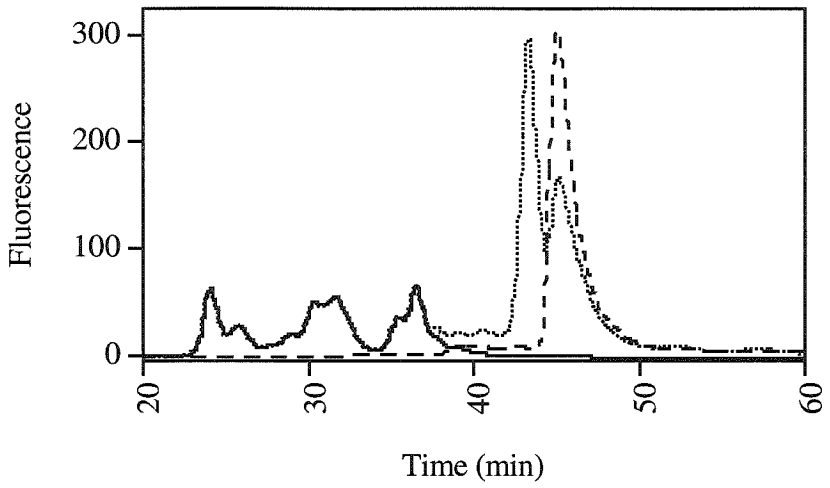

Figure 2.1 Fluorescence detection chromatograms of plasmin-degraded peptide-containing fibrin gels and free peptide.
Size exclusion chromatography of a degraded fibrin gel with the $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ peptide incorporated (–) and with the same peptide free added to the degraded fibrin gel containing incorporated peptide (···), and free peptide alone (- -), are shown. The free peptide eluted at longer times, corresponding to a lower molecular weight, than did the peptide incorporated into the fibrin gel during polymerization, demonstrating covalent attachment to degraded fibrin and thus covalent incorporation via the action of factor XIIIa activity.

2.4.2 Neurite extension in fibrin gels

When DRGs were cultured in three dimensions within fibrin gels, they extended neurites in all directions. Extension of neurites continued until such time when the neurites degraded the fibrin gel such that it no longer provided enough mechanical support for neuronal adhesion. Prior to this point, neurons extended neurites vigorously through fibrin gels. During the first two days of culture, growth was generally more rapid on the second day than on the first, with an average neurite extension of 0.25 mm at 24 hr and 0.74 mm at 48 hr. The morphology of the neurites was generally more fasciculated near the ganglion body and less fasciculated near the corona of growth cones. Images of DRGs near the ganglion body and near the growth cones are shown in Figure 2.2 (A and B).

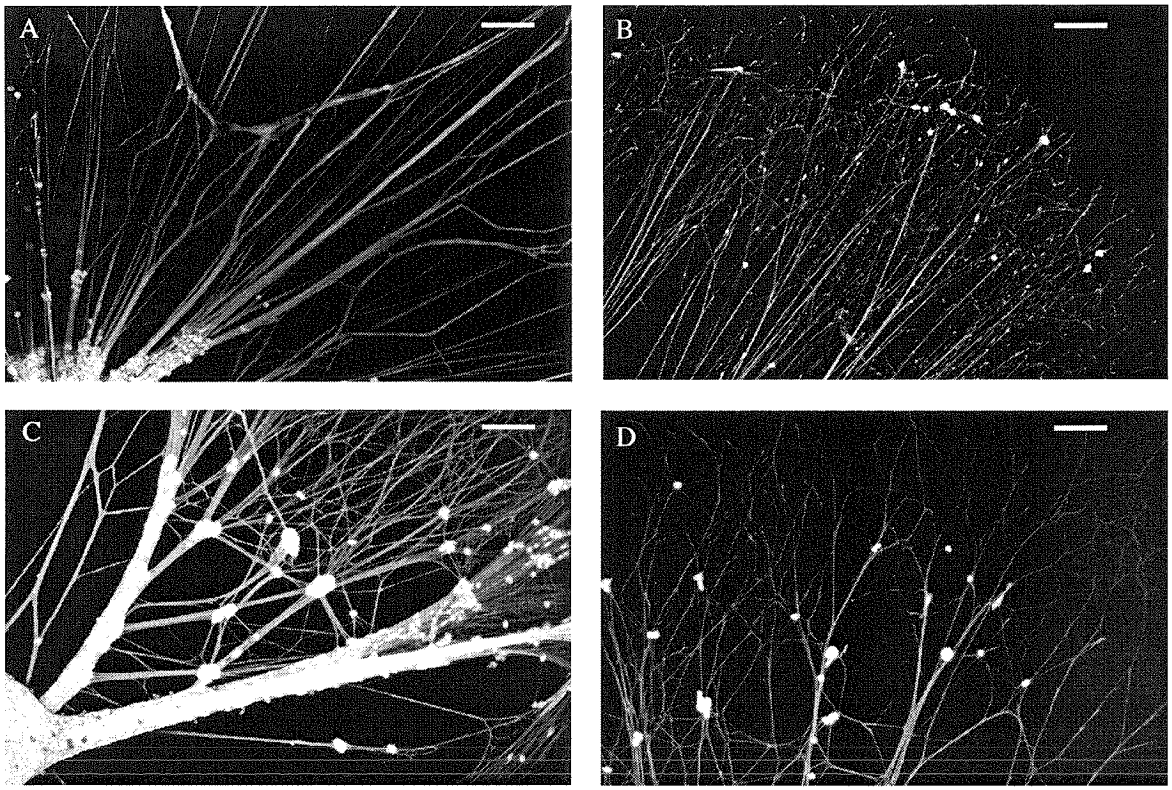

Figure 2.2 Images of DRGs cultured within fibrin gels with and without heparin-binding peptide.
A) Unmodified fibrin near ganglion body. B) Unmodified fibrin near growth cones. C) Fibrin $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ heparin-binding peptide near ganglion body. D) Fibrin containing $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ peptide near growth cones. Confocal scanning laser microscopy of DRGS was performed using 10x magnification. The scale bar represents 100 μm. Cells were stained with fluorescein diacetate prior to imaging.

2.4.3 Neurite extension through fibrin gels containing heparin-binding domains

The effect of incorporating heparin-binding domains on three-dimensional neurite extension through fibrin was determined by incorporating bi-domain peptides using factor XIIIa catalyzed cross-linking. These peptides contained one of four different heparin-binding domains, as shown in Table 2.1. Each of the heparin-binding sequences tested promoted more vigorous neurite extension compared to unmodified fibrin gels when incorporated at 8 moles of peptide per mole fibrinogen (Fig. 2.3). At 48 hr of culture, incorporation of $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ induced an increase in neurite extension of 73± 7%, $\alpha_2PI_{1\text{-}7}$-$NCAM_{133\text{-}146}$ induced an increase of 25± 6%, and $\alpha_2PI_{1\text{-}7}$-$PF4_{60\text{-}67}$ induced an increase of 20± 3 % (all $p<0.05$) relative to native fibrin. A peptide with a variation

on the heparin-binding domain of ATIII, in which all lysine residues were changed to arginine, denoted $\alpha_2PI_{1\text{-}7}$ -$ATIII_{121\text{-}134,\ K^{121,\ 125,\ 133}\ \rightarrow R}$, induced an increase of 41 ± 6% ($p<0.05$) relative to native fibrin. The observation that all of the tested heparin-binding peptides promoted more vigorous neurite extension suggests that the observed enhancement in neurite extension is not specific to one particular sequence, but rather is a result of the affinity for heparin and heparan sulfate proteoglycans of the various peptide sequences tested.

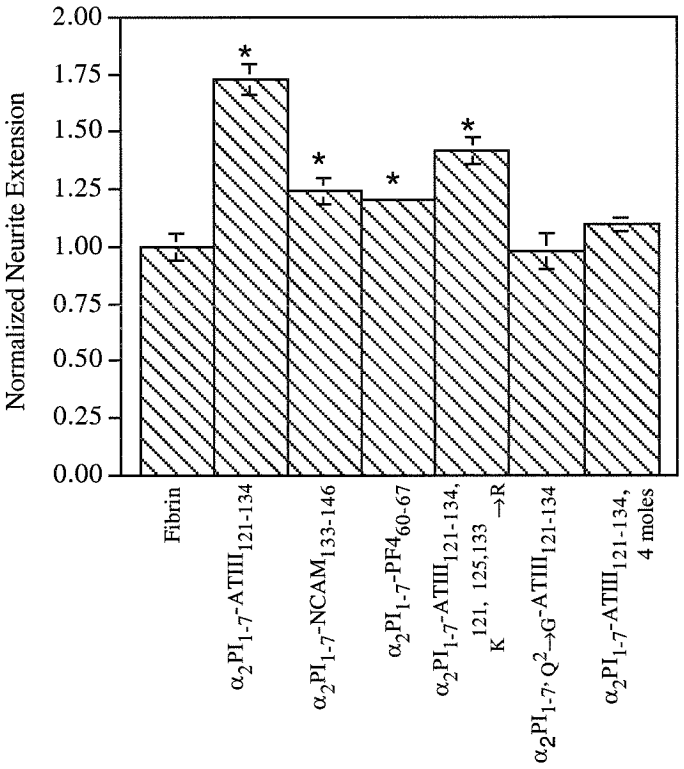

Figure 2.3 Effect of incorporated heparin-binding peptides on neurite extension within three-dimensional fibrin gels.
Mean values and standard error of the mean are shown. * denotes $p<0.05$ compared to unmodified fibrin. Incorporation of heparin-binding peptides into fibrin gels at 8 moles per mole fibrinogen increased neurite extension in all cases. When incorporation was prevented by employing the inactive factor XIIIa substrate $\alpha_2PI_{1\text{-}7,\ Q^2\rightarrow G}$, and when the amount of the ATIII peptide incorporated was reduced to 4 moles per mole of fibrinogen, no such statistically significant increase was observed.

Images of DRGs cultured within fibrin gels containing the $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ heparin-binding peptide are shown in Fig. 2.2 (C and D). DRGs cultured within fibrin

containing heparin-binding peptides were observed to display more extensive fasciculation near the ganglia bodies relative to unmodified fibrin. In some cases, more glial cells appeared to migrate toward the growth cones relative to that in unmodified fibrin.

To ensure that the increase in neurite extension induced by heparin-binding peptides was due to cross-linked peptide and not free peptide in solution, which was not covalently incorporated during polymerization, a bi-domain peptide that contained the ATIII heparin-binding domain but an α_2-plasmin inhibitor factor XIIIa substrate in which the glutamine residue that is required for cross-linking had been replaced with glycine ($\alpha_2PI_{1\text{-}7,\ Q2\rightarrow G}$-$ATIII_{121\text{-}134}$) was tested. This peptide was present during polymerization and was subjected to the standard washing and culture protocols described above. Gels thus treated with this peptide showed no significant increase in neurite extension over unmodified fibrin ($p=0.78$) (Fig. 3.3). This result demonstrates that the enhancement of neurite extension by heparin-binding domains is due to peptide that is covalently cross-linked into the fibrin gel.

To test the effect of peptide concentration on the ability of the ATIII heparin-binding domain to promote neurite extension, lower concentrations of peptide were incorporated into fibrin gels. At 48 hr of culture, incorporation of $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ at a concentration of approximately 4 moles of peptide per mole fibrinogen did not induce an increase in neurite extension ($10\pm 3\%$, $p=0.17$) relative to unmodified fibrin. This observation demonstrates that there is some minimum concentration of the exogenous heparin-binding peptide required to significantly enhance neurite extension.

To test whether soluble proteins or cell surface proteoglycans were responsible for the interaction leading to enhanced neurite extension, heparin was added during polymerization or in culture. The addition of heparin during cross-linking or in the culture medium inhibited the effect of the three heparin-binding domains tested to increase neurite length, such that outgrowth was not statistically different from the native

fibrin control ($p>0.75$ for each) (Fig. 3.4). The addition of heparin during polymerization decreased cross-linking of the peptide to 7.1 ± 0.1 moles of peptide per mole fibrinogen, a difference that is statistically different ($p \leq 0.0001$) but probably not particularly important compared to the value of 8.7 ± 0.2 for the peptide cross-linked in the absence of heparin.

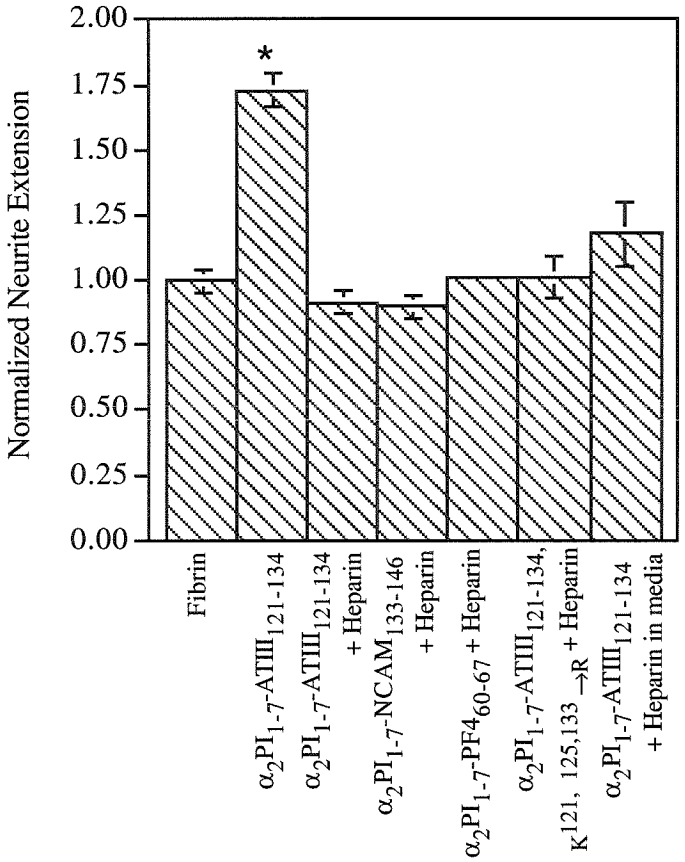

Figure 2.4 Effect of heparin addition during polymerization and to medium on neurite extension within fibrin gels containing heparin-binding peptides.
Mean values and standard error of the mean are shown. * denotes $p<0.05$ compared to unmodified fibrin. The addition of heparin either during polymerization or to the cell culture medium inhibited the ability of heparin-binding peptides to enhance neurite extension through fibrin gels.

To probe the role of direct interaction with cell-surface proteoglycans, enzymatic removal before and during culture was employed. The enzymatic cleavage of cell surface

proteoglycans resulted in decreased neurite outgrowth. The addition of chondroitinase ABC or heparitinase to fibrin gels containing the peptide $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ at 8 moles of peptide per mole fibrinogen resulted in increases of neurite extension of only 37±4 % and 35±5%, respectively relative to native fibrin ($p< 0.05$), compared to 73±7% in the absence of either enzyme ($p<0.0001$) (Fig. 2.5). Together, the results obtained with the addition of bound heparin during polymerization, free heparin during culture, and proteoglycan degrading enzymes before and during culture suggest that a proteoglycan located on the cell surface, rather than a soluble factor, is involved in the enhancement of neurite extension.

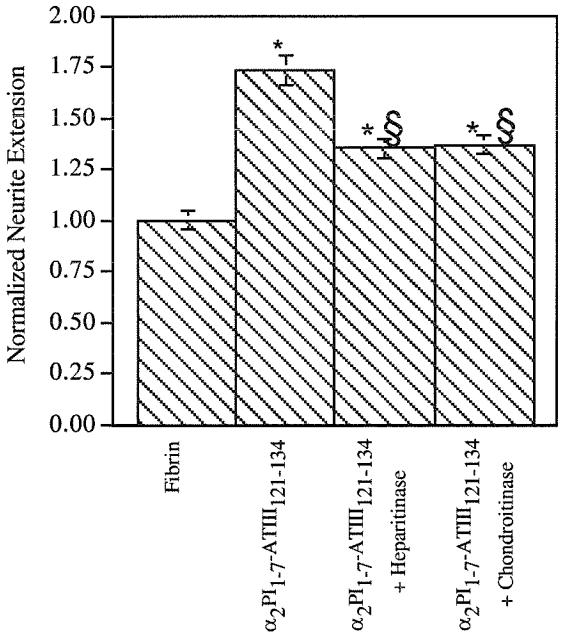

Figure 2.5 Effect of enzymatic cleavage of glycosaminoglycans on neurite extension through fibrin gels containing heparin-binding peptides.
Mean values and standard error of the mean are shown. * denotes $p<0.05$ compared to unmodified fibrin. § denotes $p<0.05$ compared with fibrin gels containing covalently attached $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ peptide. Enzymatic cleavage of either glycosaminoglycan decreased the ability of the $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$ peptide to enhance neurite extension through fibrin gels, suggesting an adhesive role for cell-surface proteoglycans in interaction with the immobilized exogenous heparin-binding peptides.

2.4.4 Heparin-affinity chromatography

The relative affinity of the four bi-domain heparin-binding peptides was determined by heparin-affinity chromatography. Bi-domain peptides containing the α_2-plasmin inhibitor factor XIIIa substrate domain at the amino terminus and heparin-binding domains from PF4 ($\alpha_2PI_{1\text{-}7}$-$PF4_{60\text{-}67}$), NCAM ($\alpha_2PI_{1\text{-}7}$-$NCAM_{133\text{-}146}$), modified ATIII ($\alpha_2PI_{1\text{-}7}$ -$ATIII_{121\text{-}134,\ K121,\ 125,\ 133\ \rightarrow R}$), and ATIII ($\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$) and are listed here from weakest to strongest heparin-binding affinity. The $ATIII_{121\text{-}134}$ variant in which the three arginine residues were substituted for lysine was synthesized and tested to explore the affinity vs. activity of the peptide with the strongest heparin-binding and the greatest ability to promote neurites, but to do so with the minimum number of structural changes possible. The NaCl concentrations at which each peptide eluted are given in Table 2.2. The observed influence on neurite extension was the same as the heparin-binding affinity, with the PF4 domain having the least influence and the ATIII domain having the greatest influence. Thus, the degree of neurite extension enhancement was observed to correlate closely with the relative heparin-binding affinity of the peptide incorporated into the fibrin gel, stronger binding leading to greater enhancement.

Table 2.2: NaCl concentration required to elute heparin-binding peptides from a heparin-affinity column.

Peptide	Source of Heparin - Binding Domain	[NaCl] require to elute peptide
$\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$	antithrombin III	0.67 M
$\alpha_2PI_{1\text{-}7}$ -$ATIII_{121\text{-}134,\ K121,\ 125,\ 133\ \rightarrow R}$	antithrombin III, modified	0.46 M
$\alpha_2PI_{1\text{-}7}$-$NCAM_{133\text{-}146}$	neural cell adhesion molecule	0.35 M
$\alpha_2PI_{1\text{-}7}$-$PF4_{60\text{-}67}$	platelet factor 4	0.34 M

2.5 Discussion

The goal of this work was to design biomaterials for use as fillers for nerve guide tubes to enhance peripheral nerve regeneration. Fibrin was chosen as the base material to

be used for these studies because it is the natural biomaterial of nerve regeneration and is readily penetrated by the proteolytic activity of the neurite growth cone as it extends through the three-dimensional fibrin gel (Herbert et al., 1996). We sought to enhance the ability of fibrin to promote nerve regeneration by covalently attaching neurite-outgrowth promoting peptides, in this case heparin-binding domains, thus endowing fibrin with the bioactivity of non-fibrin proteins.

Heparin-binding domains are found in a variety of proteins and can promote neurite outgrowth. Heparin-binding domains from a variety of adhesion proteins have been shown to promote neurite extension when adsorbed to two-dimensional surfaces, including domains from the extracellular matrix proteins laminin, fibronectin, HB-GAM, and the cell-cell adhesion protein NCAM (Cole et al., 1985; Edgar et al., 1984; Nolo et al., 1996; Rogers et al., 1985). For example, the heparin-binding domain of NCAM has been shown to promote neurite extension when adsorbed to surfaces. Furthermore, the neurite outgrowth induced by this domain could be inhibited by the addition of heparin to the cell culture media, digestion with heparitinase, or inhibition of cell proteoglycan synthesis, suggesting that the proteoglycan interacting with this domain was located on the cell surface. Moreover, heparin-binding proteins that are not naturally involved in supporting cell adhesion have also been shown to influence neurite outgrowth, e.g., the chemokine PF4, the anticoagulant protein ATIII, and the chemokine midkine (Borrajo et al., 1997; Carri et al., 1988; Kaneda et al., 1996). The heparin-binding domain of PF4 has been employed as a mimic of the heparin-binding domain of fibronectin and has been shown to promote neurite extension from various types of neurons on two-dimensional surfaces (Carri et al., 1988; Perris et al., 1989). The heparin-binding domain from ATIII was mimicked by Borrajo et al. and in the soluble form it was found to inhibit neurite extension on polylysine adsorbed to surfaces (Borrajo et al., 1997). However, when itself adsorbed to a surface, this ATIII domain mimic promoted neurite extension.

