

5 cm GAP INJURY REPAIR: GROWTH FACTOR SUPPORT AND FUNCTIONAL ANALYSIS  
IN A RABBIT COMMON PERONEAL NERVE MODEL

by

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## ABSTRACT

### 5 cm GAP INJURY REPAIR: GROWTH FACTOR SUPPORT AND FUNCTIONAL ANALYSIS IN A RABBIT COMMON PERONEAL NERVE MODEL

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Nerve conduits have shown to successfully bridge nerve gaps of  $\leq 3$  cm; however repair of longer, mixed or motor nerve gaps still remains a clinical challenge. In order to study regeneration techniques in clinically relevant long gaps, larger animal models are required. Standardized methods to evaluate functional outcome are necessary to clinically translate experimental treatments. In this study, the capability of the biodegradable and transparent cross-linked doped polyester elastomer (CUPE) to serve as a nerve conduit material in a multiluminal nerve conduit across a 5 cm gap in a rabbit common peroneal nerve model was evaluated. The effect of growth promoting factor, pleiotrophin released from microparticles and from fibers within a synthetic multiluminal conduit in bridging a 5 cm long gap, was also investigated. Functional outcome was analyzed using toe spread, sensory parameters, force and muscle weights at the end of twelve weeks in the injured and uninjured hindlimbs of each rabbit. Qualitatively CUPE demonstrated better function than polyurethane in supporting the structure of agarose microchannels, however histological analysis revealed no axonal regeneration across the gap in any of the conduits. This summarizes the results of the first two aims. Variance in gait pattern is one of the most significant observations after peripheral nerve injury. Video gait analysis, together with joint kinematics, was used to characterize the gait and

ankle angle changes in the injured rabbits during treadmill locomotion. Foot drop, exaggerated knee flexion characteristic of peroneal injury was observed. In summary, this pilot study revealed the necessity of regenerative substrate and trophic support across a 5 cm long gap, established the changes in kinematic parameters after peroneal nerve injury in a rabbit model.

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## CHAPTER 1

### INTRODUCTION

“The central objective of nerve repair is to assist regenerating axons to re-establish useful functional connections with the periphery”- Sir Sydney Sunderland

#### 1.1 Clinical Problem

Each year more than 700, 000 people suffer traumatic peripheral nerve injuries in the United States alone, with trauma to the upper extremities being more common (Wiberg and Terenghi 2003). In addition, it is reported that over 300,000 cases of peripheral nerve injury occur annually in Europe (Mohanna, Young et al. 2003). The socioeconomic impact of these traumatic injuries is dramatic, given that they result in over 8.5 million restricted activity days and almost 5 million bed/disability days each year (Kehoe, Zhang et al. 2012). Nerve injuries can be caused by motor vehicle collisions, gunshot wounds, construction casualties, natural disaster, war damage, athletic injuries and iatrogenic side effects of surgery (Gu, Ding et al. 2011). Close to 5% of all open wounds in the extremities is complicated by peripheral nerve trauma, resulting in nerve gaps (J, Jansen et al. 2004). Nerve injuries can also be inherited in cases such as tomoculus or neurofibrosacroma that are passed down from one generation to another.

Peripheral nerve injuries can occur due to stretch (most predominant), compression and partial or complete transection (laceration). The severity of these PNS injuries can range from mild motor and sensory deficits to permanent paralysis or intractable neuropathic pain. When injury occurs close to the cell body in adult motor neurons, the chance of axonal survival is greatly reduced; moreover it has been reported that sensory neurons undergo extensive cell death after injury (Terenghi, Hart et al. 2011). Unlike the central nervous system, peripheral nerves possess the capacity to regenerate after traumatic injury; though, this potential is limited.

## 1.2 Physiology and Organization of the Nervous System

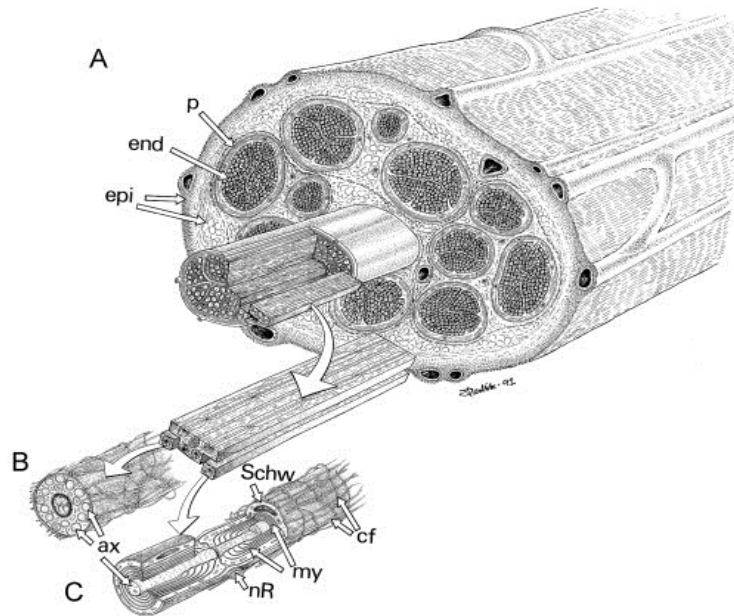


Figure 1.1 Peripheral nerve anatomy. A: Endoneurium-covered axons (end) are bundled by perineurium (p) into fascicles. These fascicles are in turn covered by epineurium (epi). B, C: Specializations of the Schwann cells that surround unmyelinated (B) and myelinated (C) axons. Numerous unmyelinated axons (ax) are embedded by invagination into the cytoplasm of a single Schwann cell (Sch). Each myelinated axon has multiple layers of Schwann cell membrane wrapped around it, forming myelin (my), interrupted at regular intervals by nodes of Ranvier (nR). Figure adapted from (Lundborg 1988).