Based on reports in the literature demonstrating the ability of heparin-binding peptides to promote neurite extension in two dimensions, we decided to explore the ability of immobilized heparin-binding peptides to promote three-dimensional neurite extension, when enzymatically incorporated into fibrin gels. Such exploration might lead to better understanding of neurite extension in a more realistic three-dimensional context, and such exploration might also lead to the development of therapeutically useful materials. Incorporation of bio-functional peptides was accomplished by constructing bi-domain peptides containing a factor XIIIa substrate, α_2PI_{1-7} , at one end and a heparin-binding domain at the other end. The transglutaminase factor XIIIa then cross-links the bi-domain peptide, via the Q_2 residue in α_2PI_{1-7}, to the fibrin gel during polymerization. The amount of peptide covalently incorporated into fibrin gels by factor XIIIa was quantified and found to be 8.7 ± 0.2 moles of peptide per mole of fibrinogen without heparin bound and to be 7.1 ± 0.1 moles per mole of fibrinogen with heparin present, agreeing well with previous results for the same factor XIIIa substrate sequence, but a vastly different carboxyl terminus (Schense and Hubbell, 1999). These results show that a previously developed method for the incorporation of bi-functional peptides into fibrin gels during polymerization can be easily adapted to attach peptides with very different carboxyl termini and even with large molecules complexed to the carboxyl terminus. The incorporated peptides have been shown both here and previously (Schense and Hubbell, 1999) to retain their bioactive function upon cross-linking. The cross-linked peptides are displayed in a manner such that their active domain is accessible for interactions with cell surface receptors.

Peptides containing the heparin-binding domains, α_2PI_{1-7}-$ATIII_{121-134}$, α_2PI_{1-7}-$PF4_{60-67}$, α_2PI_{1-7}-$NCAM_{133-146}$, and α_2PI_{1-7} -$ATIII_{121-134,\ K121,\ 125,\ 133\ \rightarrow R}$, were incorporated within fibrin gels via a substrate for factor XIIIa. The quantitative degree of enhancement of neurite extension resulting from this incorporation was observed to increase with increasing heparin affinity, as determined by heparin-affinity

chromatography. Of the three naturally occurring heparin-binding domain sequences studied, $ATIII_{121-134}$ bound heparin most strongly and also induced the highest degree of neurite extension, an increase of 73± 7% at 48 hr. This ATIII domain mimic is somewhat special, in that Tyler-Cross et al. engineered the peptide sequence, modifying it to mimic more closely the native structure of the heparin-binding domain in intact ATIII and to induce α-helix formation in the presence of heparin (Tyler-Cross et al., 1994). This modified ATIII domain has substitutions at positions F_{121} and F_{122} for K_{121} and $(\beta A)_{122}$ to better mimic the effect of R_{47} in the native protein, and $(\beta A)_{122}$ was added to increase flexibility of K_{121}. Substitutions at positions N_{127} and C_{128} for alanine at both positions were made to prevent di-sulfide bond formation between peptides and to potentially induce α-helix formation when interacting with heparin. Such modifications were not made with the heparin-binding domain mimics from other proteins, and thus it is difficult to extrapolate results on the potency of neurite promotion of the peptides studied to the native intact proteins.

A close correlation between heparin affinity and enhancement of neurite extension was observed with the peptides explored. The $PF4_{60-67}$ and $NCAM_{133-146}$ peptides eluted at lower salt concentrations than $ATIII_{121-134}$, and similar to each other. Likewise, neurite extension was statistically lower than with $ATIII_{121-134}$, and was statistically similar between $PF4_{60-67}$ and $NCAM_{133-146}$. As a more direct probe of the effect of binding affinity, a variant on $ATIII_{121-134}$ was synthesized, with all of the lysine residues substituted with arginines, α_2PI_{1-7} -$ATIII_{121-134, K121, 125, 133 \rightarrow R}$. This modification resulted in a lower heparin-binding affinity and a proportionally lower potential to enhance neurite extension. This suggests that even greater enhancement of neurite outgrowth would be possible in gels containing a heparin-binding peptide with higher heparin-affinity than the α_2PI_{1-7}-$ATIII_{121-134}$ peptide. The increase in neurite extension with increased ligand affinity is similar to results seen by Lauffenberger and coworkers, who demonstrated that increasing ligand-receptor affinity could result in maximal cell

migration at lower ligand concentration (Paleck et al., 1997). At some higher heparin-binding affinity, this effect may saturate or go through a maximum, such that beyond some point increased heparin-binding affinity at a constant ligand concentration may actually decrease neurite outgrowth.

The influence of the nature of the adhesive interactions between the heparin-binding domain peptide and the cell can be seen also in the influence of the amount of the peptide. When the concentration of the $ATIII_{121-134}$ peptide incorporated was reduced by half to about 4 moles of peptide per mole of fibrinogen, the extent of neurite extension was reduced proportionally from 73± 7% to 10± 3%, the latter of which was not statistically different from neurite extension in native fibrin. Thus, it may also be the case that incorporation of a greater number of heparin-binding peptides can lead to greater enhancements in neurite extension.

Taken together, the results of this study are consistent with the primary nature of the interactions between the cell surfaces and the immobilized peptides being adhesive: addition of soluble inhibitor (heparin, either during or after polymerization), cleavage of the presumed receptors (with enzymatic treatment), and prevention of immobilization of the presumed ligand (with the use of an non-functional factor XIIIa substrate analog) all demonstrated results consistent with this hypothesis. Candidate receptors include phosphacan, a chondroitin sulfate proteoglycan that interacts with HB-GAM and can be inhibited by heparin and chondroitin sulfate C (Maeda et al., 1994; Maeda and Noda, 1998), and N-syndecan, a heparin sulfate proteoglycan which in soluble form can inhibit neurite outgrowth on HB-GAM (Kinnunen et al., 1996; Raolo et al., 1994). Both of these proteoglycan receptors for heparin-binding domains play an important role in adhesion and associated signaling, and one can not distinguish based on the results presented herein which of these effects is more important, if they can be separated.

The materials described in this paper may have broad usefulness in tissue engineering. A variety of applications in tissue engineering could benefit from novel

three-dimensional matrices. These include the use of matrices for cell seeding to generate tissues in vitro for ultimate transplantation and the use of matrices for cell invasion to generate and regenerate tissue in vivo. Examples of tissue morphogenesis include generation of tissue-engineered skin, blood vessels, liver, nerve and cartilage (Bellamkonda et al., 1995; Black et al., 1998; Borkenhagen et al., 1998a; L'Heureux et al., 1998; Mooney et al., 1997; Sims et al., 1998; Thompson et al., 1988). In these approaches, a matrix component plays a key role, and its interactions with cells either in vitro or in vivo take an important part in the process of morphogenesis. Requirements of the characteristics of such matrices include *(1)* the ability of cells to infiltrate and remodel these matrices, *(2)* the ability to tailor the adhesive nature of the matrix, and *(3)* the ability to present other exogenous bioactive signals, such as polypeptide growth factors, from the matrix. Each of these characteristics potentially can be met in the context of engineered fibrin: *(1)* As to cell infiltration and remodeling, the activation of plasminogen to plasmin is localized to the surface of neurite growth cones via receptors for plasminogen activators (Pittman et al., 1989). Through this localization, an intact fibrin structure has been observed to within 100 nm of the growth cone surface as it penetrates the fibrin matrix in neurite extension (Herbert et al., 1996). *(2)* As to tailoring the adhesive character of fibrin matrices, Schense and Hubbell (Schense and Hubbell, 1999) provided one example of incorporation of the RGD sequence, and the present paper provides a second example in which cell-surface proteoglycans serve as the targeted receptors. *(3)* As to incorporation of other bioactive species, such as polypeptide growth factors, one can incorporate heparin into fibrin gels via the peptides described herein, and then employ heparin to bind heparin-binding growth factors and release them biomimetically as occurs naturally in the extracellular matrix (S.E. Sakiyama and J.A. Hubbell, unpublished observations).

Chapter 3 Development of Fibrin Derivatives for Controlled Release of Heparin-Binding Growth Factors*

3.1 Abstract

The goal of this work was to develop a growth factor delivery system for use in wound healing that would provide localized release of heparin-binding growth factors in a biomimetic manner, such that release occurs primarily in response to cell-associated enzymatic activity during healing. A key element of the drug delivery system was a bi-domain peptide with an N-terminal transglutaminase substrate and a C-terminal heparin-binding domain, based on antithrombin III. The bi-domain peptide was covalently cross-linked to fibrin matrices during coagulation by the transglutaminase activity of factor XIIIa and served to immobilize heparin electrostatically to the matrix, which in turn immobilized the heparin-binding growth factor and slowed its passive release from the matrix. Basic fibroblast growth factor (bFGF) was considered as an example of a heparin-binding growth factor, and cell culture experimentation was performed in the context of peripheral nerve regeneration. A mathematical model was developed to determine the conditions where passive release of bFGF would be slow, such that active release could dominate. These conditions were tested in an assay of neurite extension from dorsal root ganglia to determine the ability of the delivery system to release bioactive growth factor in response to cell-mediated processes. The results demonstrated that bFGF, immobilized within fibrin containing a 500-fold molar excess of immobilized heparin relative to bFGF, enhanced neurite extension by up to about 100% relative to unmodified fibrin. A variety of control experiments demonstrate that all components of the release system are necessary and that the bi-domain peptide must be covalently bound to the fibrin matrix. The results thus suggest that these matrices could serve as

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therapeutic materials to enhance peripheral nerve regeneration through nerve guide tubes and may have more general usefulness in tissue engineering.

3.2 Introduction

The objective of this research was to develop a growth factor delivery system for use in wound healing that would provide localized release of heparin-binding growth factors in an active manner, such that release occurs primarily in response to cellular activity during healing. A natural wound healing matrix, namely fibrin, was selected as the base material for this delivery system, in order to provide a three-dimensional cell infiltration scaffold, the adhesive and drug delivery characteristics of which could be tailored for the wound healing model of interest.

Wound healing matrices provide a three-dimensional scaffold, which can serve as a substrate for cell adhesion and migration into the wound bed (Gailit et al., 1997). Fibrin presents unique opportunities as a generally applicable matrix both in wound regeneration and in other applications in tissue engineering (Horch et al., 1998; Hunziker and Rosenberg, 1996; Meana et al., 1998; Romanos and Strub, 1998). Fibrin serves as a natural wound healing matrix in several types of tissue, including neuronal and vascular tissues (Isogai et al., 1988; Williams and Varon, 1985). This wound-healing scaffold is remodeled during cell infiltration by proteolytic degradation in a highly localized manner (Pittman et al., 1989). Plasmin-mediated proteolysis of fibrin is regulated through cell-derived plasminogen activator (Krystosek and Seeds, 1984). Cell surface receptors for plasminogen activator allow highly localized plasminogen activation, resulting in localized fibrinolysis without bulk matrix degradation (Herbert et al., 1996).

Growth factors play an important role in regeneration, both naturally and therapeutically. Numerous polypeptide growth factors bind heparin with high affinity, and this binding activity is important in sequestering growth factors in the extracellular

matrix (ECM), serving to localize growth factor activity, to prevent growth factor degradation, and in some cases enhancing binding to cell surface receptors (Roghani et al., 1994). Heparinase has been shown to release heparin-binding growth factors from ECM (Rifkin and Moscatelli, 1989; Vlodavsky et al., 1991). Two examples of heparin-binding growth factors are acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF). Both function rather broadly and induce mitosis and modulate function in a wide range of cell types, including fibroblasts, smooth muscle cells, chondrocytes, endothelial cells, astrocytes, and Schwann cells (Baird and Walicke, 1989).

Both aFGF and bFGF enhance the growth of neurites (Baird and Walicke, 1989). In vivo, both aFGF and bFGF have been added to nerve growth guides, resulting in an increase in axon growth when delivered from polymer-based depots or when present in the free form (Aebischer et al., 1989b; Cordeiro et al., 1989; Danielsen et al., 1988; Otto et al., 1987). Based on these studies, it appears that growth factors from the FGF family have the potential to enhance peripheral nerve regeneration. However, in many of these studies, the authors point out difficulty in maintaining growth factor release over the rather long duration of nerve regeneration. It seems likely that a delivery system in which active regeneration-mediated release, rather than passive release, dominated would be more effective at delivering growth factor over the duration of wound healing. Such a paradigm would mimic the natural situation, in which FGFs exist in the sequestered state and are released, to become bioavailable, by active, cell-mediated processes.

The heparin-binding affinity of FGFs has been used to develop drug delivery systems for the controlled release of heparin-binding growth factors. For example, Edelman and colleagues have utilized heparin affinity to modulate the rate of bFGF release from heparin-sepharose conjugates (Edelman et al., 1992). Heparin-based delivery systems can serve as reservoirs to store growth factors in a manner similar to the ECM, allowing for active release of growth factor controlled by cell-mediated degradation of the reservoir.

Our goal in this study was to harness the protective and binding capacity of heparin so as to deliver bFGF by a cell-mediated active release mechanism to enhance peripheral nerve regeneration. To provide controlled delivery of exogenous heparin-binding growth factors, fibrin matrices were modified to contain immobilized heparin. The fibrin was modified utilizing a previously developed method of covalently cross-linking bi-domain peptides to fibrin matrices using the transglutaminase activity of factor XIIIa (Schense and Hubbell, 1999). This method was used previously to incorporate exogenous bi-domain peptides into fibrin matrices by placing the factor XIIIa substrate from α2-plasmin inhibitor at the amino terminus and a heparin-binding domain at the carboxyl terminus (Sakiyama et al., 1999). Clotting in the presence of the bi-domain peptide spontaneously covalently incorporated the peptide at a concentration of up to 8 moles of peptide per mole of fibrinogen in the clot. These covalently bound heparin-binding peptides were used in this study to immobilize heparin within fibrin matrices based on electrostatic interactions. The bound heparin in turn served to sequester heparin-binding growth factors within fibrin matrices, which can be released by enzymatic factors, e.g., heparinase (Presta et al., 1989; Vlodavsky et al., 1991) or plasmin from migrating cells (Herbert et al., 1996), as well as by passive release. Mathematical modeling was performed to determine the conditions required for slow passive release that would allow cell-mediated active release of growth factor to dominate. The delivery system was then tested in a neuronal cell culture assay to determine the ability of such a system to deliver active bFGF in a biomimetic manner.

3.3 Materials and Methods

All materials were obtained from Fluka (Buchs, Switzerland) unless otherwise specified.

3.3.1 Mathematical Modeling of bFGF Release

A mathematical model was developed to describe the release of bFGF (or other heparin-binding growth factors) from fibrin matrices containing heparin electrostatically bound to covalently immobilized heparin-binding peptide (see Fig. 3.1A). The model was employed to estimate the rate of passive bFGF release, i.e., release in the absence of invading cells, based on that reasoning that, if passive release were fast, the bFGF would not have the opportunity to be released actively, i.e., by local cellular enzymatic activity during invasion. Thus, the model was employed to determine concentrations of covalently immobilized heparin-binding growth factor and heparin in the system required to obtain slow passive bFGF release. Six species were considered in the model: free growth factor (G), free heparin (H), immobilized heparin-binding peptide free of heparin (P), the growth factor - heparin complex (GH), the heparin - peptide complex (HP), and the growth factor - heparin - peptide complex (GHP). These species existed in a reaction network (see Fig. 3.1B) in which all possible complexation reactions were considered. It was assumed that heparin complexed to the growth factor with the same kinetic rate constants, regardless of pre-complexation to peptide or not, and likewise that heparin complexed to the peptide with the same kinetic rate constants, regardless of pre-complexation to the growth factor or not. Previously reported kinetic constants for bFGF and antithrombin III binding to heparin and diffusion coefficients for proteins in hydrogels were employed to estimate the various parameters used in the model (given with references in Table 3.1).

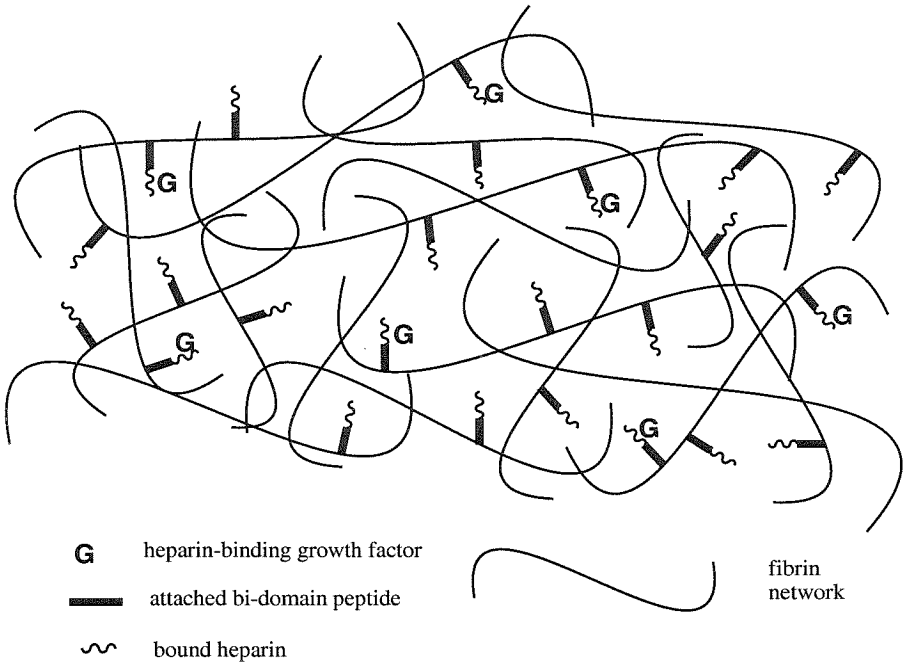

Figure 3.1A Fibrin containing heparin-binding growth factor delivery system.
A bi-domain peptide, containing a factor XIIIa substrate and a heparin-binding domain, is covalently cross-linked to the fibrin matrix during coagulation. Heparin is immobilized to heparin-binding domain of the peptide by electrostatic interactions. Heparin-binding growth factor is immobilized by binding to the immobilized heparin within the fibrin matrix.

$$G + H \underset{k_r}{\overset{k_f}{\rightleftharpoons}} GH \qquad G + HP \underset{k_r}{\overset{k_f}{\rightleftharpoons}} GHP$$

$$H + P \underset{\kappa_r}{\overset{\kappa_f}{\rightleftharpoons}} HP \qquad GH + P \underset{\kappa_r}{\overset{\kappa_f}{\rightleftharpoons}} GHP$$

Figure 3.1B The reaction network for release system components, heparin-binding peptide, heparin and heparin-binding growth factor.
All six possible species are shown: free growth factor (G), free heparin (H), immobilized heparin-binding peptide free of heparin (P), the growth factor - heparin complex (GH), the heparin - peptide complex (HP), and the growth factor - heparin - peptide complex (GHP). The kinetic rate constants shown are k_F and k_R representing the association and dissociation rate constants for bFGF binding to heparin and κ_F and κ_R representing the association and dissociation rate constants for peptide binding to heparin. It was assumed that heparin complexes to the growth factor with the same kinetic rate constants, regardless of pre-complexation to peptide or not, and likewise that heparin complexes to the peptide with the same kinetic rate constants, regardless of pre-complexation to the growth factor or not.

Table 3.1: Kinetic rate constants and diffusion coefficients employed in the mathematical modeling of passive release.

Symbol	Value	Parameter description
k_F	0.9×10^8 M^{-1} min^{-1}	association rate constant for bFGF binding to heparin (Nugent and Edelman, 1992)
k_R	0.68 min^{-1}	dissociation rate constant for bFGF and heparin (Nugent and Edelman, 1992)
κ_F	9.63×10^9 M^{-1} min	association rate constant for ATIII to heparin (Kridlel et al., 1996; Tyler-Cross et al., 1994; Tyler-Cross et al., 1996)
κ_R	78.0 min^{-1}	dissociation rate constant for ATIII and heparin (Olson et al., 1981)
D_G	6×10^{-5} cm^2 min^{-1}	diffusion coefficient of bFGF in fibrin matrix (approximation based on literature (Saltzman et al., 1994)
D_H	3.13×10^{-5} cm^2 min^{-1}	diffusion coefficient of heparin in fibrin matrix (Gaigalas et al., 1995)
D_{GH}	1.0×10^{-5} cm^2 min^{-1}	diffusion coefficient for heparin bound bFGF in fibrin matrix (approximation based on literature)(Saltzman et al., 1994)

The model consisted of six partial differential equations describing the mass balances for each of the six possible species in the system. For those species which are diffusible (free growth factor, free heparin, and the growth factor - heparin complex), terms describing diffusion and the association and dissociation kinetics were included, whereas in the case of the non-diffusible species (all species involving the peptide, which is always covalently immobilized to the fibrin network), only the association and dissociation terms were included. Modeling was performed assuming that the peptide-decorated fibrin matrix was acting as a filler for a 6 mm long nerve guide tube with impermeable walls and open ends. At the beginning of the release period, all reactions in the network were assumed to be at equilibrium. The area outside the tube, at both ends, was assumed to be a well-mixed sink, and the release from the open-ended tube was assumed to be symmetric, with equal rates at both ends.

The model is described by the following equations:

$$\frac{\partial c_G}{\partial t} = D_G \frac{\partial^2 c_G}{\partial x^2} - k_F c_G c_H + k_R c_{GH} - k_F c_G c_{HP} + k_R c_{GHP} \quad (1)$$

$$\frac{\partial c_H}{\partial t} = D_H \frac{\partial^2 c_H}{\partial x^2} - k_F c_G c_H + k_R c_{GH} - \kappa_F c_H c_P + \kappa_R c_{HP} \quad (2)$$

$$\frac{\partial c_P}{\partial t} = -\kappa_F c_H c_P + \kappa_R c_{HP} - \kappa_F c_{GH} c_P + \kappa_R c_{GHP} \quad (3)$$

$$\frac{\partial c_{GH}}{\partial t} = D_{GH} \frac{\partial^2 c_{GH}}{\partial x^2} + k_F c_G c_H - k_R c_{GH} - \kappa_F c_{GH} c_P + \kappa_R c_{GHP} \quad (4)$$

$$\frac{\partial c_{HP}}{\partial t} = \kappa_F c_H c_P - \kappa_R c_{HP} - k_F c_G c_{HP} + k_R c_{GHP} \quad (5)$$

$$\frac{\partial c_{GHP}}{\partial t} = k_F c_G c_{HP} - k_R c_{GHP} + \kappa_F c_{GH} c_P - \kappa_R c_{GHP} \quad (6)$$

where c_i is the concentration of species i, for i being G, H, P, GH, HP or GHP (the mass balances for which are represented by Eq. 1 - 6, respectively). The kinetic rate constants are as shown in Fig. 3.1B, namely with k_F and k_R representing the association and dissociation rate constants for bFGF binding to heparin, and with κ_F and κ_R representing the association and dissociation rate constants for peptide binding to heparin. D_G is the diffusion coefficient of free bFGF through fibrin, D_H is the diffusion coefficient of free heparin through fibrin, D_{GH} is the diffusion coefficient of the bFGF - heparin complex through fibrin. The independent variable x is distance from the midline of the nerve guide tube, and t is time from the initiation of release.

The system of equations were solved numerically using the FORTRAN program PDECOL, which has been designed to solve coupled systems of non-linear partial differential equations by a finite element collocation procedure (Hopkins, 1992; Madsen and Sincovec, 1979). The software was downloaded from <http://netlib.bell-labs.com>.

The initial condition to the model, namely that all reactions are at equilibrium, is represented by the following equations:

$$c_G^{eq} c_H^{eq} k_f = c_{GH}^{eq} k_r \tag{7}$$

$$c_P^{eq} c_H^{eq} K_f = c_{HP}^{eq} K_r \tag{8}$$

$$c_P^{eq} c_{GH}^{eq} K_f = c_{GHP}^{eq} K_r \tag{9}$$

$$c_P^{TOT} = c_P^{eq} + c_{HP}^{eq} + c_{GHP}^{eq} \tag{10}$$

$$c_G^{TOT} = c_G^{eq} + c_{GH}^{eq} + c_{GHP}^{eq} \tag{11}$$

$$c_H^{TOT} = c_H^{eq} + c_{HP}^{eq} + c_{GH}^{eq} + c_{GHP}^{eq} \tag{12}$$

This system of coupled nonlinear algebraic equations was solved numerically outside of PDECOL, and the equilibrium concentrations were simply input to the model as at $t = 0$, for $x \geq 0$,

$$c_i = c_i^{eq}, \text{ for } i\text{= G, H, P, GH, HP or GHP} \tag{13}$$

The boundary condition to the model, namely that the concentrations of all diffusible species just outside the ends of the tube are zero, are represented as c_i and for $t \geq 0$, at x = L (where L is the half-length of the tube),

$$c_i = 0, \text{ for } i = \text{G, H, GH} \tag{14}$$

This boundary condition is discontinuous with the initial condition at $t = 0$ and x = L, and the solver PDECOL does not allow such discontinuities. To address this, Eq. (14) was replaced with a functional equivalent, namely for $t \geq 0$, at x = L,

$$c_i = c_i^{eq} e^{\frac{-t}{\tau}}, \text{ for } i = \text{G, H, GH} \tag{15}$$

where $\tau = 0.001$ min. Thus, the initial condition at $t = 0$ and $x = L$ was exponentially forced to the boundary condition at $t > 0$ and $x = L$ over a characteristic period that was very short compared to the dissociation kinetics of the fastest dissociating species. (The value of τ was chosen such that further decreases in its value had no effect on the model results.)

The remaining boundary condition necessary to complete the model, namely symmetry about $x = 0$, is represented by for $t \geq 0$, at $x = 0$,

$$\frac{\partial c_i}{\partial x} = 0, \text{ for } i = \text{G, H, GH} \tag{16}$$

Because of the symmetry of the problem, only the range $0 \geq x \geq L$ was considered.

Because the mathematical model describes only passive release, and because the actual experimental model system is active, cell-mediated release only the first hour of release was calculated. As stated above, the model was employed to determine concentrations of peptide and heparin in the fibrin matrix that would be expected to yield slow passive release, such that active release would have the opportunity to dominate the overall release dynamics. To provide this guidance, the release rate of bFGF, either in free form (G) or bound to heparin (GH), was calculated from the concentration profiles obtained by solution of the model as

$$Flux_{G+GH} = D_G \left.\frac{\partial c_G}{\partial x}\right|_{x=L} + D_{GH} \left.\frac{\partial c_{GH}}{\partial x}\right|_{x=L} \tag{17}$$

evaluated at $x = L$. For purposes of comparison, the flux at t = 1 hr was calculated.

3.3.2 Synthesis of peptides

Two bi-domain peptide chimeras were synthesized, both containing a heparin-binding domain at the carboxyl terminus and either a functional or a nonfunctional factor XIIIa substrate domain from α_2-plasmin inhibitor at the amino terminus, as shown in Table 3.2. For clarity, the heparin-binding domains are shown in italics herein. In the first peptide, denoted $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$, the peptide contains the functional factor XIIIa substrate, NQEQVSP (Ichinose et al., 1983), in its amino-terminal domain. The carboxyl-terminal domain contains a modified sequence from the heparin-binding domain of antithrombin III (ATIII) (Tyler-Cross et al., 1994), as shown in Table 3.2. This modified ATIII domain has substitutions at positions F_{121} and F_{122} for K_{121} and $(\beta A)_{122}$ to better mimic the effect of R_{47} in the native protein, and $(\beta A)_{122}$ was added to increase flexibility of K_{121}(Tyler-Cross et al., 1994). Substitutions at positions N_{127} and C_{128} to A at both positions were made to prevent di-sulfide bond formation between peptides and to potentially induce α-helix formation when interacting with heparin (Tyler-Cross et al., 1994). The second peptide, denoted $\alpha_2PI_{1\text{-}7,\,Q^2\rightarrow G}$-$ATIII_{121\text{-}134}$, is similar, but contains a non-functional factor XIIIa substrate obtained by substitution of G for Q in the transglutaminase cross-linking site.

Table 3.2: Sequences and notation for bi-domain heparin-binding peptides.

Denotation	Sequence	Factor XIIIa substrate source	Heparin-binding domain source
$\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$	dLNQEQVSP*K(βA)FAK LAARLYRKA*-NH_2	α_2-plasmin inhibitor, functional	antithrombin III
$\alpha_2PI_{1\text{-}7,\,Q^2\rightarrow G}$-$ATIII_{121\text{-}134}$	LNGEQVSP*K(βA)FAK LAARLYRKA*-NH_2	α_2-plasmin inhibitor, nonfunctional	antithrombin III

Peptides were synthesized on solid amide resin (NovaSyn TGR, Novabiochem, Laüfelfingen, Switzerland) using an automated peptide synthesizer (Pioneer, PerSeptive Biosystems, Framingham, MA) with standard 9-fluorenyl-methyloxycarbonyl chemistry

(Fields and Noble, 1990). Peptides used in cross-linking quantification were labeled with a fluorescent probe by placing an α-dansyl leucine at the amino terminus of the sequence. All solvents for peptide synthesis were obtained from Paul Bucher Company (Basel, Switzerland). All other reagents for peptide synthesis were obtained from Novabiochem, unless noted. Peptides were cleaved using 88% trifluoroacetic acid (Paul Bucher Company), 0.5% phenol, 0.5% water, and 0.2% triisopropylsilane for 2-3 hr. Following cleavage, the peptides were precipitated into 10 volumes of cold ethyl ether, and the precipitate was recovered by filtration and washed twice with ethyl ether to remove hydrophobic protecting groups and scavengers. The peptides were dried for 4 hr under vacuum and dissolved into 20 mL of deionized water. They were dialyzed against 4 L of deionized water for 24 hr and lyophilized. Peptides were dissolved in Tris-buffered saline (TBS) (33 mM Tris, 8 g/L NaCl, 0.2 g/L KCl, pH 7.4) at a concentration of 0.01 M and syringe filtered (0.22 μm) prior to use.

3.3.3 Preparation of fibrin matrices

Fibrinogen solutions were prepared exactly as described previously, using plasminogen-free fibrinogen from pooled human plasma (Schense and Hubbell, 1999); this fibrinogen preparation contains factor XIII, the zymogen of the transglutaminase factor XIIIa. Fibrin matrices (400 μL per well) were made by mixing the components to obtain the following final solution concentrations: 3.5 mg/mL fibrinogen, 2.5 mM Ca^{++}, 2 NIH units/mL of thrombin, 0.25 mM peptide or 0.125 mM peptide (to obtain 8 moles of cross-linked peptide per mole fibrinogen or 4 moles of cross-linked peptide per mole fibrinogen, respectively), 62.5 μM heparin (sodium salt from porcine intestinal mucosa, 176 USP U/mg, 18,000 average MW, when used), and bFGF and VEGF (recombinant human, Peprotech EC, London, England) when used. The amount of peptide cross-linked into fibrin matrices during coagulation was quantified exactly as previously described (Sakiyama et al., 1999; Schense and Hubbell, 1999). The polymerization mixture was

placed into a well of a flat 24-well tissue culture plate and was incubated for 60 min at 37 °C, 95% relative humidity, and 5% CO_2.

After polymerization of the fibrin matrix, 1 mL of TBS was added to each well to wash any unbound components of the delivery system from the matrix. The matrices were washed a total of five times over 24 hr, four times with TBS and one time with 1 mL of modified neural basal medium, consisting of insulin (5 μg/mL), transferrin (100 μg/mL), progesterone (6.4 ng/mL), putrescine (16.11 μg/mL), selenite (5.2 ng/mL) (all from Gibco, Basel, Switzerland), 5 μg/mL fibronectin, 0.1% bovine serum albumin, 20 ng/mL mouse nerve growth factor, 0.5 mM L-glutamine, 25 μM L-glutamate, and 1% antibiotic-anti-mycotic solution (Gibco) added to neural basal medium (Gibco).

3.3.4 DRG culture and analysis

Dorsal root ganglia (DRGs) were dissected from embryonic day 8 (E8) White Leghorn chicken embryos (Varon, 1972) and placed in Hanks-buffered salt solution (HBSS) (Gibco). The DRGs were placed inside the fibrin matrix with dissection forceps, one per matrix. The fibrin matrices containing DRGs were incubated for 60 min at 37 °C, 95% relative humidity, and 5% CO_2, and then 1 mL of modified neural basal medium was added to each well. The medium was changed at 24 hr. Experiments with 1.0 μg/mL bFGF were performed in quadruplicate, and all other experiments were performed in duplicate, each with 6 DRGs per replication. DRGs were cultured within fibrin matrices without peptide, heparin, or heparin-binding growth factors, and all measurements of neurite extension were normalized with respect to this level.

Brightfield images of the DRGs were taken at 24 and 48 hr with 4.0x Achroplan (24 hr) and 2.5x Plan (48 hr) objective (Zeiss, Zurich, Switzerland). The images were digitized with a Shimatsu chilled color 3-chip CCD camera, Matrox Meteor PCI frame grabber (Matrox Electronic Systems, Dorval, Quebec, Canada), and Leica Qwin software (both from Leica, Zurich, Switzerland). These images were then analyzed to determine

the average length of neurite extension, which was calculated as the radius of an annulus between the DRG body and the outer halo of extending neurites, exactly as described previously (Herbert et al., 1996). Neurite length for each experiment was normalized by the average neurite extension through unmodified fibrin matrices from the same experiment at the same time point.

3.3.5 Statistics

Statistical analysis was performed using Statview® 4.5 (Abacus Concepts, Berkeley, CA). Comparative analyses were completed using the Scheffe's F post-hoc test by Analysis of Variance (ANOVA) at a 95% confidence level. Mean values and standard error of the mean are reported.

3.4 Results and Discussion

The goal of this research was to develop a growth factor delivery system to enable localized delivery during wound healing, such that heparin-binding growth factor is released in response to cellular activity during healing. Fibrin was selected as the base material for this delivery system, in order to provide a three-dimensional scaffold for wound repair, the adhesive and drug delivery characteristics of which could be tailored for the wound healing model of interest. The drug delivery system developed in these studies consisted of heparin-binding peptides covalently immobilized to fibrin, heparin bound to these immobilized peptides, and heparin-binding growth factor, as shown in Figure 3.1A. Release from such a system can occur through three different mechanisms: *i)* the dissociation of growth factor from matrix-bound heparin and the subsequent diffusion of free heparin-binding growth factor from the matrix, *ii)* proteolytic degradation of the fibrin matrix, or *iii)* enzymatic degradation of heparin. The first mechanism of release described above is passive and occurs in the presence or absence of cells, while the second two mechanisms are active and occur only in the presence of cells.

To determine the effect of the drug delivery system parameters (such as the relative concentrations of heparin, peptide and growth factor) on passive release, mathematical modeling was performed. Parameters were selected based on this modeling to obtain slow passive release, based on the logic that only then could active release dominate. Fibrin matrices formed under these conditions were then tested in a neuronal cell culture model to determine if cell-mediated release of bioactive growth factor was possible.

3.4.1 Modeling

In order to determine an optimal ratio of heparin to growth factor, a mathematical model of the release system was developed. Mass balances were written for all six possible species (free growth factor, free heparin, immobilized heparin-binding peptide free of heparin, the growth factor - heparin complex, the heparin - peptide complex, and the growth factor - heparin - peptide complex). All possible reactions between the system components were considered (shown in Figure 3.1B), and diffusion of all free species (free growth factor, free heparin, and the growth factor - heparin complex) through the fibrin matrix was also taken into account. Reaction rate constants and diffusion coefficients were estimated from values previously reported in the literature (Table 3.1). The mass balances were then solved numerically for each set of delivery system parameters, as described above.

Because the mathematical model describes only passive release of growth factor and not cell-mediated active release, modeling results were calculated only for the first hour of release, in order to determine the conditions required for slow initial passive release of growth factor. Because the growth factor concentration within the tube is always greatest initially and the concentration outside the tube is assumed to be zero, the driving force for diffusion of growth factor out of the tube is the greatest initially for all cases. Model results for times up to 1 hr, under conditions when the ratio of heparin to growth factor is high, showed that amount of bFGF bound to heparin-peptide complex in

the fibrin matrix remains constant at the initial equilibrium concentration at all locations in the matrix except in the region closest to the end of the tube (see Figure 3.2). A decrease in the concentration of bound growth factor was observed only within the endmost 1% of the tube, suggesting that very little bFGF is lost due to passive release. As one would expect, the decreasing concentration profile of bound growth factor propagates inward as time increases.

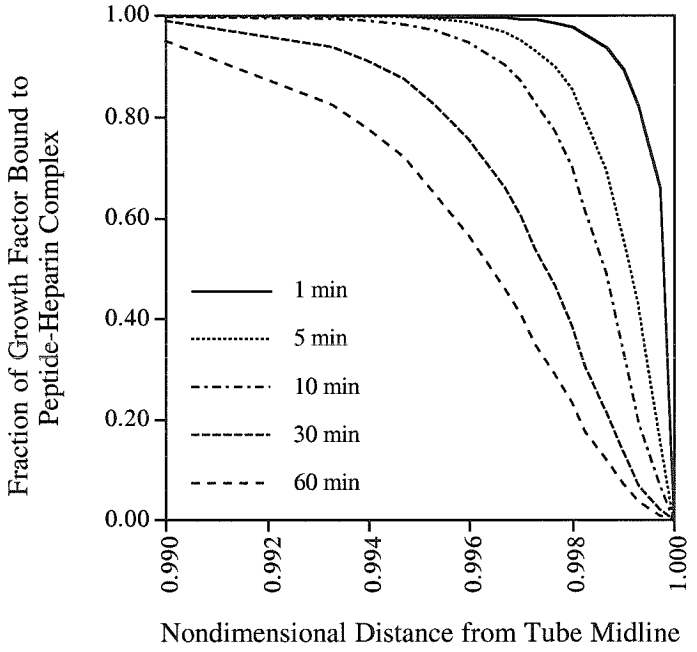

Figure 3.2 Theoretical concentration of matrix-bound bFGF as a function of distance from the midline of a model tubular nerve growth guide, 6 mm long and open at both ends.
Concentration is shown as percentage of the initial bound concentration, which was 5.7 x 10^{-8} M. The ratio of heparin to growth factor modeled was approximately 500. The decreasing concentration profile propagates inward over time, as one would expect.

Because the rate of passive diffusion is controlled in part by the kinetics of heparin binding to heparin-binding growth factor, we went on to test the effects of varying the ratio of heparin to growth factor on the total flux of growth factor out of the fibrin matrix. The rate of release is highly dependent on the concentration of heparin sites within the fibrin matrix, as is demonstrated by the results in Figure 3.3, which show that the initial flux of bFGF at the end of the tube increases as the ratio of heparin to

growth factor decreases. For low ratios of heparin to growth factor (0.1), the flux of bFGF out of the matrix at 1 hr was 3.00×10^{-14} mol/cm^{2}min, which is 93% of flux in the case of simple diffusion (when there is no heparin in the system). As the ratio of heparin to growth factor is increased to 1, the flux drops to approximately 50% of that seen in the case of simple diffusion. As the ratio of heparin to growth factor is increased further to 10, 100 and 1000, the flux at 1 hr drops to 12%, 4%, and 1.4% of that due to simple diffusion, respectively. The dependence of the flux of growth factor on the ratio of heparin to growth factor is not surprising, because at a constant growth factor concentration, increasing the concentration of heparin will increase the overall rate of growth factor binding (preventing diffusion from the matrix) and will shift the overall reaction equilibrium toward the bound state.

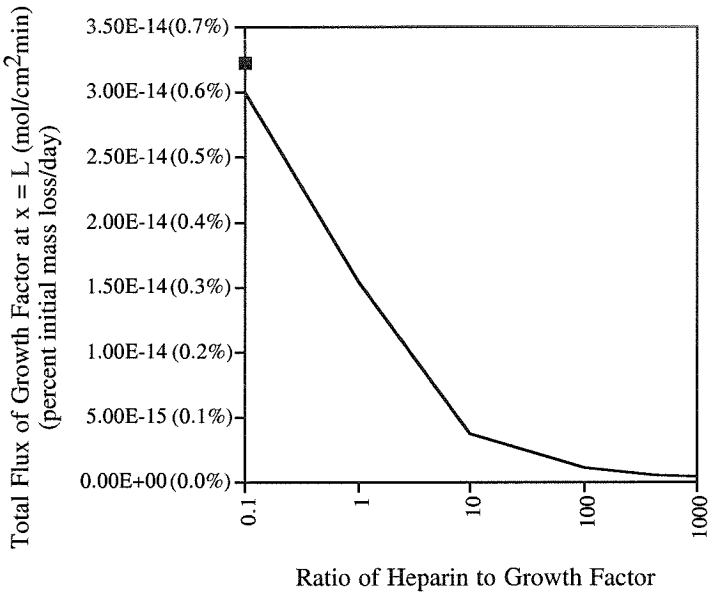

Figure 3.3 Theoretical flux of bFGF at the end of a model tubular nerve growth guide at 1 hour as a function of the ratio of heparin to growth factor.
The percent initial mass lost per day is given in parentheses on the ordinate. The ■ symbol represents the flux in the case of simple diffusion, where no heparin is present. As the ratio of heparin to growth factor was decreased, the rate of passive release of bFGF increased.

Based on these results, we selected a heparin-bFGF ratio of approximately 500 for testing in cell culture experiments. At this ratio of heparin to bFGF, the initial flux of

bFGF out of the model tube at 1 hr was calculated to be 6.10×10^{-16} mol/cm^{2}min (1.9% of the flux in the case of simple diffusion), and if release continued at this same rate, approximately 0.1% of the initial bFGF dose would be lost by passive release over 30 days. Given this initial rate of release, the goal of release of bFGF over a period of one month by cell-mediated processes rather than by passive release seemed feasible, as long as a high excess of heparin sites is maintained.

3.4.2 DRG culture with heparin-binding peptides

After mathematically determining the conditions necessary for slow passive release of heparin-binding growth factor, we went on to experimentally test fibrin matrices formed under these conditions using neurite extension from DRGs as a cell culture model to assay the availability of active bFGF. The release of matrix bound growth factor was not measured directly, but rather the stimulation of enhanced neurite extension was taken as a measure of the ability of the delivery system to release bioactive bFGF. Bi-domain peptides with a modified heparin-binding domain from antithrombin III at the carboxy terminus and the factor XIIIa substrate domain from α_2-plasmin inhibitor at the amino terminus ($\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$) were cross-linked into fibrin matrices by the action of factor XIIIa during coagulation. The maximal incorporation of peptides by this method has been previously quantified to be 7.1 ± 0.1 moles of peptide per mole fibrinogen with heparin in the polymerization mixture (Sakiyama et al., 1999) and 8.7 ± 0.2 moles of peptide per mole fibrinogen without heparin in the polymerization mixture (Sakiyama et al., 1999; Schense and Hubbell, 1999). The addition of bFGF did not significantly effect the incorporation of peptide in the presence of heparin. The amount of peptide incorporated in the presence of heparin and bFGF was quantified to be 7.9 ± 0.5 moles peptide per mole fibrinogen. (The peptide to heparin ratio used to quantify incorporation was 4:1 and the heparin to bFGF ratio used was 500:1, the same as was used for the DRG experiments.) These cross-linked heparin-binding peptides were used

to immobilize heparin, which was also present in the polymerization mixture. The immobilized heparin in turn bound bFGF, similar to the way it is sequestered to the ECM in natural tissues.