The nervous system is classified into the central nervous system (CNS) and the peripheral nervous system (PNS). Peripheral nerves carrying motor and sensory fibers enable the cross talk between the central nervous system and the peripheral organs. Both systems differ in their physiology and ability to regenerate following injury. Following injury, peripheral nerves can spontaneously regenerate, to a certain extent, unlike the CNS. The functional unit of a peripheral nerve is the axon (Figure 1.1). Axons are accompanied by Schwann cells, the glia of the peripheral nervous system, which are responsible for myelination. Adjacent Schwann cells are separated from one another by nodes of Ranvier. Each node is lined by paranodes and juxtaparanodes (Bove 2008). Peripheral axons are covered with a series of connective

tissue layers to form a peripheral nerve. Axons are supported by a collagenous endoneurium and grouped into fascicles. The fascicles are then defined by the perineurial sheath, and the epineurium forms an outer protective coating. The outermost tissue that connects the epineurium to the surrounding tissue is known as mesoneurium. The nerves are supplied with blood vessels entering through the mesoneurium and running within the epi and perineurium. Peripheral nerves may be divided into sensory, motor or mixed fiber composition. The sensory (afferent) neurons are responsible for stimulus recognition, and motor (efferent) neurons, control end organs and tissues, such as glands and muscles. The motor unit (MU) consists of a lower motor neuron cell body in the spinal cord, the axon of that neuron, and the multiple myofibers it innervates. Together they constitute the working unit of the neuromuscular system.

### 1.3 Neural Injury and Response

The success of peripheral nerve repair depends on the type and severity of the injury. Surgical intervention is chosen based on grading systems that correlate microscopic changes occurring after nerve injury with the patient's symptoms. Seddon classified nerve injury into three broad categories: neurapraxia, axonotmesis and neurotmesis (Seddon 1975). Neuropraxia is a physiological block of nerve conduction without the physical disruption of nerve tissues or axons. Axonotmesis refers to anatomical interruption of the axons, with preservation of the perineurium and epineurium. Neuropraxia and axonotmesis injuries are typically due to compression, traumatic crush, and stretch or neuroma formation. The most severe injury, neurotmesis, is characterized by a complete transection of the nerve and surrounding tissue continuity. Sunderland expanded Seddon's classification system into 5 degrees of nerve injury by refining axonotmesis injury depending on the source, duration and severity of the anatomical block (Sunderland 1978). Figure 1.2 shows the classification of nerve injury illustratively along with a normal nerve. In both fourth and fifth degree injuries, repair does not happen without surgical intervention. S.E Mackinnon characterized a mixed pattern of injuries to the multiple fascicles in the nerve and introduced it as the 6<sup>th</sup> degree of injury (Mackinnon 1988)

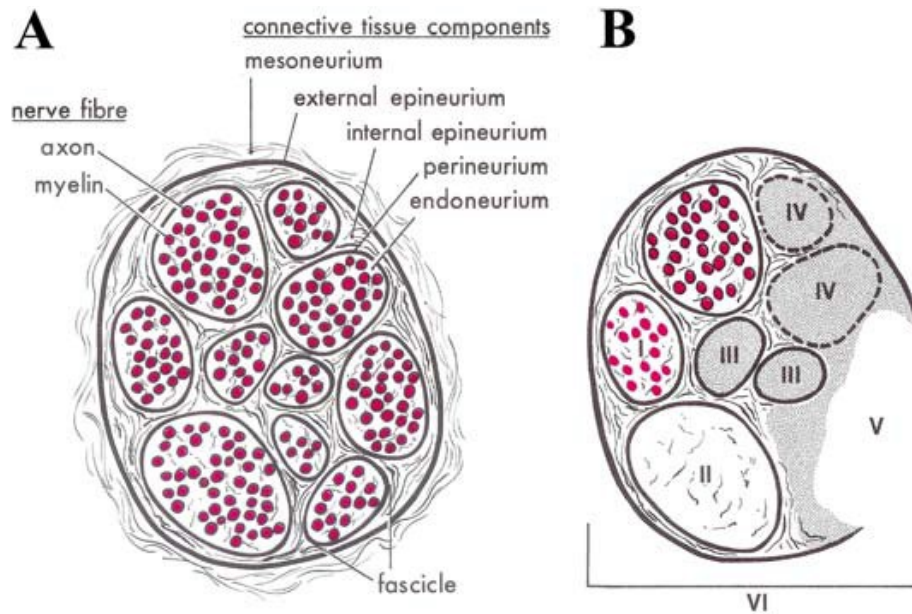


Figure 1.2 Nerve injury classification. (A) Cross-section of a normal nerve. (B) Illustration of injury classifications. Type I: myelin disruption with axons intact. Type II: axon disruption with intact perineurium. Type III: damaged Schwann cell basal lamina and endoneurial scarring inhibiting regeneration. Type IV: nerve fascicle disruption and loss of the perineurium sheath; repair required. Type V: disruption of the entire nerve; repair required. Type VI: mixed injury of all types along the damaged nerve. Figure adapted from (Mackinnon 1988)