To determine the ability of such materials to deliver bioactive growth factors in a controlled manner, heparin-binding peptide, heparin and bFGF were added during the polymerization of fibrin matrices. The matrices were washed for 24 hr and then whole DRGs were placed inside the matrices. When all three components of the delivery system (heparin-binding peptide, heparin, and bFGF) were present during polymerization, matrix-bound bFGF was found to enhance neurite extension in a dose dependent manner (Fig. 3.4). Enhancement of neurite extension by up to 100% relative to unmodified fibrin was observed (Fig. 3.4). The dose response effect appeared to be biphasic, with significant enhancement in neurite length at 1 μg/mL and 5 μg/mL bFGF. However, no significant enhancement of neurite length was observed at 10 μg/mL bFGF. Similar dose saturation effects with growth factors have been previously observed (Wang and Aspenberg, 1996), and are attributed to the saturation of the cell-surface receptors for the growth factor. Therefore, we were not surprised to observe a biphasic dose response effect that increased up to some optimal dose, beyond which the addition of more bFGF did not enhance neurite extension.

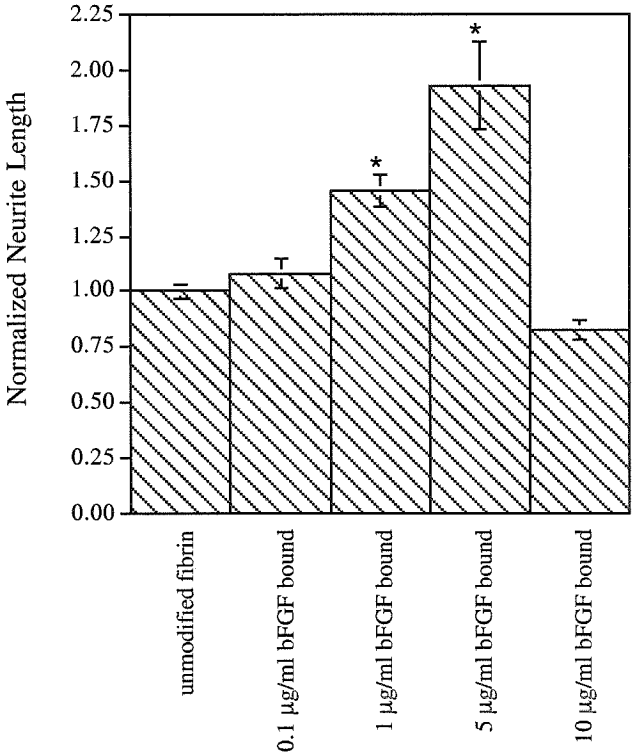

Figure 3.4 Effect of matrix-bound bFGF on DRG neurite extension through fibrin matrices, containing the complete delivery system (bi-domain peptide with a functional factor XIIIa domain and a heparin-binding domain, together with heparin).
* denotes $p<0.05$ compared to unmodified fibrin. These results demonstrated that neurite extension increased as the amount of bFGF immobilized in the matrix was increased up to some optimal dose, beyond which the addition of more bFGF did not enhance neurite extension.

To determine the ability of our delivery system to immobilize bFGF within fibrin matrices, we tested the ability of fibrin matrices lacking the delivery system to retain and deliver active bFGF. When only free bFGF (no heparin or peptide) was added to the polymerization mixture, no enhancement of neurite extension was seen (Fig. 3.5). This result demonstrated that in the absence of the delivery system, bFGF diffused out the fibrin matrix during washing and was lost due to rapid passive release. This demonstrated that the drug delivery system must be present in order for bFGF to enhance neurite extension in our culture model.

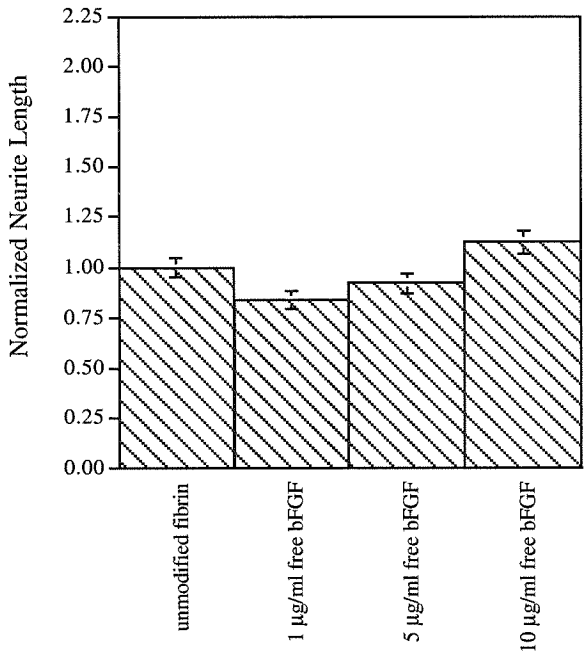

Figure 3.5 Effect of non-matrix-bound bFGF on DRG neurite extension within fibrin matrices, containing no peptide or heparin.
These results demonstrated that neurite extension was not enhanced by the addition of free bFGF in the polymerization mixture, suggesting that bFGF added in the polymerization mixture must be retained within the matrix in order to enhance neurite extension through fibrin matrices in our culture model.

3.4.3 Additional control experiments

The ability of matrix-bound bFGF to enhance neurite extension was compared at 1 µg/mL with the activity of bFGF in the medium (Fig. 3.6) to determine if the delivery system affected the activity of the growth factor being delivered. Matrix-bound bFGF and free bFGF in the culture media showed no statistically significant difference in their ability to promote neurite extension. Free bFGF in the culture media at this dose also showed no statistically significant difference relative to unmodified fibrin cultured without bFGF in the media. No enhancement of neurite extension was observed when doses of bFGF less than 1 µg/mL were added to the medium (data not shown), and doses above 1 µg/mL in the medium were not tested. These results suggested that immobilizing bFGF to the fibrin matrix using our drug delivery system did not have an effect, positive or negative, on growth factor activity per se, but rather merely prevents

the rapid passive release that would otherwise take place in vivo thus allowing for delivery of active growth factor over a sustained period.

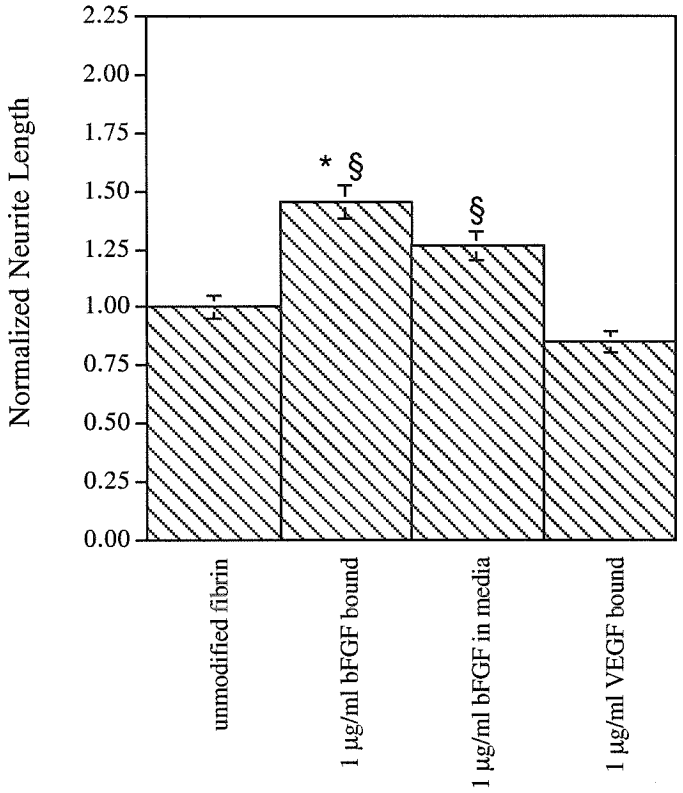

Figure 3.6 Effect of bFGF and VEGF on DRG neurite extension through fibrin matrices, containing the complete delivery system.
* denotes $p<0.05$ compared to unmodified fibrin. § denotes $p>0.05$ versus 1.0 µg/mL matrix-bound bFGF. These results demonstrated that matrix-bound bFGF and free bFGF present in culture medium promoted similar enhancement of neurite extension; however, a heparin-binding growth factor with no known effects in neural culture (VEGF) had no effect on neurite extension.

As a control on the identity of the growth factor, matrix-bound VEGF was tested for its ability to promote neurite extension (Fig. 3.6). VEGF is a heparin-binding growth factor, but it is not know to promote neurite extension in culture. Matrix-bound VEGF did not enhance neurite extension relative to unmodified fibrin. This result demonstrated that in order to enhance neurite extension, the growth factor of interest must be both capable of binding to heparin and capable of promoting neurite extension.

Some precipitate was observed when heparin and peptide were mixed in the polymerization mixture (with or without growth factor), and it was for this reason that we performed further control experiments to ensure that the enhancement of neurite extension seen was due to the delivery of growth factors from the system described above and not due to release from the precipitate. When only heparin and peptide were added to the polymerization mixture (without bFGF) no enhancement of neurite extension was observed (Fig. 3.7), suggesting that the delivery system without growth factor did not enhance neurite extension on its own. When only heparin and bFGF (without peptide) were added to the polymerization mixture, no enhancement of neurite extension was observed (Fig. 3.7), suggesting that heparin-binding peptide is required to immobilize the heparin and growth factor components of the delivery system. To test whether the heparin-binding peptide had to be covalently cross-linked to fibrin matrix to enhance neurite extension, a peptide containing a non-functional transglutaminase cross-linking domain ($\alpha_2PI_{1\text{-}7,\,Q2\rightarrow G}$-$ATIII_{121\text{-}134}$) was used ("non-functional" peptide bar in Fig. 3.7). No enhancement of neurite extension was observed, suggesting that the heparin-binding peptide must be covalently immobilized for sustained delivery to occur, even though precipitate was observed with the "non-functional" peptide.

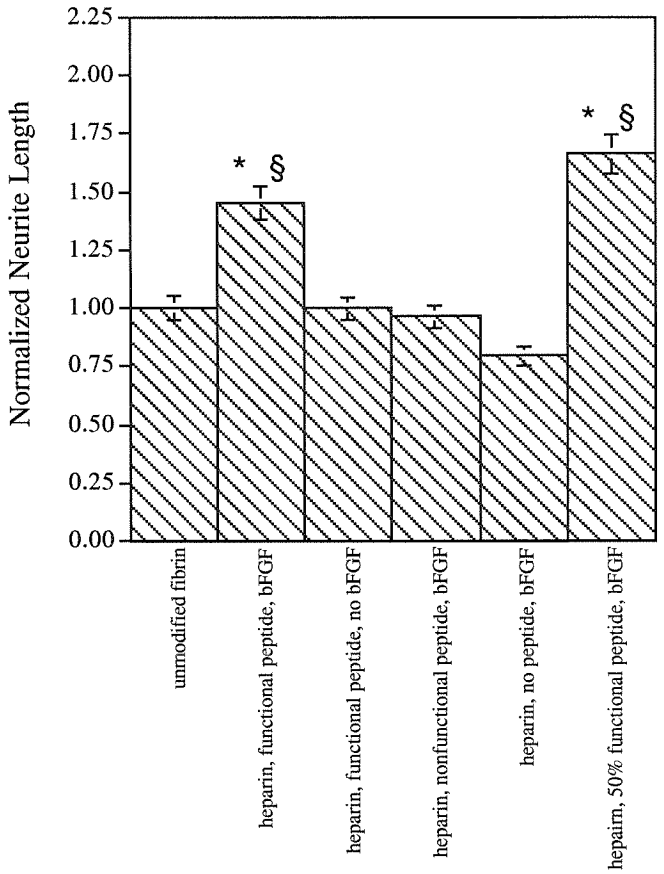

Figure 3.7 Effect of the removal of delivery system components on DRG neurite extension through fibrin matrices.
Experiments where bFGF was added to the polymerization mixture were performed at a dose of 1.0 μg/mL. * denotes $p<0.05$ compared to unmodified fibrin. § denotes $p>0.05$ versus 1.0 μg/mL matrix-bound bFGF. The removal of either bFGF or peptide from the delivery system resulted in no enhancement of neurite extension. The use of a peptide containing a non-functional cross-linking substrate demonstrated that the peptide component of the delivery system must be covalently immobilized within the fibrin matrix. Decreasing the amount of peptide cross-linked to the fibrin matrix by 50% did not significantly decrease neurite extension promoted by the dose of bFGF used, suggesting that a large excess of heparin-binding peptide was present in the fibrin matrix. These results suggested that heparin, functional peptide, and growth factor are all necessary components of the drug delivery system.

To determine if a high excess of heparin-binding peptide was present, the amount of heparin binding peptide was reduced by half in the polymerization mixture (Fig. 3.7). This did not lead to a decrease in the enhancement of neurite extension at a dose of 1

μg/mL bFGF. This suggests that there was indeed a high excess in the amount of heparin-binding peptide, such that the loss of half of the peptide did not reduce the efficacy of the delivery system. This is consistent with the results of Figure 3.3, which suggest that we are operating under conditions of high heparin, such that a decrease of 50% in the amount of peptide or heparin would have little effect on the flux of growth factor out of the fibrin matrix.

Taken together, the results of the additional control experiments suggested that the heparin-based delivery system described above can sequester heparin-binding growth factors and prevent their diffusion from fibrin matrices. The results demonstrate that the growth factors delivered from such systems suffer no loss of bioactivity due to their binding interaction with the fibrin matrix. They also show that all components of the delivery system, namely heparin, covalently immobilized peptide and heparin-binding growth factor, are necessary for slow passive release.

3.4.4 Discussion

Because growth factors are such powerful regulators of biological function, their presence in tissues is highly regulated in both time and space. One means by which this is accomplished is by binding to ECM components, as is observed with the heparin-binding growth factors and their interaction with ECM glycosaminoglycans. This high excess of ECM proteoglycans very effectively sequesters the FGFs and other heparin-binding growth factors; FGFs that passively dissociate from the matrix are quickly recaptured by unoccupied heparan sulfate sites. FGFs that are in solution must be attached to a heparin-like molecule in order to diffuse through the ECM; otherwise, they are sequestered by the matrix and do not diffuse (Vlodavsky et al., 1991). Thus, although the dissociation of FGF from heparin is rapid (k_D equal to approximately 1 min^{-1}), provision of excess immobilized heparin or heparan sulfate can dramatically slow FGF release, both naturally as well as in therapeutic systems. We have developed a growth

factor delivery system, which mimics the ECM and its ability to provide slow passive release of growth factor, which allows cell-mediated active release to dominate in the context of wound healing and tissue regeneration.

The results of this study demonstrate that matrices modified to contain heparin-binding sites can be used to deliver heparin-binding growth factors in manner that is dependent on cell-mediated active, rather than passive, release. Cell-mediated active release can be especially beneficial for long term delivery applications, such as nerve regeneration and angiogenesis, when the healing process may require weeks to months and may proceed from one side of an implanted matrix to another. Materials, such as the ones described above, can release growth factor at different rates in different locations, depending on the location of cells or cell processes within the material. For example, as a neurite extends from a neuron in a regenerating nerve, the proteolytic activities associated with the nerve growth cone will liberate sequestered growth factor, freeing it to be active on that very neurite. The binding sites in the material may also serve to protect the bioactivity of the growth factor while it is bound within the matrix.

3.5 Conclusions

The materials described in this paper may have broad usefulness in tissue engineering. A variety of applications in tissue engineering could benefit from novel three-dimensional matrices, which can act as both cell ingrowth substrates and drug delivery devices. These applications include the use of matrices for cell seeding to generate tissues in vitro for ultimate transplantation and the use of matrices for cell invasion to generate and regenerate tissue in vivo. Examples of tissue morphogenesis include generation of tissue-engineered skin, blood vessels, liver, nerve, bone and cartilage (Black et al., 1998; L'Heureux et al., 1998; Mooney et al., 1997; Schroeder-Tefft et al., 1997; Sims et al., 1998). In these approaches, a matrix component plays a key role, and its interactions with cells either in vitro or in vivo exert an important influence in the

process of morphogenesis. The fibrin-based materials described above possess three key characteristics beneficial for tissue engineering: *(1)* the ability of cells to infiltrate and remodel these matrices, *(2)* the ability to tailor the adhesive nature of the matrix, and *(3)* the ability to present other exogenous bioactive signals, such as polypeptide growth factors, from the matrix. Regarding the first point, cells can infiltrate and remodel the fibrin matrix using plasmin, and the activation of plasminogen to plasmin is localized to the surface of many cell types via receptors for plasminogen activators (Pittman et al., 1989). For example, through this localization, an intact fibrin structure has been observed to within 100 nm of the growth cone surface as it penetrates the fibrin matrix in neurite extension (Herbert et al., 1996). Regarding the second point, the adhesive character of fibrin matrices can be tailored, as has been previously demonstrated, e.g., (Schense and Hubbell, 1999) through incorporation of RGD peptides, which bind to integrin adhesion receptors, and (Sakiyama et al., 1999) through incorporation of peptides that bind to cell-surface proteoglycans. This paper demonstrates the third point, namely the ability to incorporate other bioactive species, such as polypeptide growth factors, and deliver them in a biomimetic manner.

Chapter 4 Controlled Release of Nerve Growth Factor from a Heparin-Containing Fibrin-Based Cell Ingrowth Matrix*

4.1 Abstract

The goal of this work was to develop a growth factor delivery system for use in nerve regeneration that would provide localized release of beta-nerve growth factor (β-NGF) and other members of the neurotrophin family in a controlled manner. Although β-NGF does not bind heparin with high affinity, we postulated that a basic domain found at the surface of native β-NGF could interact with heparin and slow its diffusion from a heparin-containing delivery system. To test this hypothesis, we used a heparin-containing fibrin-based cell ingrowth matrix consisting of three components, namely an immobilized heparin-binding peptide, heparin and a neurotrophin with low heparin-binding affinity. The heparin-binding peptide contained a factor XIIIa substrate and was covalently cross-linked to fibrin matrices during polymerization. This cross-linked heparin-binding peptide served to immobilize heparin within the matrix, and this immobilized heparin interacted with the neurotrophin and slowed the passive release of the growth factor from the matrix. The ability of heparin-containing fibrin matrices, with a high excess of heparin-binding sites, to slow the diffusion-based release of β-NGF from fibrin matrices was measured in the absence of cells. Conditions that provided for slow diffusion-based release of β-NGF, brain derived neurotrophic factor, and neurotrophin-3 were tested in an assay of neurite extension from dorsal root ganglia to determine the ability of the delivery system to release active growth factor. The results demonstrated that neurotrophins, interacting with fibrin matrices containing a large molar excess of heparin relative to growth factor, enhanced neurite extension by up to 100% relative to unmodified fibrin. In the absence of the delivery system, free neurotrophins within the fibrin matrix did not enhance neurite extension. The results suggest that these matrices

* The contents of this chapter have been submitted to *Journal of Controlled Release* for publication.

could serve as therapeutic materials to enhance peripheral nerve regeneration through nerve guide tubes and may have more general usefulness in tissue engineering for the delivery of non-heparin binding growth factors.

4.2 Introduction

The objective of this research was to develop a growth factor delivery system for use in tissue repair that would provide localized release of β-NGF and other members of the neurotrophin family to enhance peripheral nerve regeneration. *In vivo*, β-NGF has been added to nerve guide tubes, resulting in an increase in nerve regeneration when delivered from polymer-based depots or when present in the free form (Otto et al., 1987; Rich et al., 1989; Varon and Conner, 1994). Based on these studies, it appears that growth factors from the neurotrophin family have the potential to enhance peripheral nerve regeneration. However, in many of these studies, the authors point out difficulty in maintaining growth factor release over the rather long duration of nerve regeneration. It seems likely that a delivery system that provided prolonged release of active growth factor would be more effective at delivering growth factor over the duration of wound healing.

Previously, heparin-containing delivery systems have been used to immobilize high affinity heparin-binding growth factors and protect them from degradation (Edelman et al., 1992; Sakiyama-Elbert and Hubbell, 2000). Specifically, we have demonstrated the ability of heparin-containing delivery systems to release heparin-binding growth factors, such as basic fibroblast growth factor (bFGF), from fibrin matrices in an active manner, such that release occurs primarily in response to cellular activity during healing (Sakiyama-Elbert and Hubbell, 2000). However, the use of heparin-containing delivery systems to deliver growth factors with low heparin-binding affinity, such as β-NGF, has not been reported previously in the literature.

We considered the primary structures of three members of the neurotrophin family, β-NGF, brain derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). None of these three growth factors possess strong heparin-binding affinity under physiological conditions. For example, β-NGF has even been used as a negative control in experiments regarding heparin-binding proteins (Lee and Lander, 1991). The carboxyl-termini of these three proteins all contain a basic cluster of amino acid residues, as shown in Table 4.1. Each of these basic domains contains three or four basic residues, which are underlined, flanked by hydrophobic residues, shown in bold. These basic domains follow the general pattern of heparin-binding domains, namely of basic residues flanked by hydrophobic residues, as described by Cardin and Weintraub (Cardin and Weintraub, 1989). Furthermore, each of these domains is known to be surface-accessible in the native protein. The three-dimensional structure of β-NGF is shown in Figure 4.1, and the basic domain on the surface of the protein is illustrated, demonstrating the accessibility of this domain in the native protein.

Table 4.1: C-terminal amino acid sequences of β-NGF, BDNF, and NT-3.

Growth Factor	Species	C-terminal sequence
β–NGF	human	SRKAVRRA
	mouse	SRKATRRG
	rat	SRKAARRG
	pig	SRKAGRRA
BDNF	human	TLTIKRGR
	mouse	TLTIKRGR
	rat	TLTIKRGR
	pig	TLTIKRGR
NT-3	human	LSRKIGRT
	mouse	LSRKIGRT
	rat	LSRKIGRT

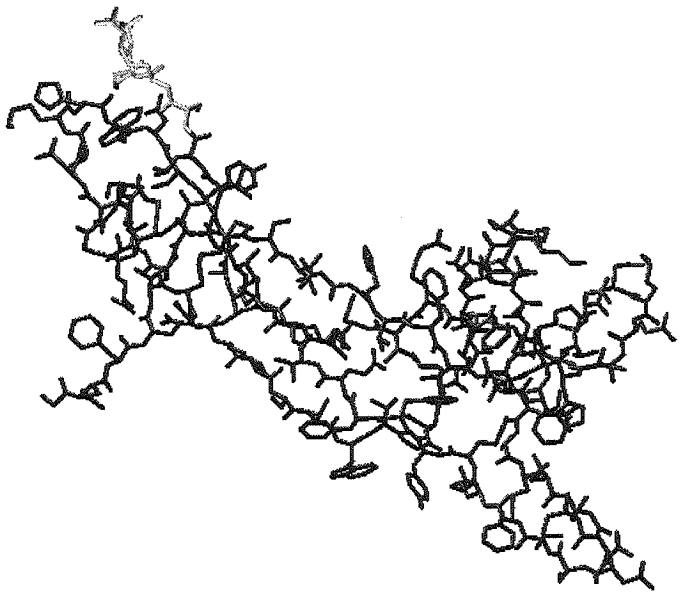

Figure 4.1 Three-dimensional structure of β-NGF in a stick model.
The basic domain, which can interact with heparin, is shown in gray. Structure was downloaded from SWISSPROT (http://www.expasy.ch/sprot/) (Bairoch and Apweiler, 1999) and is based on the crystal structure of mouse β–NGF (McDonald et al., 1991). The model was generated using Swiss Pdb software (downloaded from http://www.expasy.ch/spdbv/) using the SWISSPROT structure file 1BET (http://www.expasy.ch/cgi-bin/get-pdb.pl?1bet).

We hypothesized that by providing a high molar excess of heparin sites within a three-dimensional matrix delivery system, we could slow the diffusion-based release of β-NGF, BDNF and NT-3. To test this hypothesis, we utilized a heparin-containing delivery system that has been shown to deliver heparin-binding growth factors in controlled manner and that allows the immobilization of a high excess of heparin-binding sites. This drug delivery system consisted of heparin-binding peptides covalently immobilized within a fibrin matrix, heparin bound to these immobilized peptides, and growth factor. The fibrin was modified utilizing a previously developed method of covalently cross-linking bi-domain peptides during fibrinogen polymerization using the transglutaminase activity of factor XIIIa (Schense and Hubbell, 1999). This method has been used to incorporate exogenous heparin-binding peptides into fibrin matrices by placing a factor XIIIa substrate from α_2-plasmin inhibitor (Sakiyama et al., 1999;

Sakiyama-Elbert and Hubbell, 2000) at the N-terminus of the peptide, and polymerizing the fibrin matrix in the presence of the bi-domain peptide (Schense and Hubbell, 1999). The bi-domain peptide could be covalently incorporated at concentrations of up to 8 moles of peptide per mole of fibrinogen. The second domain of these peptides was designed to possess heparin-binding character, based on a domain of antithrombin III, and these covalently bound heparin-binding peptides were used to immobilize heparin within fibrin matrices based on electrostatic interactions. The heparin thus bound to the fibrin matrix in turn served to immobilize neurotrophins within the fibrin, or at least to delay their release by multiple, low-affinity binding interactions. These growth factors can then be released by enzymatic factors, e.g., heparinase (Presta et al., 1989; Vlodavsky et al., 1991) or plasmin from migrating cells (Herbert et al., 1996), as well as by passive diffusion-based release. The release of β-NGF from the heparin-containing delivery system in the absence of cells was quantified to determine the ability of the delivery system to slow diffusion-based release of β-NGF. The delivery system was then tested in a neuronal cell culture assay to determine the ability of such a system to deliver active non-heparin binding members of the neurotrophin family in a controlled manner.

4.3 Materials and Methods

All materials were obtained from Fluka (Buchs, Switzerland) unless otherwise specified.

4.3.1 Synthesis of peptides

A bi-domain heparin-binding peptide was synthesized with the sequence dLNQEQVSP*K(βA) FAKLAARLYRKA*-NH_2, where dL denotes dansyl leucine. This peptide, denoted $\alpha_2PI_{1\text{-}7}$-$ATIII_{121\text{-}134}$, contains a factor XIIIa substrate, NQEQVSP (Ichinose et al., 1983), in its amino-terminal domain, derived from the N-terminal seven residues of α_2-plasmin inhibitor. The carboxyl-terminal domain contains a modified sequence from the heparin-binding domain of antithrombin III (ATIII) (Tyler-Cross et

al., 1994), as shown above in italics. This modified ATIII domain has substitutions at positions F_{121} and F_{122} for K_{121} and $(\beta A)_{122}$ to better mimic the effect of R_{47} in the native protein, and $(\beta A)_{122}$ was added to increase flexibility of K_{121} (Tyler-Cross et al., 1994). Substitutions at positions N_{127} and C_{128} to A at both positions were made to prevent di-sulfide bond formation between peptides and to potentially induce α-helix formation when interacting with heparin (Tyler-Cross et al., 1994).

Peptides were synthesized on amide resin (NovaSyn TGR, Novabiochem, Laüfelfingen, Switzerland) using an automated peptide synthesizer (Pioneer, PerSeptive Biosystems, Framingham, MA) with standard 9-fluorenyl-methyloxycarbonyl chemistry (Fields and Noble, 1990). Peptides used in cross-linking quantification were labeled with a fluorescent probe by placing an α-dansyl leucine at the amino terminus of the sequence. All solvents for peptide synthesis were obtained from Paul Bucher Company (Basel, Switzerland). All other reagents for peptide synthesis were obtained from Novabiochem, unless noted. Peptides were cleaved using 88% trifluoroacetic acid (Paul Bucher Company), 0.5% phenol, 0.5% water, and 0.2% triisopropylsilane for 2-3 hr. Following cleavage, the peptides were precipitated into 10 volumes of cold ethyl ether, and the precipitate was recovered by filtration and washed twice with ethyl ether to remove hydrophobic protecting groups and scavengers. The peptides were dried for 4 hr under vacuum and dissolved into 20 mL of deionized water. They were dialyzed against 4 L of deionized water for 24 hr and lyophilized. Peptides were dissolved in Tris-buffered saline (TBS) (33 mM Tris, 8 g/L NaCl, 0.2 g/L KCl, pH 7.4) at a concentration of 0.01 M and syringe filtered (0.22 μm) prior to use.

4.3.2 Preparation of fibrin matrices

Fibrinogen solutions were prepared exactly as described previously, using plasminogen-free fibrinogen from pooled human plasma (Schense and Hubbell, 1999); this fibrinogen preparation contains factor XIII, the zymogen of the transglutaminase

factor XIIIa, at approximately 27 μg per mg of fibrinogen (Schense et al., 2000). Fibrin matrices (400 μL per well) were made by mixing the components to obtain the following final solution concentrations: 3.5 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), 62.5 μM heparin (sodium salt from porcine intestinal mucosa, 176 USP U/mg, 18,000 average MW), and β-NGF, BDNF, or NT-3, as indicated (all recombinant human, Peprotech EC, London, England). The amount of peptide cross-linked into fibrin matrices during polymerization was previously quantified in the presence of heparin or both heparin and growth factor (Sakiyama et al., 1999; Sakiyama-Elbert and Hubbell, 2000; Schense and Hubbell, 1999). The polymerization mixture was placed into a well of a flat 24-well tissue culture plate was incubated for 60 min at 37 °C, 95% relative humidity, and 5% CO_2.

After polymerization of the fibrin matrix, 1 mL of TBS was added to each well to wash any unbound components of the delivery system from the matrix. The matrices were washed a total of five times over 24 hr, four times with TBS and once with 1 mL of modified neural basal medium, consisting of insulin (5 μg/mL), transferrin (100 μg/mL), progesterone (6.4 ng/mL), putrescine (16.11 μg/mL), selenite (5.2 ng/mL) (all from Gibco, Basel, Switzerland), 5 μg/mL fibronectin, 0.1% bovine serum albumin, 20 ng/mL mouse nerve growth factor (added only in the case of unmodified fibrin), 0.5 mM L-glutamine, 25 μM L-glutamate, and 1% antibiotic-anti-mycotic solution (Gibco) added to neural basal medium (Gibco).

4.3.3 Quantification of β-NGF release

Fibrin matrices containing β-NGF were made as described above, with and without the heparin-containing delivery system. Matrices were washed 5 times in the first 24 hr and once every 24 hr subsequently, with 1 mL of TBS per wash. The washes from three matrices were collected and stored in silanized Eppendorf tubes at –20 °C.

After 15 days, the matrices were degraded enzymatically by adding 0.2 U of porcine plasmin in 100 μL of TBS and incubating at 37 °C for 3 hr. The amount of β-NGF present in the washes and remaining in the matrices was quantified using a sandwich enzyme-linked immunosorption assay (ELISA) (Roche Diagnostics, Rotkreuz, Switzerland). The absorbance at 580 nm of the samples was read using an EL311SX plate reader from Bio-Tek Instruments (Winooski, Vermont). The concentration of each sample was determined from a calibration curve based on known concentrations of β-NGF.

4.3.4 Dorsal root ganglia culture and analysis

Dorsal root ganglia (DRGs) were dissected from embryonic day 8 (E8) White Leghorn chicken embryos (Varon, 1972) and placed in Hanks-buffered salt solution (HBSS) (Gibco). The DRGs were placed inside the fibrin matrix with dissection forceps, one per matrix. The fibrin matrices containing DRGs were incubated for 60 min at 37 °C, 95% relative humidity, and 5% CO_2, and then 1 mL of modified neural basal medium was added to each well. The medium was changed at 24 hr. Experiments were performed in duplicate, each with 6 DRGs per replicate. DRGs were also cultured within unmodified fibrin matrices in medium containing 20 ng/mL 7S NGF, and all measurements of neurite extension were normalized with respect to this level.

Brightfield images of the DRGs were taken at 24 and 48 hr with 4.0x Achroplan (24 hr) and 2.5x Plan (48 hr) objective (Zeiss, Zurich, Switzerland). The images were digitized with a Shimatsu chilled color 3-chip CCD camera, Matrox Meteor PCI frame grabber (Matrox Electronic Systems, Dorval, Quebec, Canada), and Leica Qwin software (both purchased from Leica, Zurich, Switzerland). These images were then analyzed to determine the average length of neurite extension, which was calculated as the radius of an annulus between the DRG body and the outer halo of extending neurites, exactly as described previously (Herbert et al., 1996). Neurite length for each experiment was

normalized by the average length of neurite extension through unmodified fibrin matrices from the same experiment, at the same time point.

4.3.5 Statistics

Statistical analysis was performed using Statview® 4.5 (Abacus Concepts, Berkeley, CA). Comparative analyses were completed using the Scheffe's F post-hoc test by analysis of variance at a 95% confidence level. Mean values and standard error of the mean are reported, unless otherwise noted.

4.4 Results

The goal of this research was to develop a growth factor delivery system to enable the controlled release of β-NGF and other non-heparin binding neurotrophins during nerve regeneration. Fibrin was selected as the base material for this delivery system, to provide a three-dimensional cell ingrowth matrix for wound repair, the adhesive and drug delivery characteristics of which could be tailored for the wound healing indication of interest. The drug delivery system used in these studies consisted of heparin-binding peptides covalently immobilized to fibrin, heparin bound to these immobilized peptides, and growth factor. Heparin-binding peptides were cross-linked into fibrin matrices by the action of factor XIIIa during polymerization. The incorporation of peptides by this method has been previously quantified to be 7.9 ± 0.5 moles of peptide per mole fibrinogen with heparin and growth factor in the polymerization mixture (Sakiyama et al., 1999; Sakiyama-Elbert and Hubbell, 2000). Covalently immobilized heparin-binding peptides were used to sequester heparin within the fibrin matrix. The immobilized heparin in turn served to interact with neurotrophins of low heparin-binding affinity, such as β-NGF, BDNF, and NT-3, and to slow their release from fibrin matrices.

4.4.1 Release profiles for β-NGF

To determine the ability of the heparin-containing delivery system to prolong the release of neurotrophins in the absence of cells, the release profile of β-NGF from fibrin matrices with and without the cross-linked heparin-binding peptides and immobilized heparin to form a delivery system was quantified using a sandwich ELISA. The release profile of β-NGF from matrices containing the delivery system was compared with the profile from matrices lacking the delivery system (Fig. 4.2). Without the delivery system present, almost all of the β-NGF was released from the matrix within the first day. However, in the presence of the delivery system, approximately 50% of the β-NGF initially present was released in the first day and 30% of the β-NGF initially present still remained in the matrix after 15 days. These results demonstrated that the release of β-NGF from fibrin matrices was prolonged by the heparin-containing delivery system.

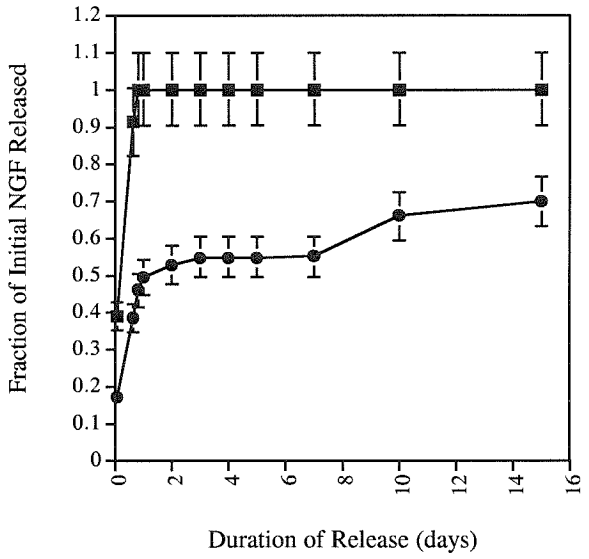

Figure 4.2 Release profile of β-NGF diffusion from fibrin matrices in the presence or absence of the heparin-containing delivery system.
■ denotes unmodified fibrin with 100 ng/mL of β-NGF in the culture medium. ● denotes heparin-containing matrices with 100 ng/mL of matrix-interacting β-NGF. Mean values and standard deviation are shown. These results demonstrate that despite β-NGF's weak heparin-binding affinity, the release of β-NGF from fibrin matrices is prolonged by a heparin-containing drug delivery system.

4.4.2 Delivery of neurotrophins with heparin-containing delivery system

To determine the ability of the heparin-containing delivery system to deliver bioactive neurotrophins in a controlled manner, heparin-binding peptide, heparin and β-NGF, BDNF, or NT-3 were added during the polymerization of fibrin matrices, and the delivery of neurotrophins from these matrices was assayed in a neuronal cell culture model. In our previous evaluation of the heparin-containing delivery system, we determined the conditions required for slow passive (diffusion-based) release of heparin-binding growth factors by mathematical modeling (Sakiyama-Elbert and Hubbell, 2000). We used similar conditions to experimentally test whether neurotrophins could be delivered in a controlled manner from the heparin-containing delivery systems. A molar ratio of heparin to growth factor of approximately 5000 was used to slow diffusion-based release of the growth factor from the matrix. The matrices were washed thoroughly for 24 hr and then whole DRGs were placed inside the matrices.

When all three components of the delivery system (heparin-binding peptide, heparin, and growth factor) were present during polymerization, each of the neurotrophins tested (at an initial concentration of 100 ng/ml in the polymerization solution) was found to enhance neurite extension (Fig. 4.3). Enhancement of neurite extension by approximately 100%, 75%, and 50% relative to unmodified fibrin was observed in the case of matrix-interacting BDNF, β-NGF and NT-3, respectively (striped bars, Fig. 4.3). In all cases, this enhancement was statistically significant versus unmodified fibrin with NGF in the cell culture medium ($p<0.05$). (DRGs in matrices containing matrix-interacting neurotrophins were cultured with no additional NGF in the medium.) The release of matrix-interacting growth factor was not directly measured in the presence of cells, but rather the stimulation of enhanced neurite extension from chick DRGs was taken as a measure of the ability of the delivery system to release bioactive neurotrophins. These results demonstrated the ability of the heparin-containing delivery system to deliver low heparin-affinity neurotrophins in a bioactive form.

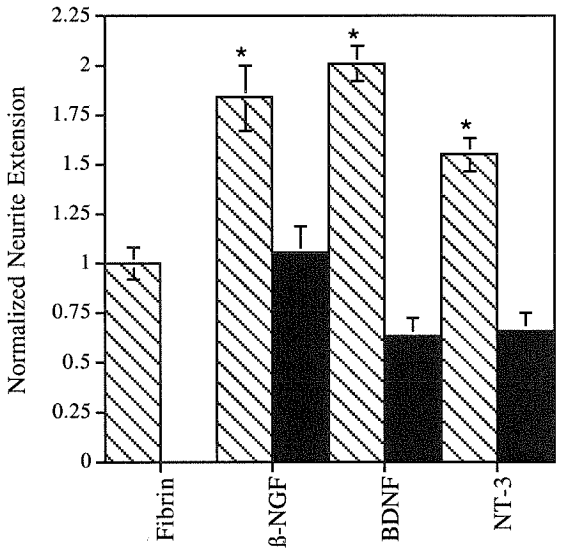

Figure 4.3 Effect of neurotrophin delivery on neurite extension through fibrin matrices in the presence or absence of the heparin-containing delivery system. Solid bars denote neurite extension through matrices containing only neurotrophin (no heparin-containing delivery system) and striped bars denote neurite extension through heparin-containing matrices with matrix-interacting growth factors. Neurite extension was enhanced by up to 100% over unmodified fibrin matrices with 20 ng/mL of 7S NGF in the culture medium. This demonstrates that the heparin-containing delivery system can effectively deliver neurotrophins with low heparin-binding affinity, which possess basic domains accessible in the native protein structure.

As a comparison in determining the ability of the heparin-containing delivery system to interact with low heparin-binding affinity neurotrophins and slow their release from heparin-containing fibrin matrices, we tested the ability of fibrin matrices lacking the delivery system to interact with and to prolong the release of active neurotrophins. When only free neurotrophins in the absence of the delivery system (no heparin or peptide) were added to the fibrin matrix polymerization mixture at a concentration of 100 μg/mL, no enhancement of neurite extension versus unmodified fibrin was observed (solid bars, Fig. 4.3). In the absence of the delivery system, the growth factor diffused out of the fibrin matrix prior to cell seeding and was lost due to rapid passive (diffusion-based) release. This result demonstrated that the heparin-containing delivery system must be present in order for neurotrophins to enhance neurite extension versus unmodified fibrin in the DRG culture model employed.

Previously, we have tested the ability of the heparin-containing delivery system to promote neurite extension in the absence of immobilized growth factors. We found that the presence of immobilized heparin-binding peptide and heparin within fibrin matrices lacking any growth factors did not enhance neurite extension versus unmodified fibrin, when DRGs in both matrices were cultured in the presence of 20 ng/mL 7S NGF in the medium (Sakiyama-Elbert and Hubbell, 2000). Therefore, it appears that the enhancement of neurite extension in the presence of matrix-interacting β-NGF, BDNF, or NT-3 must be due the interaction of the growth factor with the heparin-containing delivery system, which slows the escape of the growth factor from the fibrin matrix into the culture medium.

4.4.3 Dose Response Study for β-NGF

To determine the dose response effect of matrix-interacting β-NGF, we varied the concentration of β-NGF in the polymerization mixture. The dose response effect appeared to be biphasic, with significant enhancement in neurite length at 100 ng/mL (Fig. 4.4). However, no significant enhancement of neurite length was observed at 50 ng/mL or 250 ng/mL β-NGF versus unmodified fibrin. Similar dose saturation effects with growth factors have been previously observed (Wang and Aspenberg, 1996) and are attributed to the saturation of the cell-surface receptors for the growth factor. Therefore, we were not surprised to observe a biphasic dose response effect that increased up to some optimal dose, beyond which the addition of more β-NGF did not further enhance neurite extension.