Based on the severity of the injury, the axonal damage is followed by disruption of neuronal tissue with or without a gap, leading to the loss of motor and sensory function. A series of degenerative events must take place before regeneration of nerve fibers can occur (Burnett 2004). Gap injuries induce the distal part of the axon to degenerate; this is a calcium-mediated process known as anterograde or Wallerian degeneration. This triggers the physical fragmentation of both axon and myelin at the distal stump, and ultra-structurally, the axoplasmic microtubules and neurofilaments become disintegrated (Seckel 1990). Schwann cells play a key role in Wallerian degeneration. Within 24 hours post injury, axons of the distal stump are reduced to debris, and conduction impulses are lost (Chaudhry, Glass et al. 1992). By 48 hours, myelin disintegration occurs, and large fibers are degenerated (Chaudhry, Glass et al. 1992).

Schwann cells undergo a shift in gene regulation whereby expression of myelin proteins is down-regulated and expression of regeneration associated genes (RAG) is up-regulated. While axons of the proximal stump undergo die back up to the first node of Ranvier, chromatolytic changes occur in the lesioned cell body (Keith Fenrich 2004). These changes include movement of the nucleus from the center of the periphery, pronounced prominence of the nucleolus and Nissl substance from the cytoplasm. Along with Schwann cells, infiltrating macrophages play an essential role in clearing the axonal, myelin and tissue debris by phagocytosis (Perry, Brown et al. 1987). The orchestrated pattern of anterograde changes in the distal segment are followed by Schwann cells forming aligned columns, known as Bands of Bungner (Bradley and Asbury 1970). The growth cones of the proximally sprouting axons use these columns as regenerative substrates that provide neurotrophic support and contact guidance.

Repair of nerve gaps caused by neurotmesis poses the greatest challenge. The failure of regenerating axons to extend along the endoneurial tubes results in atrophic Schwann cell changes, increased deposition of endoneurial collagen and reduction of endoneurial tube diameter. Chronic axotomy and long term denervation impede the efficiency of surgical repair significantly, leading to reduced or no functional recovery.

#### 1.4 Clinical Alternatives for Nerve Repair

Over short nerve gaps, spontaneous regeneration occurs. The best method of nerve repair is the one that yields the most consistent and optimum functional result. Manipulative nerve procedures like direct nerve repair (end-to-end suturing, end-end repair or end-to-end coaptation) are performed in the situation of no nerve tissue loss or tension-free suturing of the injury site. These surgical interventions are usually deployed for gaps <5 mm and are preferred clinically (Lee and Wolfe 2000). Beyond this short gap, alternative tissue engineering strategies are needed to bridge the gap between the two stumps to facilitate nerve regeneration.



#### 1.4.1 Nerve Grafting: Autograft and Allograft

Currently, autografts remain the more preferred method to bridge gaps precluded from direct nerve repair. The presence of a permissive and stimulating scaffold, along with Schwann cell basal laminae, neurotrophic factors and adhesion molecules, make nerve autografts the ideal conduit for nerve repair (Almgren 1975). The grafts are usually taken from less critical nerve segments of sensory nerves, such as sural, superficial cutaneous or lateral and medial antebrachii nerves. The diameter of the grafted nerve segment and vascularity of the surrounding tissue bed influence the success of nerve repair significantly. Unfortunately, autografts are limited by inherent drawbacks, such as donor site morbidity, development of neuropathic pain, limited supply and mismatch between sensory nerve grafts and motor nerves. When the gaps are longer than the available autograft material, allografts from cadavers are the only clinical option currently available. Host immunogenicity or allograft rejection is the main concern with nerve allografts. Major histocompatibility complex (MHC), graft pretreatment, host immunosuppression and tolerance induction are the main strategies that have been studied and used to prevent allograft rejection. In 2001, Mackinnon et.al. published a thorough report on 10 clinical cases treated with nerve allografts and immunosuppressive therapy (Mackinnon, Doolabh et al. 2001). Patients treated with immunosuppression become susceptible to other fatal diseases; this, along with prolonged surgery duration encouraged the development of nerve conduits for bridging a gap following nerve injury.

#### 1.4.2 Conduit Repair: Biological and Artificial Conduits

The various disadvantages encountered with nerve gap repair using autografts have encouraged the development of conduit/nerve tube-based strategies for repair. Various biological and artificial materials have been investigated for the fabrication of a nerve conduit. Tubed nerve repair utilizes a cylindrical substance to bridge the gap between 2 nerve ends (Figure 1.3). This tube can be fabricated to serve a number of critical purposes: mechanical support and guidance of the regenerating axons, diffusion of neurotropic and neurotrophic