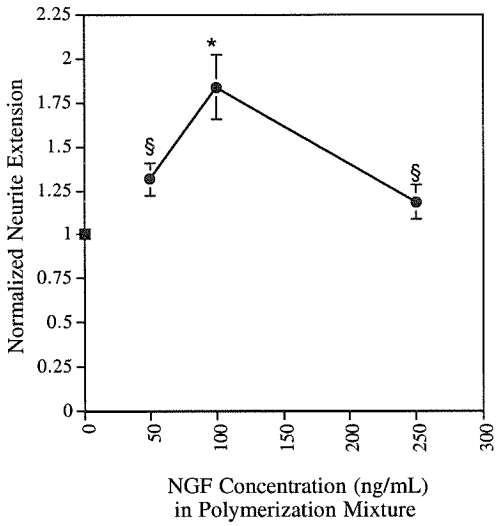

Figure 4.4 Effect of β-NGF dose on neurite extension through fibrin matrices in presence of the heparin-containing delivery system.
■ denotes unmodified fibrin with 20 ng/mL of 7S NGF in the culture medium. ● denotes heparin-containing matrices with matrix-interacting β-NGF. A biphasic effect to β-NGF dose is observed for β-NGF interacting with the heparin containing delivery system. The optimal dose for this cell culture model was observed at 100 ng/mL β-NGF.

4.4.4 In vitro release profile of β-NGF

To determine how long the release of active β-NGF could be sustained from the heparin-containing delivery system, fibrin matrices containing the delivery system and 100 ng/mL β-NGF were washed for a prolonged period of time prior to placement of DRGs within the matrices. Matrices washed for 4 days prior to cell seeding showed no difference in their ability to promote neurite extension from matrices washed for 1 day (Fig. 4.5). However, fibrin matrices washed for 7 days prior to seeding showed a decreased ability to promote neurite extension, and the neurite extension in the these matrices was not statistically different from unmodified fibrin.

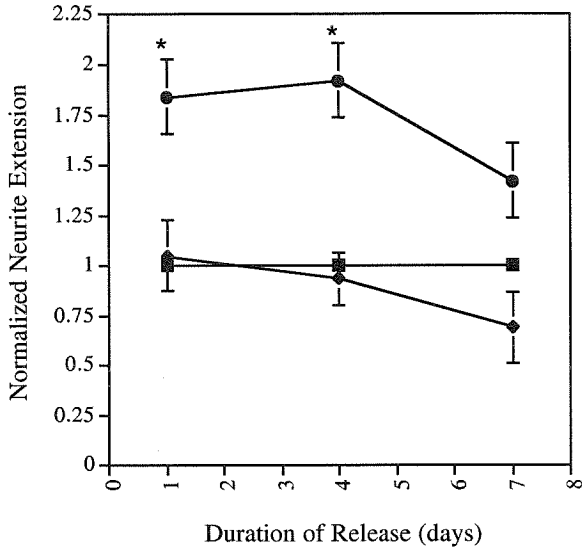

Figure 4.5 Duration of β-NGF release with and without the heparin-containing delivery system *in vitro*.
■ denotes unmodified fibrin with 20 ng/mL of 7S NGF in the culture medium. ● denotes heparin-containing matrices with matrix-interacting β-NGF. ◆ denotes matrices containing β-NGF in the absence of the heparin-containing delivery system. The effect of prolonged washing prior to cell seeding was determined by washing fibrin matrices containing β-NGF for 1, 4, and 7 days prior to placing DRG within the matrix. These results demonstrate that bioactive β-NGF was released for at least 4 days in the presence of cells.

4.5 Discussion

Because growth factors are such powerful regulators of biological function, their presence in tissues is highly regulated in both time and space. One means by which this is accomplished is by binding to extracellular matrix (ECM) components, as is observed with the heparin-binding growth factors and their interaction with ECM glycosaminoglycans. The high excess of ECM proteoglycans very effectively sequesters the fibroblast growth factors and other heparin-binding growth factors. Heparin binding growth factors that passively dissociate from the ECM are quickly recaptured by unoccupied heperan sulfate sites and must be attached to a heparin-like molecule in order to diffuse through the ECM. Otherwise, heparin-binding growth factors are sequestered by the matrix and do not diffuse (Vlodavsky et al., 1991). Thus, although the dissociation

of heparin-binding growth factors from the ECM is rapid, an excess of immobilized heparin or heperan sulfate can dramatically slow their release, both naturally as well as in therapeutic systems. We sought in the present research to determine whether the same principles could be employed with growth factors that bind heparin with low affinity. We have developed a growth factor delivery system, which can slow the diffusion of low heparin-affinity neurotrophins through a fibrin matrix that contains a high excess of immobilized heparin-binding sites, in manner similar to the way the ECM functions *in vivo* with growth factors that possess high heparin-binding affinity.

The results of this study demonstrate that heparin-containing drug delivery systems can be used to deliver low heparin-binding affinity growth factors, such as β-NGF, BDNF and NT-3, in an active form over a longer period than is observed with simple diffusion-based release. Prolonged release can be especially beneficial for applications in nerve regeneration, when the healing process may require weeks to months and may proceed from one side of an implanted matrix to another. These materials could also prove useful for many applications including the use of matrices for cell seeding to generate tissues in vitro for ultimate transplantation and the use of matrices for cell invasion to generate and regenerate tissue in vivo. Other low heparin-binding affinity growth factors that could be delivered from heparin-containing delivery systems include transforming growth factor-β2 (TGF-β2) for bone regeneration, the chemoattractant insulin-like growth factor 1, and TGF-β3 and epidermal growth factor for healing in skin (Grande, 1997; Lyon et al., 1997; Prigent and Lemoine, 1992). These materials may therefore have broad usefulness in tissue engineering.

The use of heparin-containing delivery systems to deliver growth factors with low heparin-binding affinity allows the use of previously developed heparin-based systems for the delivery of a new class of growth factors. Despite the very low affinity interactions between these growth factor and heparin, prolonged release can be attained through the use of delivery systems that possess a high excess of heparin sites. This high

excess is necessary due to the transient nature of the binding interactions under physiological conditions. Heparin affinity chromatography was performed to demonstrate that low affinity interactions between heparin and β-NGF do occur. β-NGF eluted from a heparin affinity column after several column volumes of buffer with a sub-physiological salt concentration (50 mM), demonstrating that interactions with heparin do occur but that under physiological conditions they are relatively short in duration. By contrast, bFGF eluted from the heparin-affinity column at a salt concentration of 2.0 M, demonstrating that it has a much higher affinity for heparin than NGF.

We speculate that interactions between the basic domains found at the carboxyl-termini of neurotrophins and the ECM may serve to regulate the physiological function of neurotrophins, similar to the way that interactions between the ECM and bFGF regulate that physiological function of bFGF. Recently, the crystal structure for β-NGF and the ligand-binding domain from its high affinity receptor, TrkA, has been obtained, and it was found that it is the amino-terminus of β-NGF, not the carboxyl-terminus, that is involved in receptor binding (Wiesmann et al., 1999). However, it has also been observed that a mutant β-NGF lacking the seven most carboxyl-terminal amino acids of β-NGF shows no biological activity (Drinkwater et al., 1993), implicating an important role for the carboxyl-terminus, as well as the amino-terminus, in growth factor activity. Together these results suggest that the carboxyl-terminus of β-NGF may be important physiologically, perhaps in the localization of β-NGF to the ECM, based on low affinity interactions between β-NGF and heparan sulfate proteoglycans.

Chapter 5 Development of Novel Growth Factor Fusion Proteins with Exogenous Cross-linking Substrates for Cellularly Triggered Drug Delivery

5.1 Abstract

The goal of this research was to develop an approach to growth factor delivery for use in tissue regeneration. We sought to stably incorporate the growth factor within a three-dimensional cell ingrowth matrix in a manner such that local enzymatic activity associated with cell invasion would free the growth factor to become bioavailable to the invading cells. In such a manner, the rate of growth factor release would be modulated by the rate of tissue regeneration. To immobilize the growth factor to an ingrowth matrix, the transglutaminase activity of factor XIIIa was utilized, within a platform matrix of fibrin. To provide cell-mediated release, cell-associated plasmin was exploited. The work was conducted in the context of nerve regeneration, and thus a fusion protein was constructed and expressed with an N-terminal factor XIIIa substrate, a C-terminal beta-nerve growth factor (β-NGF) domain, and an intervening plasmin substrate domain. We compared the relative activity of the β-NGF fusion proteins and native NGF in two *in vitro* assays, using pheochromocytoma (PC12) cells and chick dorsal root ganglia (DRGs). The ability of the immobilized NGF fusion proteins to promote neurite extension from chick DRGs through fibrin matrices was enhanced by approximately 50% compared to free NGF in the culture medium, and the response was 350% greater than that observed in unmodified fibrin matrices with no NGF in the medium. These results suggest that this novel approach to growth factor delivery could serve to enhance nerve regeneration *in vivo* and may have more general usefulness in tissue engineering for the delivery of growth factors.

5.2 Introduction

The objective of this research was to develop a novel approach to growth factor delivery for use in wound healing that would provide localized release in a manner that is triggered by cellular activity. Protein drugs are commonly delivered from polymer-based implants in which the rate of release is controlled by the diffusion of the drug from the delivery system or by the timed degradation of the depot (Langer, 1998). Our goal was to develop an approach to permit the rate of drug release to be varied at different locations within the delivery system, depending on cellular activity at that location. This approach could be particularly useful when long-term drug delivery is desired, for example in the case of nerve regeneration, where one would like to vary the rate of drug release spatially as a function of regeneration, e.g., rapidly near the living tissue interface and more slowly farther into the injury zone. This type of delivery system might allow a lower total drug dose to be incorporated within the delivery system, and spatial regulation of release could permit a greater percentage of the drug to be released at the time of greatest cellular activity.

To develop a delivery system where the rate of growth factor release could be locally controlled by cellular processes, we designed growth factor fusion proteins that could be covalently immobilized within a three-dimensional cell ingrowth matrix via an exogenous substrate for enzymatic cross-linking to the matrix material. To prevent a loss of growth factor activity due to covalent attachment to the matrix, we placed an enzymatically-degradable linker between the cross-linking substrate and the growth factor domain in our fusion proteins. This enzymatically degradable linkage allowed for release of growth factor in response to cellular remodeling of the matrix, and permitted the localized rate of release within the delivery system to vary depending on the location of cells within the system and their associated enzymatic activity.

To test whether this method for growth factor delivery was feasible, we selected β-NGF as a model growth factor, the delivery of which is of interest for the development of therapies to enhance peripheral nerve regeneration (Otto et al., 1987; Rich et al., 1989; Santos et al., 1999; Varon and Conner, 1994). We expressed β-NGF fusion proteins with an exogenous cross-linking substrate that would allow the β-NGF fusion proteins to be enzymatically cross-linked to fibrin matrices, which served as the base material for the drug delivery system. We have previously used a factor XIIIa substrate from the N-terminus of α_2-plasmin inhibitor to covalently immobilize short peptides within fibrin during fibrinogen polymerization (Sakiyama et al., 1999; Sakiyama-Elbert and Hubbell, 2000; Schense and Hubbell, 1999). In this study, we utilized the same transglutaminase substrate and tested its ability to covalently immobilize proteins, as well as short peptides, within fibrin matrices. A plasmin substrate was placed between the cross-linking substrate and the β-NGF domain in the fusion protein, which served as a degradable linker and allowed β-NGF to be released from the matrix in a form almost identical to its native sequence by enzymatic cleavage. The β-NGF fusion proteins were covalently attached to fibrin by the transglutaminase activity of factor XIIIa and were tested in an *in vitro* model of nerve regeneration to determine the ability of the delivery system to release active growth factors in response to cell-associated enzymatic activity.

5.3 Materials and Methods

All materials were obtained from Fluka (Buchs, Switzerland) unless otherwise specified.

5.3.1 Gene synthesis

Two β-NGF fusion proteins were made by recombinant protein expression. Each protein contained a cross-linking substrate at the N-terminus of the protein that consisted of the transglutaminase (TG) substrate from α_2-plasmin inhibitor, NQEQVSPL (Ichinose et al., 1983). Each β-NGF fusion protein also contained the native β-NGF sequence at

the C-terminus of the protein (Ullrich et al., 1983). One of two plasmin substrates (P) was placed between the cross-linking substrate and the β-NGF domain of the fusion protein, either a functional plasmin substrate (LIK/MKP, where / denotes the cleavage site) or a non-functional plasmin substrate (LINMKP) (Takagi and Doolittle, 1975), in which the lysine residue at the cleavage site in the plasmin substrate was changed to an asparigine residue to render the plasmin substrate non-functional. The fusion protein containing a functional plasmin substrate was denoted TG-P-NGF, and the fusion protein containing a non-functional plasmin substrate was denoted TG-P_i-NGF.

In order to synthesize the β-NGF fusion protein by recombinant protein expression, the gene encoding for the protein was cloned. This was accomplished by optimizing the DNA sequence coding for the β-NGF fusion protein for bacterial codon usage (Andersson and Kurland, 1990), and then the gene encoding the fusion protein was divided into 5 fragments of about 100 bp in length. The choice of fragment size was determined based on the occurrence of unique restriction enzyme sites within the gene. If a unique site was not present within about 20 bp of the desired location, then alternate codons for the amino acids near the desired site were tested until a unique enzyme site was created.

Once the gene fragments were designed, Eco RI and Hind III sites were added to the end of each fragment, to allow the cloning of these fragments into the poly-cloning linker of pUC18 (Gibco, Basel, Switzerland) (Yanish-Perron et al., 1985). The 3' and 5' single-stranded oligonucleotides of each gene fragment were synthesized by Microsynth (Balgach, Switzerland) with sticky ends for the two cloning restriction sites. The single-stranded oligonucleotides were purified using denaturing poly-acrylamide gel electrophoresis (PAGE), the highest molecular weight band for each fragment was extracted from the gel, and the corresponding 3' and 5' oligonucleotide fragments were annealed. The annealed fragments were phosphorylated with T4 DNA kinase (Boehringer Mannheim, Rotkreuz, Switzerland) and ligated into pUC18. Following

ligation, the plasmids were transformed into DH5α-F' competent cells and plated on isopropyl β-D-thiogalactopyranoside (IPTG)/ 5-bromo-4chloro-3-indolyl β-D-galactopyranoside (X-gal)/ampicillin (Amp) plates to screen for insertion of the gene fragments into the plasmid. Plasmids from colonies containing inserted gene fragments were sequenced to identify colonies containing gene fragments with the correct sequence. After the correct sequence for each gene fragments was obtained, the fragments were assembled to form the complete gene for the fusion protein. Briefly, plasmids that contained fragment 2 were digested with the enzymes EcoR V and Hind III (Boehringer Mannheim), and the fragments were purified by non-denaturing PAGE. Plasmids containing fragment 1 were digested with the enzymes Eco RV and Hind III, dephosphorylated with alkaline phosphatase (Boehringer Mannheim), and the digested plasmids were purified by agarose gel electrophoresis. Fragment 2 was ligated into the digested plasmids that contained fragment 1 to obtain a plasmid that contained both fragments 1 and 2. This plasmid was transformed into DH5α-F' competent cells, and plated on Amp plates. The resulting colonies were screened for plasmids containing both fragments, and these plasmids were sequenced. This process was repeated until the complete gene for the β-NGF fusion protein was assembled.

The gene for the TG-P_i-NGF fusion protein was made from the TG-P-NGF gene by site directed mutagenesis. Polymerase chain reaction (PCR) was performed to modify the region of the fusion protein gene coding for the plasmin substrate, using primers containing the desired modification of the gene (Ho et al., 1989; Horton et al., 1993). Using the TG-P-NGF gene as a template, two reactions were performed, one with primer A and primer B, and the other with primer C and primer D (see Appendix for primer sequences). The products from the two reactions were purified by agarose gel electrophoresis, and used as primers for the third reaction. Primers A and D were also added to the third reaction to amply the desired product. The final reaction product was

digested with Eco RI and Hind III and purified by agarose gel electrophoresis. The PCR fragment was cloned into pUC18, and sequenced to identify the correct PCR product.

5.3.2 Protein Expression

The complete gene for each of the β-NGF fusion proteins was digested out of pUC18 and ligated into the expression vector, pET14b (Novagen, Madison, Wisconsin) (Studier et al., 1990). The expression vector was transformed into the expression host, BL21 (DE3) pLysS, to allow for tight regulation of fusion protein expression. The fusion protein was expressed by growing the *E. coli* until they reached mid-log phase growth (optical density at 600 nm of 0.4-0.6) and then inducing protein expression by the addition of 0.4 mM IPTG to the culture medium. The bacteria were harvested after 2 hr by centrifugation at 5500x**g**. After harvesting, the cells were suspended in 1/10 the culture volume of 20 mM Tris HCl, 250 mM NaCl, pH 8.0. Lysozyme (0.4 mg/mL) and DNase (5 ng/mL) were added to the harvested cells, and the solution was incubated at 37 °C for 30 min. The inclusion bodies were collected from the cell lysate by centrifugation at 10,000x**g** for 15 min. The pellet containing the inclusion bodies was resuspended in 40 mL/ liter culture volume of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9) containing 6 M guanidine hydrochloride (GuHCl) at room temperature for 90 min. Insoluble material in the solution was collected by centrifugation for 20 min at 20,000x**g**, and the supernatant, which contained the solubilized fusion protein, was saved for further purification.

5.3.3 Protein Purification

The fusion protein contained a thombin-cleavable histidine tag for purification at the N-terminus of the protein, because the gene for the β-NGF fusion protein was inserted in pET14b between the Nde I and Bam HI sites. Nickel affinity chromatography was used to purify the β-NGF fusion protein. His Bind resin (Novagen) was packed into a chromatography column (2.5 mL bed volume per liter culture volume), charged with

Ni^{++} and equilibrated with binding buffer containing 6M GuHCl (according to the manufacturer's instructions). The supernatant, which contained the fusion protein, was filtered with a 5 μm syringe filter and loaded on the column. The column was washed with 10 column volumes of binding buffer containing 8 M urea and 6 column volumes of wash buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9) containing 8 M urea. The fusion protein was eluted with 4 column volumes of elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9) containing 8 M urea. The presence of β-NGF fusion protein in the elution fractions was confirmed by sodium dodecyl sulfate (SDS)-PAGE.

5.3.4 Protein Refolding

The β-NGF fusion protein was refolded by adding a 5-fold excess of ice cold refolding buffer (20 mM Tris HCl, 250 mM NaCl, 2 mM reduced glutathione and 0.2 mM oxidized glutathione, pH 8.0) to the purified β-NGF fusion protein slowly, until a final urea concentration of 1.3 M was attained. The fusion protein was refolded for 48 hr at 4 °C while stirring. The refolded fusion protein was dialyzed against a 50-fold excess of storage buffer (20 mM Tris HCl, 500 mM NaCl, pH 8.0) containing 10% glycerol at 4 °C overnight. The fusion protein was concentrated by centrifugation using Vivaspin concentrators (5000 MW cutoff, Vivascience, Lincoln, UK) to a concentration of about 300-400 μg/mL, as measured by Bradford assay.

5.3.5 Incorporation of β-NGF Fusion Protein into Fibrin Matrices

To determine the efficiency of β-NGF fusion protein incorporation into fibrin matrices, TG-P_i-NGF fusion protein was labeled with biotin to allow β-NGF quantification by a direct enzyme-linked immunosorption assay (ELISA). A 20-fold molar excess of Sulfo-N-hydroxysuccinimide (NHS)-LC Biotin (Pierce, Lausanne, Switzerland) was added to the β-NGF fusion protein in phosphate buffered saline (PBS, 0.01 M phosphate buffer, 8 g/L NaCl, 0.2 g/L KCl, pH 7.4) at a concentration of 1

mg/mL from a stock solution of 10 mg/mL biotin in N,N-dimethylformamide (dissolved for 10 min at 37 °C). The reaction was allowed to proceed for 2 hr at room temperature. The unreacted biotin was then removed by gel filtration chromatography using a PD-10 column (Amersham Pharmacia, Dubendorf, Switzerland).

A known amount of labeled β-NGF was adsorbed to 96 well plates overnight in coating buffer (0.1 M $NaHCO_3$, pH 8) at 4 °C. The wells were blocked with 1% bovine serum albumin (BSA) in PBS for 2 hr at room temperature. The wells were washed three times in PBS with 0.5% Tween-20 (PBST buffer). Horseradish peroxidase (HRP)-conjugated streptavidin was diluted to 1 μg/mL in PBS and added to each well for 1 hr. The wells were washed three times with PBST buffer and then incubated in ABTS (2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) developing solution (0.1M $NaC_2H_3O_2$, 0.05 M NaH_2PO_4, 0.1% ABTS, 0.01% H_2O_2, pH 4.2). After 1-5 min, the reaction was stopped by adding an equal volume of 0.6% SDS, and the absorbance of each well was measured at 405 nm using an EL311SX plate reader from Bio-Tek Instruments (Winooski, Vermont). A standard curve of β-NGF concentration versus absorbance at 405 nm was made using these measurements from the direct ELISA assay.

Plasminogen-free fibrinogen was dissolved in water and dialyzed versus Tris-buffered saline (TBS, 33 mM Tris, 8 g/L NaCl, 0.2 g/L KCl) at pH 7.4 for 24 hr, as described previously (Schense and Hubbell, 1999). β-NGF fusion protein was incubated with 5 mM Ca^{++} and 4 NIH units/mL thrombin for 1 hr at 37 °C to remove the histidine tag used for purification. The β-NGF fusion protein solution was mixed in equal ratio with fibrinogen at a concentration of 8 mg/mL and polymerized at 37 °C for 60 min. The fibrin matrices were washed five times over 24 hr, and each wash was saved to determine the total amount of β-NGF washed out of the matrix. After 24 hr, the fibrin matrices containing β-NGF were degraded with 0.1 U of porcine plasmin. The amount of β-NGF in the washes and remaining in the matrix was quantified as described above by direct ELISA, and a β-NGF standard curve was constructed for each ELISA performed.

A Western blot was performed to show directly that β-NGF fusion proteins were covalently coupled to fibrin matrices. Fibrin matrices were made and washed as described in the incorporation quantification assay. The matrices were washed five times over 24 hr and then degraded with plasmin, as described above. The degradation products were separated by SDS-PAGE using a 13.5% denaturing gel. The proteins from the gel were transferred to an activated Immobilon–P polyvinylidene difluoride (PVDF) membrane (Millipore, Volketswil, Switzerland) with a current of 400 mA for 1 hr. The membrane was dried overnight. The proteins transferred to the membrane, including the molecular weight marker, were visualized by staining with 0.2% Ponceau S. Non-specific protein binding to the membrane was blocked with 3% BSA in TBS for 2 hr. The membrane was incubated with goat anti-human β-NGF antibody (R&D Systems, Minneapolis, Minnesota) at a concentration of 0.2 μg/mL in 3% BSA for 1 hr. The membrane was washed three times with TBS for 5 min and incubated with the secondary antibody, HRP-conjugated rabbit anti-goat immunoglobulin (Dako Diagnostics, Zug, Switzerland) at a concentration of 0.5 μg/mL in 3% BSA for 30 min. The membrane was washed three times with TBS, and then incubated for 5 min with an enhanced chemi-luminescent HRP substrate (Pierce, Lausanne, Switzerland) diluted 1:5 in TBS. Excess liquid was removed from the membrane and it was covered with plastic and was exposed to X-ray film for 5-60 sec.

5.3.6 Bioactivity Assay for β-NGF Fusion Proteins

PC12 cell neurite extension was used as a measure of the relative activity of β-NGF fusion proteins compared with native β-NGF. Poly-L-Lysine was coated onto 48 well tissue-culture plates using a 0.01% solution in PBS at room temperature for 1 hr. The wells were rinsed twice with PBS and then coated with laminin at a concentration of 20 μg/mL in PBS overnight at 4 °C. The wells were rinsed once with PBS. PC12 cells were harvested from suspension culture in a 75 cm^2 flask by centrifugation for 5 min at

500x**g**. The cells were resuspended in 2 mL of medium (RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 1% antibiotic-antimycotic, all from Gibco) and triturated with a 5 mL pipette to break up cell aggregates. The cells were further passed through a 25 gauge needle to break up aggregates, diluted 1 to 32 in medium, and seeded in laminin-coated 48 well plates. Images were taken at 24 and 48 hr using 20x Achroplan Ph2 phase contrast objective (Zeiss, Zurich, Switzerland). The percentage of cells extending neurites was determined by counting the number of cells extending neurites and dividing by the total number of cells in each field. For each well, a total of about 100 cells were counted. Experiments were performed in duplicate, with four wells per replicate.

Chick dorsal root ganglia (DRGs) neurite extension was also used as a measure of relative β-NGF activity. DRGs were dissected from embryonic day 8 White Leghorn chicken embryos (Varon, 1972) and placed in Hanks-buffered salt solution (Gibco). The DRGs were pipetted into the bottom of flat 24-well tissue culture plates (1 per well) and fibrin matrices (prepared as described above) were polymerized around the ganglia such that they were three-dimensionally embedded within the matrix. Fibrin matrices (400 μL per well) were made by mixing the components to obtain the following final solution concentrations: 3.5 mg/mL fibrinogen, 2.5 mM Ca^{++}, 2 NIH units/mL of thrombin. The polymerization mixture was incubated for 60 min at 37 °C, 95% relative humidity, and 5% CO_2.

After polymerization of the fibrin matrices was complete, 1 mL of modified neural basal medium was added to each well, consisting of insulin (5 μg/mL), transferrin (100 μg/mL), progesterone (6.4 ng/mL), putrescine (16.11 μg/mL), selenite (5.2 ng/mL) (all from Gibco), 5 μg/mL fibronectin, 0.1% BSA, 10 ng/mL mouse 7S NGF (for use in standard samples only), 0.5 mM L-glutamine, 25 μM L-glutamate (all from Fluka), and 1% antibiotic-antimycotic solution (Gibco) added to neural basal medium (Gibco). DRGs were cultured within fibrin matrices in medium containing 10 ng/mL mouse NGF,

and all measurements of neurite extension were normalized with respect to this level. Experiments were performed in duplicate, each with 6 DRGs per replicate.

Brightfield images of the DRGs were taken at 24 and 48 hr with 4.0x Achroplan (24 hr) and 2.5x Plan (48 hr) objective (Zeiss). The images were digitized with a Shimatsu chilled color 3-chip CCD camera, Matrox Meteor PCI frame grabber (Matrox Electronic Systems, Dorval, Quebec, Canada), and Leica Qwin software (both from Leica, Zurich, Switzerland). These images were then analyzed to determine the average length of neurite extension, which was calculated as the radius of an annulus between the DRG body and the outer halo of extending neurites, exactly as described previously (Herbert et al., 1996). Neurite length for each experiment was normalized by the average neurite extension through unmodified fibrin matrices from the same experiment at the same time point.

5.3.7 Bioactivity Assay of Cross-linked Protein

The histidine-tag was cleaved from β-NGF fusion protein as described above (for incorporation quantification) for 1 hr, and then fibrin matrices were made by mixing equal ratios of β-NGF solution and fibrinogen at a concentration of 8 mg/mL. The fibrin matrices were polymerized for 1 hr at 37 °C. The matrices were washed four times with TBS and once with supplemented neural basal medium prior to cell seeding. DRGs were dissected and placed inside the fibrin matrix with dissection forceps, one per matrix. The fibrin matrices containing DRGs were incubated for 60 min at 37 °C, 95% relative humidity, and 5% CO_2, and then 1 mL of medium was added to each well. The medium was changed at 24 hr. Experiments were performed in duplicate, each with 6 DRGs per replicate. DRGs were also cultured within unmodified fibrin matrices in medium containing 10 ng/mL 7S NGF, and all measurements of neurite extension were normalized with respect to this level. Neurite length was quantified as described.

5.3.8 Statistics

Statistical analysis was performed using Statview® 4.5 (Abacus Concepts, Berkeley, CA). Comparative analyses were completed using the Scheffe's F post-hoc test by Analysis of Variance at a 95% confidence level. Mean values and standard deviation are reported, unless otherwise noted.

5.4 Results

5.4.1 Protein Expression and Purification

The genes for the β-NGF fusion proteins were assembled from oligonucleotide fragments and cloned into pUC18. After confirmation of the correct sequence, each of the genes was moved to pET14b for expression. The expression of β-NGF fusion proteins in *E. coli* was induced during mid-log phase growth, and a new protein with a molecular weight of approximately 18kD was observed in the whole cell lysate after 2 hr (Fig. 5.1). Both β-NGF fusion proteins formed inclusion bodies within the bacteria and were harvested from the cell pellet following expression. The inclusion bodies were solubilized using GuHCl, and the β-NGF fusion proteins were purified from the cell lysate by nickel affinity chromatography. The chromatography fractions were analyzed by SDS-PAGE, as shown in Fig. 5.1. Following purification, the β-NGF fusion protein was refolded from 8M urea based on a protocol for refolding TGF-β (Han et al., 1997) and concentrated by centrifugation.

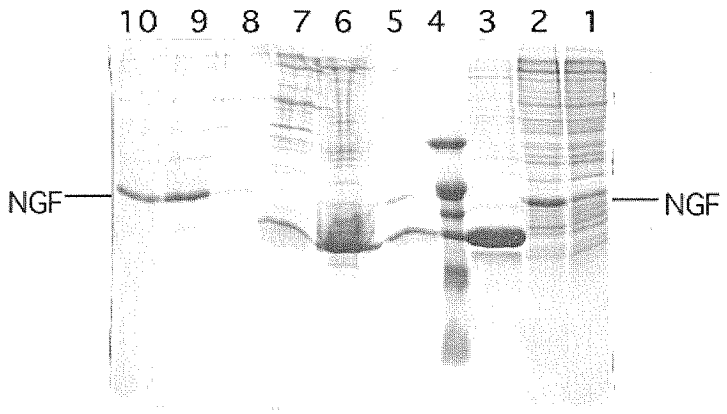

Figure 5.1 SDS-PAGE gel showing β-NGF fusion protein expression and purification.
Lane contents are as follows: 1) whole cell lysate prior to protein expression, 2) whole cell lysate 2 hr after induction of protein expression, 3) cytoplasm fraction of lysate, 4) MW marker, 5) insoluble fraction of cell lysate, 6) Ni^{++} column flow through, 7) binding buffer, 8) wash buffer, 9) elution of β-NGF fraction 1, 10) elution of β-NGF fraction 2. β-NGF expression was clearly visible after 2 hr of expression. β-NGF fusion protein was found in the insoluble fraction of the cell lysate and was purified by nickel affinity chromatography.

5.4.2 Incorporation of β-NGF Fusion Proteins into Fibrin Matrices

To determine whether the β-NGF fusion proteins were covalently coupled to fibrinogen during polymerization, immunoblotting was performed on degraded fibrin matrices that contained β-NGF fusion protein in the polymerization mixture. TG-P_i-NGF was incorporated into fibrin matrices during polymerization. After washing to remove any unbound β-NGF fusion protein, the matrices were degraded with plasmin and the degradation products were analyzed by Western blotting. If the fusion proteins were covalently cross-linked to the fibrin matrix during polymerization, then fragments of the fibrin would remain attached to the fusion proteins upon degradation of the matrix with plasmin, resulting in an increase in molecular weight of the β-NGF fusion proteins. An increase in molecular weight was indeed observed for β-NGF fusion proteins that were present during polymerization of fibrin matrices (Fig. 5.2), while in the case of β-NGF lacking a cross-linking substrate, no β-NGF was observed in the matrix after washing. This result showed directly that the β-NGF fusion protein was covalently immobilized

within fibrin matrices during polymerization via the transglutaminase activity of factor XIIIa.

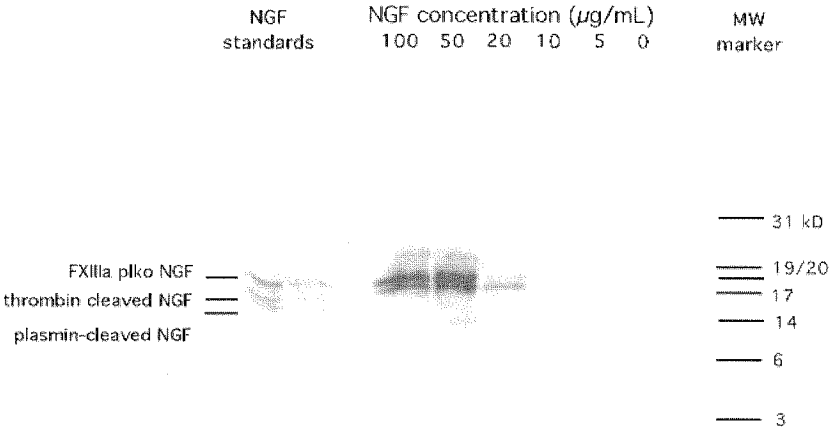

Figure 5.2A Immunoblot of plasmin-degraded fibrin matrices containing β-NGF fusion protein.
The degraded matrices containing β-NGF fusion proteins were separated by 13.5% denaturing SDS-PAGE and transferred to a PVDF membrane. Free β-NGF fusion protein ran at a lower molecular weight than did β-NGF fusion protein incorporated into the fibrin matrix during polymerization, demonstrating the covalent attachment of the β-NGF fusion proteins to degraded fibrin.

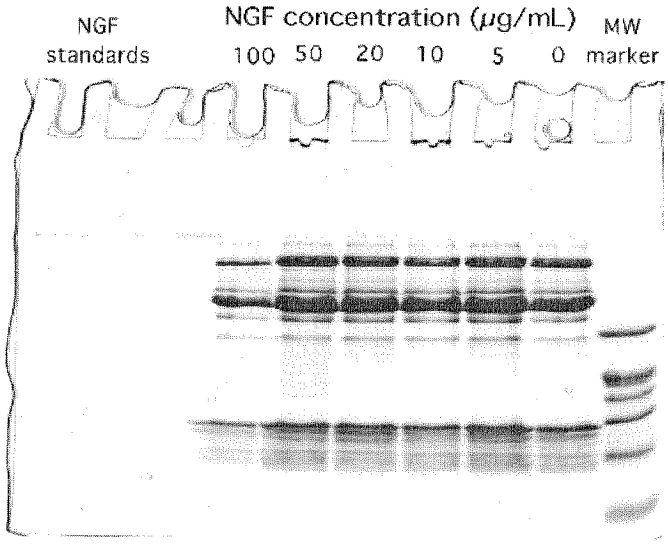

Figure 5.2 B SDS-PAGE of fibrin degradation products with β-NGF fusion protein in the polymerization mixture.
This gel is the same as the gel used for immunoblotting. It demonstrates that the β-NGF antibody staining is specific. Degradation products with an NGF concentration of 100 μg/mL were diluted 1:2 prior to loading.

5.4.3 Efficiency of β-NGF Fusion Protein Cross-linking

To determine the efficiency of β-NGF fusion protein incorporation in fibrin matrices, the protein was labeled with biotin, and a direct ELISA was performed on fibrin matrices that contained biotin-labeled β-NGF fusion protein in the polymerization mixture. Biotin-labeled β-NGF fusion proteins were incorporated into fibrin matrices during polymerization. After washing to remove any unbound β-NGF fusion protein, the matrices were degraded with plasmin and the amount of β-NGF in the degraded matrices and in the washes was quantified. The percentage of β-NGF fusion protein incorporated into the fibrin matrix is shown in Fig. 5.3 as a function of β-NGF concentration in the polymerization mixture. Over the range of β-NGF fusion protein concentrations tested, 50-60% of the fusion protein was incorporated during polymerization of the fibrin matrix. This result demonstrated that β-NGF fusion proteins were incorporated into fibrin matrices efficiently through the action of factor XIIIa.

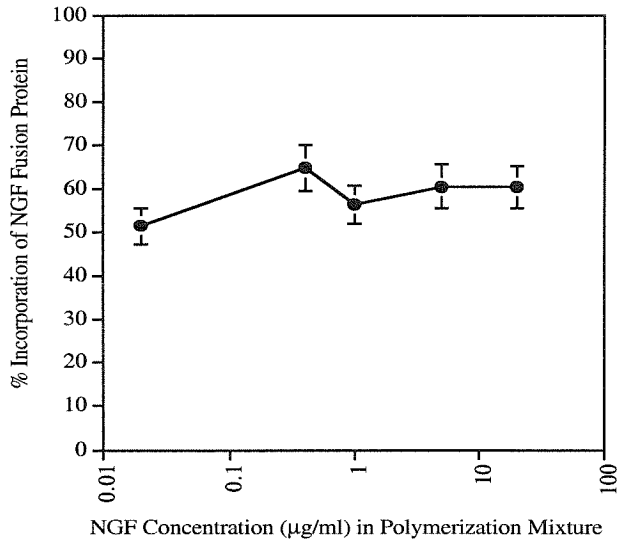

Figure 5.3. Incorporation of β-NGF fusion protein with exogenous factor XIIIa substrate into fibrin matrices.
The incorporation of β-NGF fusion protein into fibrin matrices was quantified by direct ELISA, using biotin-labeled β-NGF. The incorporation efficiency of the β-NGF fusion protein was relatively constant over the range of concentrations tested.

5.4.4 Bioactivity of β-NGF Fusion Proteins

To determine the activity of the β-NGF fusion proteins made in this study relative to the native β-NGF, PC12 cell neurite extension was measured. PC12 cells were seeded on laminin-coated tissue culture polystyrene, and the percentage of cells extending neurites was quantified for various concentrations of native β-NGF and β-NGF fusion protein in the culture medium. Native β-NGF promoted a maximal level of PC12 cell neurite extension of approximately 65% at 48 hr at a concentration of 100 ng/mL (Fig. 5.4). To attain the same level of neurite extension with the β-NGF fusion proteins, 250 ng/mL of β-NGF was required. This result suggested that the activity of the fusion protein β-NGF is about 40% of native β-NGF, but that if the lower activity is corrected for by adding 2.5 times as much β-NGF fusion protein, similar levels of neurite extension can be attained.

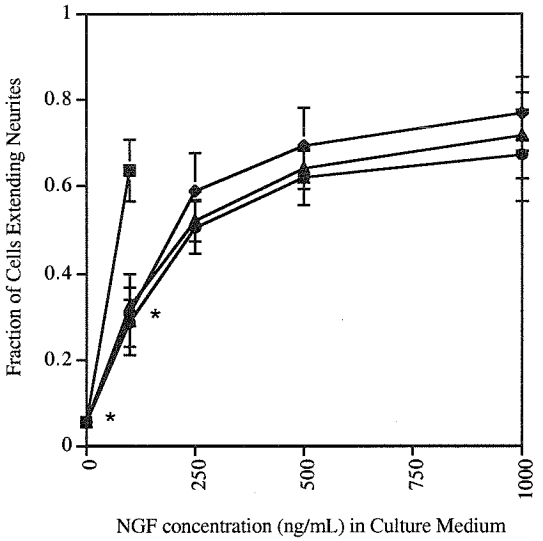

Figure 5.4 Ability of β-NGF fusion proteins to promote PC12 cell neurite extension.
The relative activity of β-NGF fusion protein was determined by finding the β-NGF concentration required for the maximal level of neurite extension from PC12 cells. ■ denotes native β-NGF. ◆ denotes TG-P-NGF. ● denotes TG-P_i-NGF. ▲ denotes plasmin-cleaved TG-P-NGF. * denotes $p<0.0001$ versus a concentration of 100 ng/mL of native NGF in the medium. These results suggest that the NGF fusion proteins have lower activity than native NGF, but that a similar level of neurite extension can be attained with a higher dose of NGF fusion protein.

A second β-NGF activity assay, neurite extension from chick DRGs, was also used. In this assay, chick DRGs were cultured within fibrin matrices, creating a three-dimensional culture model, and various concentrations of β-NGF were added to the culture medium. In this activity assay there was very little difference between the β-NGF fusion protein and native NGF in the culture medium. Similar doses of native NGF and β-NGF fusion protein promoted similar levels of neurite extension (Fig. 5.5). For both native NGF and β-NGF fusion protein, concentrations of 100 pg/mL or greater showed similar levels of neurite extension compared to a standard concentration of 10 ng/mL. Whereas, for NGF concentrations of 10 pg/mL or lower, both forms of NGF promoted levels of neurite extension that were significantly lower than that observed at a standard concentration of 10 ng/mL. This assay suggested that there is little difference in activity between the β-NGF fusion protein and native NGF. This result could be due to the use of primary cells rather than a PC12 cell line, or it may be due to the relative lack of sensitivity of this assay to differences in NGF concentration in the media (which were small over 5 orders of magnitude of NGF concentration).

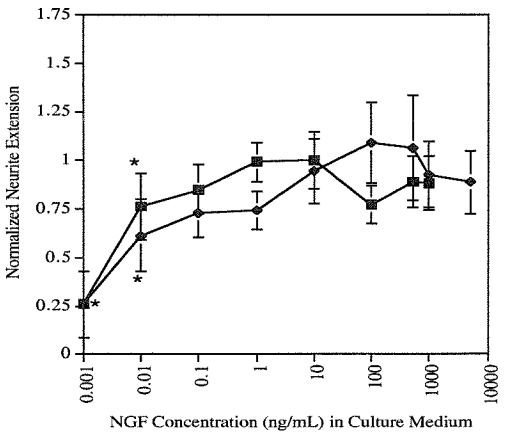

Figure 5.5 Ability of β-NGF fusion proteins to promote DRG neurite extension within fibrin matrices.
DRGs were cultured within fibrin matrices in medium containing various concentrations of NGF. ■ denotes native NGF. ◆ denotes TG-P-NGF. * denotes $p<0.05$ versus unmodified fibrin with a concentration of 10 ng/mL of native NGF in the medium. These results show very little difference in activity between native NGF and the β-NGF fusion protein.

5.4.5 Bioactivity of Immobilized β-NGF Fusion Protein

To determine the ability of covalently immobilized β-NGF fusion protein to be delivered in a controlled manner, β-NGF fusion proteins were incorporated into fibrin matrices during polymerization, and the ability of these matrices to enhance neurite extension in vitro was assayed using chick DRGs. TG-P-NGF was found to enhance neurite extension by over 350% versus unmodified fibrin with no NGF present in the culture medium and by up to 50% over unmodified fibrin with 10 ng/mL of native NGF in medium (Fig. 5.6). TG-P_i-NGF, which contained a non-functional plasmin substrate, could not be cleaved from the fibrin matrix by plasmin in a native form and did not significantly enhance neurite extension versus native NGF in the culture medium, when covalently immobilized within fibrin matrices at any of the concentrations tested. However, TG-P-NGF, which contained a functional plasmin substrate, could be cleaved from the matrix by plasmin in a form very similar to native NGF and was observed to enhance neurite extension, even when compared with similar doses of native NGF present in the culture medium. A dose response effect for TG-P-NGF fusion protein was observed, with an optimal dose attained when 1-5 μg/mL of β-NGF fusion protein was present in the polymerization mixture. These results demonstrated that the TG-P-NGF fusion protein was bioactive when immobilized within fibrin matrices, suggesting that it could be released in an active form by cell-associated matrix degradation. Despite the lower activity of the β-NGF fusion proteins in the PC12 cell activity assay, when the plasmin degradable β-NGF fusion protein was covalently coupled to fibrin, it promoted greater levels of neurite extension than the same dose of native NGF in the culture medium. These results also suggested that for the β-NGF fusion proteins to be fully active, they must be released from the fibrin matrix in a form similar to that of their native structure.