factors, prevention of soft tissue and scar formation between nerve endings and creation of an optimal microenvironment for regenerating axons (Gu, Ding et al. 2011). Biological (non-nervous system) conduits have been developed using arteries, veins, pre degenerated or fresh skeletal muscle and epineural sheaths (Deumens, Bozkurt et al.; Chiu, Janecka et al. 1982; Gu, Ding et al. 2011). Autologous, allogenic and xenogenic non-nerve tissues (blood vessels, muscles, muscle-in-vein combinations) have been used as material for neural scaffolds with limited supply (Crouzier, McClendon et al. 2009; Gu, Ding et al. 2011). Vein grafts showed good functional outcomes in pure sensory nerve repair (Risitano, Cavallaro et al. 2002). The favorable properties of autologous biological tissues, such as immunological compatibility, finely oriented microstructure and optimal donor-host integration characteristics, promoted their use in peripheral nerve repair; however, all conduits made from autologous tissues suffer from inadequate availability. Using decellularization methods to remove the immunogenic components from the allogenic or xenogenic (non-nervous) tissues have led to the development of neural scaffolds having structure comparable to that of native nerves. Avance® (AxoGen Inc. Alachua, FL) is one such commercially available acellular allogeneic nerve graft, which has shown positive results in clinical studies.

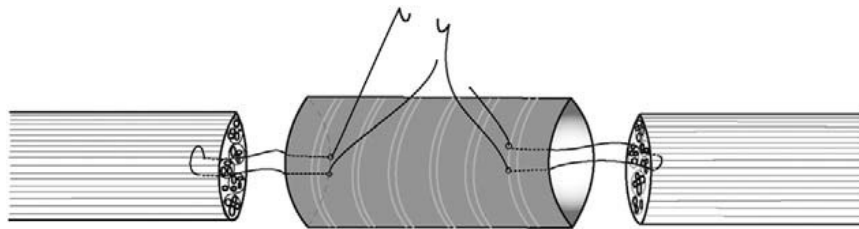


Figure 1.3: The nerve tube selected should be slightly larger than the diameter of the nerve. The freshened nerve endings are inserted 2 mm into the ends of the tube under magnification. An 8-0 horizontal mattress suture is then used to secure the outer epineurium of the nerve within the nerve tube to protect against dislodgement, with the knot lying on the conduit. Figure adapted from (Agnew and Dumanian 2010)

The advances in biomaterials and chemical engineering have enabled the exploration of different types of synthetic conduits as alternatives to autografts. Characteristics of the nerve

guidance conduit (NGC), including the biocompatibility, biodegradability, mechanical properties of the material, permeability, and surface properties influence the outcome of the nerve repair.

Among naturally-derived polymers, collagen, gelatin, laminin, fibronectin, chitin, chitosan, alginate and silk-fibroin have been used as materials to form scaffolds (Gu, Ding et al. 2011). Despite possessing good biocompatibility, only two nerve guides of natural origin (resorbable) have been FDA approved: Type I collagen (NeuraGen ; Neuroflex; NeuroMatrix; NeuroWrap and NeuroMend) and porcine small intestinal submucosa (Surgis Nerve Cuff) (Kehoe, Zhang et al. 2012).

The numerous engineering properties possessed by synthetic materials make them attractive for the development of nerve guides. The potential to tailor their degradation time and functionalize the material by incorporating signaling molecules or peptide sequences has further promoted their use. The application of non-biodegradable silicone and expanded polytetrafluoroethylene (ePTFE) has been reported in humans, but their presence in situ have been shown to cause foreign body reactions and nerve compression, inhibiting functional recovery (Dahlin, Anagnostaki et al. 2001; Battiston, Geuna et al. 2005; Ichihara, Inada et al. 2008). Nerve conduits made from these polymers did not promote good recovery over defects exceeding 4 cm (Stanec and Stanec 1998). The only FDA-approved non-resorbable nerve guides are made of polyvinylalcohol hydrogel (Salubridge; SaluTunnel) (Kehoe, Zhang et al. 2012). Preclinical and clinical studies have not yet been published for these devices.

Polymers belonging to different classes such as aliphatic polyesters, polyphosphoesters, piezoelectric polymers, and hydrogel based have also been investigated as nerve guide materials. Resorbable conduits made of polyglycolic acid polymer (PGA), polylactide-caprolactone polymer (PLCL) and PGA polymer coated with cross-linked collagen (PGA-c) have been reported for application in humans (Meek and Coert 2002; Battiston, Geuna et al. 2005; Ichihara, Inada et al. 2008). The only two FDA approved resorbable nerve conduits of synthetic origin are PGA (Neurotube) and PLCL (Neurolac) (Kehoe, Zhang et al. 2012). In all

the studies conducted, use of these resorbable artificial nerve guides to bridge nerve defects up to 3 cm had no significant differences in functional outcome, as compared to nerve autograft repair (Meek and Coert 2002).

### 1.5 Luminal Fillers

Currently, all the FDA-approved NGCs are simple empty, hollow tubes that are not functionalized in any manner. In an attempt to enhance the efficacy of short gap nerve repairs but also extend the application of nerve conduits to long gap nerve repairs, the approach of using luminal fillers has been explored. The coordinated action of haptotactic and chemotactic cues (attractive or repulsive) guides axons to their targets via growth cone signalling. Several groups proposed the idea of internal fibers and guidance channels within the conduit, in an effort to create architecture similar to that of the peripheral nerve autograft. Physical cues using ECM proteins, like collagen filaments, laminin-filled tubes, gelatin fibers, Matrigel with PLA wet-spun fibers, and collagen sponges, have all been investigated with the goal of promoting early Schwann cell alignment (Lundborg, Dahlin et al. 1997; Itoh, Takakuda et al. 2001; Hu, Gu et al. 2008). High manufacturing times, optimal packing density of fibers and reduction in luminal space are some of the problems associated with luminal fillers.

Within hours of nerve transection, a fluid is secreted from the damaged nerve ends containing various cells, structural components and neurotrophic factors essential for nerve regeneration (Seckel 1990). Based on this fact, several studies have been carried out using cellular components as luminal additives inside the conduit. Studies employing Schwann cells (Cheng and Chen 2002; Mosahebi, Wiberg et al. 2003; Phillips, Bunting et al. 2005), bone stromal cells (Cuevas, Carceller et al. 2002; Cuevas, Carceller et al. 2004; Hu, Zhu et al. 2007), fibroblasts (Phillips, Bunting et al. 2005), ectomesenchymal stem cells (Nie, Zhang et al. 2007), neural stem cells (Heine, Conant et al. 2004), hair follicle stem cells (Amoh, Li et al. 2005), and skin-derived stem cells (Marchesi, Pluderer et al. 2007) to promote growth of regenerating axons

have shown varying levels of success. Problems associated with harvesting autologous cells and the resulting time lag between injury and repair has slowed their acceptance.

The cellular activities of survival, migration, differentiation and growth of neurons in the developing nervous system are regulated through signaling molecules collectively known as neurotrophic factors. They also promote nerve regeneration in humans and animals. The construction of tissue engineered nerve grafts incorporating neurotrophins such as nerve growth factor (NGF) (He, Chen et al. 1992; Lee, Yu et al. 2003; Xu, Yee et al. 2003), brain-derived neurotrophic factor (BDNF) (Terris, Toft et al. 2001), neurotrophin-3 (NT-3) (Sterne, Brown et al. 1997; Midha, Munro et al. 2003) and neurotrophin-4/5, have been attempted. Other neurotrophic components that have been investigated are fibroblast growth factor (FGF) (Cordeiro, Seckel et al. 1989; Wang, Cai et al. 2003), ciliary neurotrophic factor (CNTF) (Zhang, Lineaweaver et al. 2004), glial growth factor (GGF) (Mohanna, Terenghi et al. 2005), vascular endothelial growth factor (VEGF) (Hobson 2002) and Glial-derived neurotrophic factor (GDNF) (Barras, Pasche et al. 2002; Boyd and Gordon 2003; Wood, Moore et al. 2009). Though growth factors have shown favorable effects on peripheral nerve regeneration, they have to be optimized for therapeutic use in humans. Several details about growth factors such as high biological activity, short half-life, slow tissue penetration, pleiotropic actions and potential toxicity at high penetration levels makes growth factor delivery important. Currently, the commonly used methods of delivering growth factors include inside the hollow lumen of NGCs, osmotic pumps, repeated injection, release from matrices inside the lumen or NGC wall, microspheres and gene-based delivery strategies (Pfister, Papaloizos et al. 2007). In order to ensure adequate effect with little adverse reactions, different modes of growth factor delivery are being investigated.

#### 1.6 Growth Factor Concentration Gradients for neurite guidance

During embryonic development of the nervous system, the developing axons are guided to their target organs by a combined action of haptotactic and chemotactic cues, to

which the growth cone reacts. Cellular activities such as cell migration, elongation, differentiation and signaling are controlled by gradients that exist within the extracellular matrix (ECM) (Li, Ma et al. 2005; Liu, Ratner et al. 2007; Singh, Berkland et al. 2008). The growth cone, a motile actin-supported network at the tip of the developing axon, samples the environment and undertakes decision-making procedures depending on concentration gradients of many guidance cues. By manipulating the concentration gradients of signaling molecules (chemoattractant and chemorepellant) artificially, axonal extension may be possible in the adult nervous systems.

Specifically, insoluble and soluble gradients of NGF, NT-3 and BDNF have been shown to induce turning of growth cones toward increasing concentrations of these neurotrophic factors (Song, Ming et al. 1997; Cao and Shoichet 2001; Cao and Shoichet 2003). In vitro studies by Cao and Stoichet et al. have evaluated the approximate concentration range and minimal gradient of NGF concentration effective at inducing PC12 cell neurite extension. Agarose hydrogels incorporated with laminin and NGF in an anisotropic manner have demonstrated successful sciatic nerve repair up 20 mm gaps (Dodla and Bellamkonda 2008). Microfabricated hydrogels, microfluidic systems and gradient printing are some of the methods that have been studied so far to create controlled concentration gradients; however, they face problems creating the same effect in vivo (Walker and Barbour 2010). Injection of viral vectors have been used to create a concentration gradient in an animal model, but this method is limited by replication – competent carriers (Ziemba, Chaudhry et al. 2008). Recently, our lab developed a method to create a programmable gradient using growth factor loaded fibers which can be coiled around the lumen of a microchannel. By programming the number of turns, the needed steepness of the gradient can be created (Lotfi 2011).