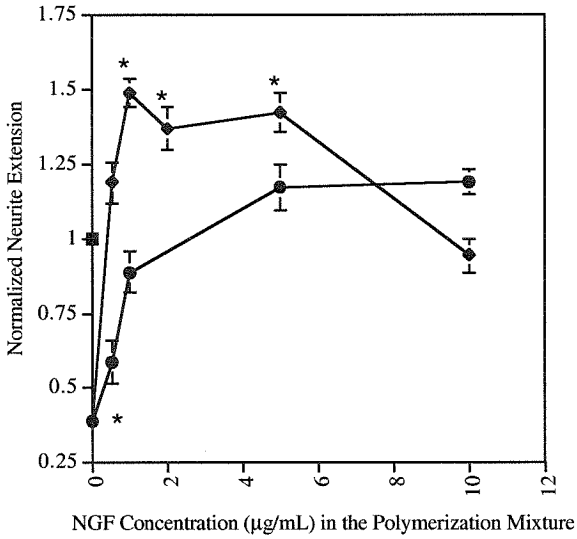

Figure 5.6 Effect of immobilized β-NGF fusion proteins on DRG neurite extension within fibrin matrices.
Mean values and standard error of the mean are shown. ■ denotes native NGF. ◆ denotes TG-P-NGF. ● denotes TG-P_i-NGF. * denotes $p<0.0001$ versus unmodified fibrin with NGF in the culture medium. This result demonstrates that matrix-bound β-NGF enhances neurite extension through fibrin matrices versus the same concentration of NGF in the medium.

5.5 Discussion

The objective of this research was to design a novel method for the delivery of growth factor proteins that allows release to be controlled by cellular processes, rather than by diffusion. Toward this goal, growth factor fusion proteins were synthesized that contain a substrate for enzymatic cross-linking at the N-terminus and a growth factor domain at the C-terminus. An enzymatically-degradable linker was placed between the cross-linking substrate and the growth factor domain, to allow the covalently immobilized growth factor to be released from the delivery system in response to localized cellular activity in a form similar to the native growth factor.

We sought to demonstrate the utility of this novel method for growth delivery in the context of peripheral nerve regeneration. β-NGF was selected as a model growth factor protein because it has been previously demonstrated to enhance nerve regeneration

in both the peripheral and central nervous system regeneration (Otto et al., 1987; Rich et al., 1989; Santos et al., 1999; Varon and Conner, 1994). Fibrin was chosen as the base material for the delivery system because it is the natural biomaterial of nerve regeneration and is readily penetrated by the proteolytic activity of the neurite growth cone as it extends through the three-dimensional fibrin matrix (Herbert et al., 1996). Others have previously demonstrated that the addition of fibrin matrices to nerve guide tubes enhanced peripheral nerve regeneration (Williams, 1987; Williams et al., 1987). The goal of this research was to enhance the ability of fibrin matrices to promote nerve regeneration by incorporating a drug delivery system into the matrix, and thus allowing the fibrin matrix to serve not only as a wound-healing scaffold but also as vehicle for controlled drug release.

The work described herein demonstrates that β-NGF fusion proteins are biologically active and that can be covalently immobilized within fibrin matrices during polymerization by factor XIIIa. The results presented above demonstrate that the method that we previously developed for the immobilization of small peptides could also be used to immobilize proteins. Furthermore, these results also show that by placing an enzymatically degradable linker between the cross-linking substrate and the growth factor domain in the fusion protein, growth factors can be delivered in an active form in response to cell-regulated processes. Through the selection of this enzymatically-degradable linker, the rate of growth factor release can presumably be tailored depending on the activity of a particular protease in the wound-healing model of interest. For example, in the case of bone regeneration, a collagenase substrate might be more useful than a plasmin substrate.

Not only did the above results demonstrate that growth factors immobilized within this growth factor delivery system could be release in an active form, we also observed an enhancement of neurite extension with our immobilized growth factor fusion proteins versus a similar growth factor concentration in the culture medium. This result

suggests that the release of immobilized growth factors in a manner that can be temporally and spatially regulated by cellular processes may be important in the context of wound healing. Thus, delivery systems that allow drug release to be regulated by the progress of wound healing through a cell ingrowth matrix could prove to be more effective in promoting successful tissue regeneration.

The materials investigated in this study may be useful in promoting peripheral nerve regeneration in vivo following transection due to trauma or surgery. This method can also be applied more generally to other growth factors and wound healing models of interest and may have broad usefulness in tissue engineering. A variety of applications in tissue engineering could benefit from novel three-dimensional matrices, which can act as both cell ingrowth substrates and drug delivery devices. These applications include the use of matrices for cell seeding to generate tissues in vitro for ultimate transplantation and the use of matrices for cell invasion to generate and regenerate tissue in vivo. Examples of tissue morphogenesis include generation of tissue-engineered skin, blood vessels, liver, nerve, bone and cartilage (Black et al., 1998; L'Heureux et al., 1998; Mooney et al., 1997; Schroeder-Tefft et al., 1997; Sims et al., 1998). In these approaches, a matrix component plays a key role, and its interactions with cells either in vitro or in vivo exert an important influence in the process of morphogenesis.

5.6 Appendix – DNA and Protein Sequences

TG-P-NGF DNA sequence 5'->3'

GAATTCCCAT GGCATATGAA CCAGGAACAG GTTAGCCCGC TGCCCGTGGA
ACTGCCGCTG ATCAAAATGA AACCCGTGGA ACTCGAGAGC TCTTCCCACC
CGATTTTCCA TCGTGGCGAG TTCTCCGTGT GTGACTCTGT CTCTGTATGG
GTAGGCGATA AAACCACTGC CACTGATATC AAAGGCAAAG AGGTGATGGT
GCTGGGAGAA GTAAACATTA ACAACTCTGT ATTCAAACAG TACTTCTTCG
AAACTAAGTG CCGTGACCCG AACCCGGTAG ACTCTGGGTG TCGCGGCATC
GATTCTAAAC ACTGGAACTC TTACTGCACC ACTACTCACA CTTTCGTTAA
AGCGTTGACT ATGGATGGTA AACAGGCTGC CTGGCGTTTC ATCCGTATCG
ATACTGCATG CGTGTGTGTA CTGTCCCGTA AAGCTGTTCG TTAAGGATCC

TG-P-NGF protein sequence (including His tag)

*MGSSHHHHHHSSGLVPRGS*HM**NQEQVSPLP**VELPLIKMKPVELESSSHPIFHRGEF
SVCDSVSVWVGDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETKCRDPNPVDS
GCRGIDSKHWNSYCTTTHTFVKALTMDGKQAAWRFIRIDTACVCVLSRKAVR

(Single underline denotes thrombin cleavage substrate. Bold type denotes factor XIIIa substrate. Double underline denotes plasmin substrate.)

Primers for PCR mutagenesis 5'->3'

Primer A AACAGCTATG ACCATG (M13 reverse)

Primer B GTTTCATGTT GATCAGCGGC AGT

Primer C TGATCAACAT GAAACCCGTG GAA

Primer D GTAAAACGACG GCCAGT (M13)

TG-P_t-NGF DNA sequence 5'->3'

GAATTCCCAT GGCATATGAA CCAGGAACAG GTTAGCCCGC TGCCCGTGGA

ACTGCCGCTG ATC**A**C**A**ATGA AACCCGTGGA ACTCGAGAGC TCTTCCCACC

CGATTTTCCA TCGTGGCGAG TTCTCCGTGT GTGACTCTGT CTCTGTATGG

GTAGGCGATA AAACCACTGC CACTGATATC AAAGGCAAAG AGGTGATGGT

GCTGGGAGAA GTAAACATTA ACAACTCTGT ATTCAAACAG TACTTCTTCG

AAACTAAGTG CCGTGACCCG AACCCGGTAG ACTCTGGGTG TCGCGGCATC

GATTCTAAAC ACTGGAACTC TTACTGCACC ACTACTCACA CTTTCGTTAA

AGCGTTGACT ATGGATGGTA AACAGGCTGC CTGGCGTTTC ATCCGTATCG

ATACTGCATG CGTGTGTGTA CTGTCCCGTA AAGCTGTTCG TTAAGGATCC

(Bold text denotes codon switch in plasmin substrate.)

TG-P_t-NGF – protein sequence

*MGSSHHHHHHSSGLVPRGS*HMN**QEQVSPLP**VELPLINMKPVELESSSHPIFHRGEF

SVCDSVSVWVGDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETKCRDPNPVDS

GCRGIDSKHWNSYCTTTHTFVKALTMDGKQAAWRFIRIDTACVCVLSRKAVR

(Single underline denotes thrombin cleavage substrate. Bold type denotes factor XIIIa substrate. Double underline denotes plasmin substrate.)

Chapter 6 Summary and Recommendations

6.1 Summary of Results

The results of this work demonstrate that the ability of fibrin matrices to promote neurite extension can be enhanced through the immobilization of exogenous adhesion sites or growth factor binding sites. The method for the incorporation of exogenous peptides in fibrin via the transglutaminase activity of factor XIIIa was shown by this work to be applicable to many different peptides and proteins.

Initially, this method for peptide incorporation was used to immobilize bi-domain heparin-binding peptides, which contained a factor XIIIa substrate, within fibrin matrices during polymerization. Exogenous heparin-binding domains from antithrombin III, neural cell adhesion molecule, and platelet factor 4 were found to enhance neurite extension within fibrin matrices, when immobilized at a ratio of 8 moles of peptide per mole of fibrinogen. Each of the four heparin-binding domains tested enhanced dorsal root ganglia (DRG) neurite extension versus unmodified fibrin, but the level of enhancement varied for the different heparin-binding domains. The relative heparin-binding affinity of these domains was determined by heparin-affinity chromatography, and the extent of enhancement of neurite extension was found to correlate positively with the heparin-binding affinity of the individual domains. These results demonstrate that the addition of exogenous heparin-binding domains enhanced neurite extension within fibrin matrices *in vitro*, and that these materials may serve a potential therapeutic for peripheral nerve regeneration.

The rest of this research focused on the development of drug delivery systems for use in tissue regeneration that would provide localized release of growth factors in an active manner, such that release occurred primarily in response to cellular activity during healing. The first delivery system developed sought to mimic the ability of the ECM to

sequester heparin-binding growth factors and release them in response to matrix degradation. Toward this goal, fibrin matrices were modified to contain immobilized heparin that could bind to heparin-binding growth factors and slow their diffusion from the matrix. Fibrin was modified using factor XIIIa to covalently cross-link bi-domain heparin-binding peptides to fibrin matrices during polymerization. These covalently bound heparin-binding peptides were used to immobilize heparin within fibrin matrices based on electrostatic interactions. The bound heparin in turn served to sequester heparin-binding growth factors within fibrin matrices, which can be released by enzymatic factors, as well as by passive release. Mathematical modeling suggested that a high excess of heparin (versus growth factor) was required for slow passive, diffusion-based release that would allow cell-mediated active release of growth factor to dominate.

The ability of the delivery system to release basic fibroblast growth factor (bFGF) in an active form was tested using neurite extension from DRGs within fibrin matrices. When all three components of the delivery system were present during fibrin polymerization (heparin, peptide and bFGF), matrix-bound bFGF was found to enhance neurite extension in a dose dependent manner by up to 100% relative to unmodified fibrin. Further experiments demonstrated that all components of the delivery system, namely heparin, covalently-immobilized peptide and heparin-binding growth factor, were necessary for slow passive release. Taken together, the results of these experiments suggest that the delivery system sequesters heparin-binding growth factors and prevent their diffusion from fibrin matrices. The results also demonstrate that the growth factors delivered from this system suffer no loss of bioactivity due to their binding interaction with the heparin-containing fibrin matrix.

It was hypothesized that this heparin-containing delivery system could also be used to deliver growth factors with low heparin-binding affinity, which contain surface-accessible basic domains, based on low affinity interactions between these basic domains and heparin. The ability of heparin-containing fibrin matrices, with a high excess of

heparin-binding sites, to slow the diffusion-based release of growth factors with low heparin-binding affinity, such as β-NGF, from fibrin matrices was measured in the absence of cells. Release of β-NGF, BDNF, and NT-3 were tested using neurite extension from DRGs to determine the ability of the delivery system to release active growth factor. The results demonstrated that neurotrophins, interacting with fibrin matrices containing a large molar excess of heparin relative to growth factor, enhanced neurite extension by up to 100% relative to unmodified fibrin. In the absence of the delivery system, free neurotrophins within the fibrin matrix did not enhance neurite extension. These results suggest that prolonged release of low affinity heparin-binding growth factors, such as NGF, is also possible with these heparin-containing delivery systems as long as high excess of heparin sites is maintained.

The second method for growth factor delivery developed in this research involves the use of recombinantly expressed growth factor fusion proteins that contain an exogenous cross-linking domain. This domain allows the fusion protein to be covalently immobilized within fibrin matrices by factor XIIIa during fibrin polymerization. A protease substrate was placed between the cross-linking domain and the growth factor domain to allow the growth factor to be released in response to cellular activity during nerve regeneration. The results of this work demonstrate that such growth factor fusion proteins have biological activity. Furthermore, immobilized growth factor fusion promoted an increased level of neurite extension within fibrin matrices versus similar concentrations of growth factor in the medium. These results suggest that such fusion proteins may serve as a potential therapeutic for nerve regeneration.

The approaches described above to enhance peripheral nerve regeneration through the use of exogenous adhesion domains or via the controlled release of exogenous growth factor were all found to enhance neurite extension *in vitro* by approximately 50-100% versus unmodified fibrin. The magnitude of this enhancement is comparable or better to enhancements reported by other researchers, who used similar assays or approaches to

promoting neurite extension. In addition, the ability of unmodified fibrin matrices to promote neurite extension is relatively good, so the baseline level of neurite extension through fibrin-based materials corresponds to neurite extension of about 700 μm in 48 hrs. Comparisions with other research to develop similar scaffolds for nerve regeneration suggests that the materials developed in this research may search of potential therapeutics to enhance peripheral nerve regeneration.

6.2 Recommendations for Future Work

The materials developed in this project could be used for many applications and could be adapted to promote tissue regeneration in many different types of tissue, including skin bone, cartilage and vascular tissues. Fibrin-based tissue engineering scaffolds can promote the adhesion and migration of many different cell types. Fibrin can be degraded by many different types of cells, because plasmin expression and activation occurs in many tissues.

The method developed for the incorporation of exogenous peptides in fibrin via the transglutaminase activity of factor XIIIa was shown by this work to be applicable to many different peptides and proteins. However, in all cases the efficiency of incorporation was less than 100%. One possibility for improving these fibrin materials is through the use of exogenous factor XIII to increase the incorporation efficiency of peptides and proteins. By adding exogenous factor XIII, peptide incorporation has been increased from 8 moles of peptide per mole fibrinogen to approximately 25 moles of peptide per mole fibrinogen (Jason Schense, unpublished results). In addition, the efficiency of protein incorporation at a ratio of about 1 mole of protein per mole fibrinogen was increased from 50-60% to 95% through the use of exogenous factor XIII (Andreas Zisch, unpublished results). These results suggest that more peptide or protein can be incorporated into fibrin matrices through the use of exogenous factor XIII.

Additional factor XIII could be used to increase the number of heparin-binding peptides incorporated with fibrin matrices. This increase in peptide concentration might enhance the ability of such materials to promote neurite extension, through the use of heparin-binding domains as cell adhesion sites, because the addition of exogenous heparin-binding domains enhanced neurite extension when 8 mole of peptide per mole of fibrinogen were incorporated into fibrin matrices, but not when 4 moles of peptide per mole fibrinogen were incorporated. The incorporation of additional peptide might also be useful for providing sites for heparin immobilization. For example, 10% of the heparin-binding sites might be used to immobilize heparin for drug delivery and the other 90% would remain unoccupied and free to serve as cell adhesion domains, thus allowing the heparin-binding peptides to be used as both cell adhesion domains and growth factor immobilization sites within the same material.

The heparin-containing delivery system could be useful for growth factor delivery in a variety of models for tissue regeneration in many tissues including the central nervous system, bone, cartilage, skin, and in the promotion of angiogenesis. Preliminary in vivo studies that have been performed suggest that these materials may be of interest for enhancing spinal cord regeneration. The controlled delivery of aFGF, platelet-derived growth factor, and NT-3 have been investigated the rat dorsal hemisection model for spinal cord regeneration. Cellular infiltration of the lesion site was increased when the fibrin-based materials were placed in the lesion site, and scar formation was greatly reduced in these cases. In some cases increased axonal sprouting was observed, with the addition of aFGF to the delivery system (Regula Müller and Martin Schwab, unpublished results). These results suggest that these materials may serve as a potential therapeutic for nerve regeneration in the central nervous system.

The ability of bone morphogenetic protein-2 (BMP-2) immobilized via the heparin-containing delivery system to promote ectopic bone formation is also being studied. The use of low ratios (1:1 or 2:1) of heparin to BMP-2 were shown to promote

ectopic bone formation when the fibrin matrices were implanted subcutaneously in rats (Shelly Sakiyama, et al., unpublished results). These results suggest that the heparin-containing delivery system could also be used to promote bone regeneration. These materials are also currently being analyzed through a number of collaborations for their ability to promote regeneration of skin via the delivery of TGF-β3 and cartilage via the delivery of BMP-2 and IGF-1, demonstrating the broad utility of these materials.

The method developed for growth factor fusion protein delivery could also be applied to promote enhanced regeneration of many tissues. Currently, studies are underway to determine the ability of NGF fusion proteins to enhance peripheral nerve regeneration in the rat sciatic nerve. In a pilot study, the ability of NGF fusion protein immobilized within fibrin matrices to promote nerve regeneration was tested in a 15 mm gap in the rat sciatic nerve. No axonal bridging of the gap was observed in unmodified fibrin or saline control, but one case of complete bridging and two cases of incomplete bridging was observed for a dose of 1 μg/mL of NGF fusion protein (Sakiyama et al., unpublished results). Testing of other NGF doses is currently underway in this model.

The fusion protein approach for growth factor delivery allows any growth factor to be incorporated into fibrin matrices and released in controlled manner. Furthermore, the release of growth factors can be regulated through control of the choice of enzymatic substrates. One can vary the rate of release based on knowledge of substrate kinetics from the literature, i.e., selecting a faster or slower substrate. In addition, substrates for proteases other than plasmin could be used, such as elastase or collagenase. The drawback of this approach is that in order to change the growth factor used or the enzymatic substrate requires more work than in the case of the heparin-containing delivery system because a new gene must be cloned and a new fusion protein must be expressed.

This type of approach has already been applied to other growth factors. A vascular endothelial cell growth factor fusion protein has been expressed with a factor

XIIIa substrate and shown to promote tube formation by endothelial cells in an in vitro model of angiogenesis (Andreas Zisch, unpublished results). These results suggest that this method for growth factor delivery can be applied to enhance the regeneration of many different tissues.

The delivery systems developed in this research may also be used with other base scaffolds than fibrin. For example, the heparin-binding peptides and heparin can be immobilized within polyethylene glycol (PEG) hydrogels and used to deliver heparin-binding growth factors to promote tissue regeneration. The ATIII heparin-binding domain used in this research was immobilized within enzymatically-degradable PEG gels with heparin and BMP-2 and implanted subcutaneously in rats. The delivery system promoted bone regeneration throughout the implant, but in the absence of the delivery system bone was only found on the periphery of the implant (Alison Pratt and Hugo Schmökel, unpublished results). These preliminary results demonstrate the utility of the heparin-containing delivery system in materials other than fibrin matrices.

The fusion protein system could also potentially be used with synthetic matrices as well. This would require exogenous factor XIIIa for cross-linking of the fusion protein and the placement of a lysine substrate for the transglutaminase in the synthetic scaffold. These requirements may render the fusion protein system more difficult to extrapolate to other scaffolds. One alternative is to use an immobilization domain within the fusion protein other than a factor XIIIa substrate. For example, one could use another enzymatic substrate for cross-linking or a functionality rarely found in folded proteins, such as a free sulfhydrol group found on unpaired cysteine residues, which could be reacted with functionalized synthetic polymers, such a PEG diacrylate.

Currently, research is underway to develop the approaches described above for clinical use. Fibrin sealants have been approved for clinical use for many years in Europe and were recently approved for use in the US. Heparin is approved for clinical use as an anticoagulant therapy. Most growth factors of interest have been tested in clinical trials,

and many are approved clinical use. Therefore, pre-clinical research should focus on demonstrating the safety of material components that are not currently approved for clinical use, namely heparin-binding peptides or growth factor fusion proteins, and the efficacy of these material in the wound-healing model of interest.

The safety of fibrin matrices modified with heparin-binding peptides is currently being evaluated. Fibrin matrices containing covalently incorporated exogenous heparin-binding peptides were implanted subcutaneously in mice, and the immune response to such materials is being analyzed. Animal studies are also underway to evaluate the ability of heparin-containing fibrin-based delivery systems to promote skin and bone regeneration through the controlled delivery of growth factors. If these materials are shown to be both effective and safe in small animal models, they will be evaluated in large animal models. If these studies also demonstrate efficacy, then clinical trials to evaluate safety and efficacy may begin.

Chapter 7 Bibliography

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