### 1.6 Biosynthetic Nerve Implant (BNI)

The Biosynthetic Nerve Implant (BNI) was developed in an attempt to mimic multifascicular anatomy and guide regenerating axons. It was designed to provide a multi-

channelled, transparent, hydrogel-based scaffold facilitating axon repair (Figure 1.4). With the aid of special casting devices, collagen channels are created throughout a micro-renathane tube filled with agarose. The conduit material is made porous for allowing nutrient/gas exchange. In a previous study conducted in our lab, it was demonstrated that 10mm long BNIs produced results comparable to autografts in a rat sciatic nerve injury model (Tansey, Seifert et al. 2011) These conclusive results supported the hypothesis that the BNI method of repair allows for better mimicry of the multifascicular anatomy of the nerve.

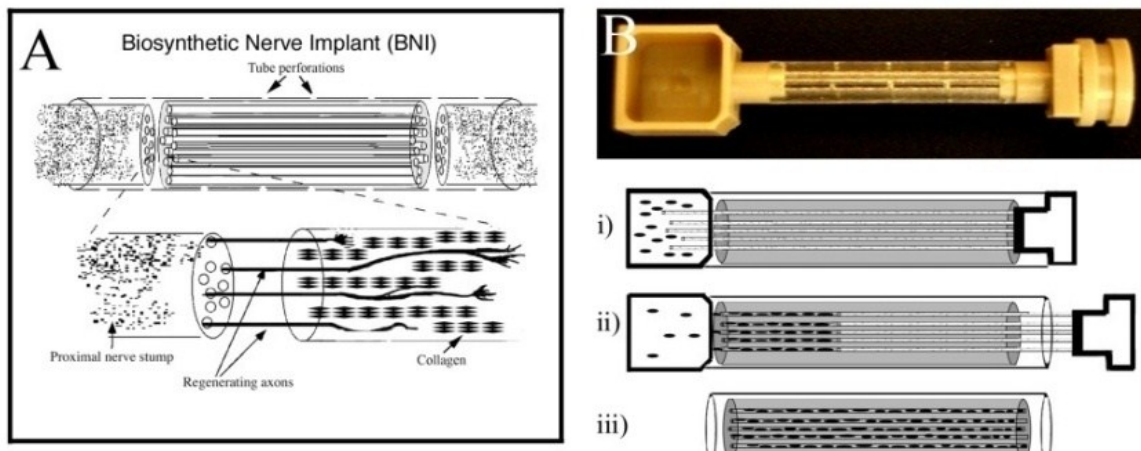


Figure 1.4 Fabrication of multiluminal nerve guides. (A) Representation of the biosynthetic nerve implant (BNI). (B) Fabrication method consisting of (i) inserting a metal brush through polyurethane tubing into a well filled with collagen or cells or microparticles. (ii) polymerization of agarose in the tube (iii) following removal of the metal fibers creates multiple microchannels simultaneously filled with collagen, cells or particles.

In a later study, neurotrophic support was provided using VEGF or PTN nanoparticles to entice axons to regenerate across long gap nerve defects (3 cm) in rabbit common peroneal nerve model. More neurofilament positive axons along with recovery of different functional parameters were observed in the BNI supplemented with VEGF or PTN. A larger study was conducted by our lab to confirm the results and to evaluate the synergistic effects of VEGF and PTN on enhancing axonal regeneration. The results demonstrated successful bridging of the

long gap (3cm) using multiluminal conduits with PTN microparticles, whereas the combined VEGF/PTN group did not have any significant impact (Nguyen 2010).

### 1.7 Crosslinked urethane-doped polyester (CUPE)

Polymers belonging to the class of 'Biodegradable elastomers' have shown excellent capability in recovering from multiple deformations without causing irritation to the surrounding tissue in a mechanically demanding environment. Citric acid, a non-toxic metabolic product of the Krebs cycle approved by the FDA has been used as a multifunctional monomer to produce citric acid derived biodegradable elastomers (CABEs). The controllable mechanical and physical properties of CABEs have made them applicable in a variety of fields such as vascular tissue engineering, wound repair and drug delivery.

In 2008, Yang and coworkers developed a class of soft but strong biodegradable elastomers: crosslinked urethane-doped polyesters (CUPE) (Dey, Xu et al. 2008). They successfully combined the advantages of a fully elastic and biocompatible crosslinked

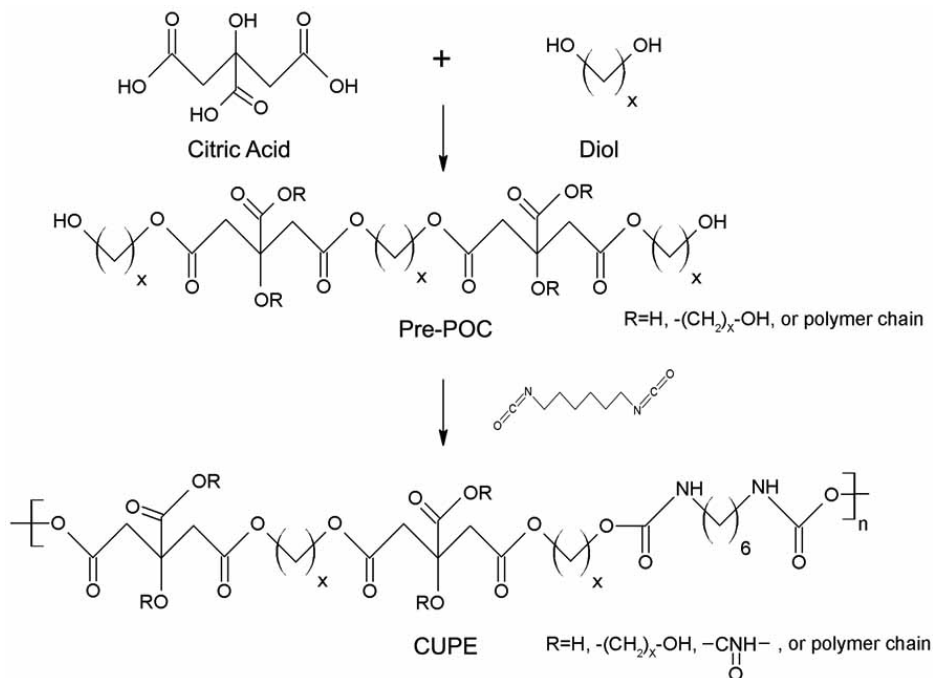


Figure 1.5 Schematic representing crosslinked urethane-doped polyester (CUPE) synthesis



polyester network with the strength of linear polyurethanes. Figure 1.5 represents CUPE synthesis. CUPE exhibited excellent mechanical properties, including tensile strength as high as  $41.07 \pm 6.85$  MPa with corresponding elongation at break of  $222.66 \pm 27.84\%$  (Dey, Xu et al. 2008). In vitro degradation studies of CUPE polymers demonstrated degradation rate to be a function of the choice of diol used in the synthesis, the isocyanate content, and the post-polymerization conditions. Good material-cell interactions were observed in biocompatibility studies using CUPE, 3T3 fibroblasts and human aortic smooth muscle cells. Our lab reported successful nerve regeneration across long nerve gaps (15 mm) using multiluminal conduits made of CUPE in a rat sciatic nerve model. The translucency of the CUPE material allowed pre and post-surgery evaluation of luminal fillers and the integrity of microchannels. The peak stress and elongation at break at 8 and 12 weeks of the study indicated specific and controlled reabsorption.

### 1.8 Pleiotrophin

Pleiotrophin (PTN), along with midline, belongs to the family of Neurite Growth-Promoting Factors. It is more commonly known by other names such as heparin-binding growth factor-8, heparin-binding growth-associated molecule, heparin-binding neurotrophic factor, heparin-affinity regulated peptide, osteoblast-specific protein-1, and p18 (Kovesdi, Fairhurst et al. 1990). PTN plays a major role in various functions such as neurite outgrowth and synaptic plasticity, liver regeneration, fertility, auditory function, wound healing and adipogenesis (Koutsoumpa, Drosou et al. 2012). Studies have reported a possible association of PTN in the blood vessel network formation of solid tumors, and up-regulation of PTN has been observed in malignant tumors. PTN acts as a mitogen to many cell types (endothelial cells, fibroblasts, epithelial cells) and encourages angiogenesis in normal and tumor cells. Jin et al. reported that PTN promoted outgrowth in neurons, glial progenitor cells and oligodendrocyte progenitors (Jin, Jianghai et al. 2009). Using microarray analysis, it was found that PTN gene expression was up regulated in denervated distal stumps immediately after sciatic nerve transection (Mi, Chen et

al. 2007). Hoke et al. reported that the expression of PTN was higher in an injured nerve, motor nerve compared to sensory and in denervated muscle than skin (Hoke, Redett et al. 2006).

The effects of PTN are mediated through four cell surface receptors: receptor protein tyrosine phosphatase (RPTP)  $\beta/\zeta$ , anaplastic lymphoma kinase (ALK), N-syndecan, and lowdensity lipoprotein receptor-related protein-5 (Jin, Jianghai et al. 2009). Studies by Mi et al. suggest that ALK might be the receptor responsible for PTN's neurotrophic activity (Mi, Chen et al. 2007). In the peripheral nervous system, PTN is involved in survival and regeneration of motor neurons and their muscle targets (Jin et al 2009; Mi et al 2007; Yeh et al 1998; Zhang et al 1999). Previously, our lab has reported that treatment of motor neuron culture with PTN produces denser and longer neurites compared to BSA (Dawood 2009). Recently, we used PTN alone or in combination with VEGF to investigate their effect on long gap nerve regeneration in a 3 cm gap in a rabbit common peroneal nerve injury model. It was observed that BNIs having PTN had better axonal regeneration compared to VEGF and combined VEGF/PTN group.

### 1.9 In vivo animal models of peripheral nerve injury

Though research efforts in peripheral nerve repair have increased, only three synthetic nerve guides have reached the clinics over the past 50 years. There are different levels of testing through which an artificial conduit must be screened through before it gets to the clinical use. The investigator has to plan carrying out sufficient preliminary in vitro investigation, choice of appropriate animal model, selection of experimental paradigm of nerve lesion and nerve model employed. With the ongoing emphasis on ethical issues in biomedical research, in vitro models of axonal elongation have been developed. Immortalized neuronal and glial cell lines, primary neuronal and glial cultures (De Paola, Buanne et al. 2007; Scanlin, Carroll et al. 2008; Geuna, Raimondo et al. 2009), and 3-D and organotypic cocultures (Fornaro, Lee et al. 2008; Gingras, Beaulieu et al. 2008) have been utilized for the first phase testing of various artificial nerve guides (Trotter 1993; Shastry, Basu et al. 2001; Sak and Illes 2005; Hara, Yasuhara et al. 2008).

### 1.9.1 Rodents

Peripheral nerve regeneration studies have been conducted in diverse animal species, including primates, sheep, goats, pigs, dogs, cats, rabbits, rats and mice (Kline, Hayes et al. 1964; Archibald, Krarup et al. 1991; Trotter 1993; Madison, Archibald et al. 1999; Strasberg, Strasberg et al. 1999). As shown in Table 1.1, the systematic review conducted by Angius et al. shows the different animal models used to evaluate nerve regeneration in tissue-engineered scaffolds.

Table 1.1 Overview of animal models used to study nerve regeneration in tissue-engineered scaffolds. Adapted from (Angius, Wang et al. 2012)

<b>Species</b>	<b>Rat</b>	<b>Mouse</b>	<b>Rabbit</b>	<b>Dog</b>	<b>Cat</b>	<b>Sheep</b>	<b>Monkey</b>	<b>Pig</b>	<b>Total</b>
<b>Number of articles</b>	308	31	31	17	14	4	10	1	416

Different evaluation methods have been used in peripheral nerve regeneration research, with qualitative histological analysis and neuromorphometry being the most commonly used. Among the different parameters evaluated nerve fiber count, nerve histological success ratio, nerve fiber density, compound muscle action potential, muscle weight and muscle force have been demonstrated to have resolving power (Vleggeert-Lankamp 2007). The rat sciatic nerve model continues to be the most studied model in peripheral nerve regeneration studies due to the several behavioral functional tests available (Varejao, Cabrita et al. 2004; Nichols, Myckatyn et al. 2005).

In most biomedical applications, rats and mice are the most commonly employed laboratory animals. A search using PubMed analysis of 1500 research papers on nerve regeneration showed that 90% of them used rat as an animal model (Wang, Tseng et al. 2011). The main reasons for this include larger nerve size compared to mice, which reduces the intricacy involved with direct repair, availability of standardized and comparable functional tests, well established nerve anatomy and fast rate of nerve regeneration. In addition, low

maintenance costs, ease of handling and faster rates of axonal elongation have led to the extensive use of smaller animal models (rats and mice) in nerve regeneration research. As shown in Table 1.2, the distance between the site of injury to the functional organ and the lag phase of regeneration is shorter in rats than other animal models and, hence, has important implications in regeneration studies.

Table 1.2 Rates of peripheral nerve regeneration. Adapted from (Rupp 2007)

Species	Lag Phase	Elongation	Return of Function
Human	2-9d		1 – 2mm/day
Rabbit	5-8d	3.5 mm/day(section) 4.4 mm/day(crush)	2.0-3.0 mm/day(motor) 3.5 4.4 mm/day (sensory)
Rat	1-4d	3.0 -4.6 mm/day(crush)	3.0 – 4.7 mm/day(sensory)
Mouse			1.5 -1.9 mm/day(motor) 2.0 - 2.9 mm/day(sensory)

Motor, sensory and also autonomic functional tests have been developed to study the degree of recovery after nerve repair in the rat sciatic nerve model, as shown in Table 3. Sensory functional tests are carried out by observing latency of the withdrawal reflex to a noxious stimulus, such as pin pricks, electric currents, small forceps, von Frey filaments, hot and cold baths. Using a force transducer, muscle tetanic force has been measured and has been shown to provide valuable information regarding functional recovery. The most common method so far used to evaluate functional recovery in the rat model is walking track analysis. The below formula using print length, hind paw toe spread and intermediate toe spread measured from the walking track is used to measure Sciatic Functional Index (SFI) (de Medinaceli, Freed et al. 1982; Bain, Mackinnon et al. 1989).

$$SFI = -38.3(EPL-NPL) / NPL + 109.5(ETS-NTS) / NTS + 13.3(EIT-NIT) / NIT - 8.8$$

Table 1.3 Different functional tests used to evaluate nerve regeneration in rat animal model

***Gap length studied in rat sciatic nerve model:5 mm- 25 mm***

<b>Nerve</b>	<b>Method</b>	<b>Functional parameter measured</b>	<b>No. of papers</b>
sciatic	Walking Track analysis using footprint measurements	SFI(Toe spread, Print length)	>120
sciatic	Video recording	Static sciatic index	>15
sciatic	Extensor postural thrust	Percentage Motor deficit	>15
sciatic, peroneal,tibial	Video recording of gait	Stance phase/Swing phase duration, ankle angle at terminal stance or mid swing. Toe-out angle/Midline deviation/Step length/Step height	>61
sciatic, peroneal,tibial	Measuring weight of dependent muscles	Muscle weight	>50
sciatic, peroneal,tibial	Isometric contractile force	Tetanic/twitch muscle force	>38
sciatic, peroneal,tibial	Electromyography	Muscle activity	>72
sciatic, peroneal,tibial	Electric, mechanical or heat/cold stimulus, pin pricks, sticky tape	Withdrawal reflex latency	>153

This group also developed functional indices for the peroneal and tibial nerve in the rat. Problems associated with obtaining walking tracks due to autotomy or contracture formation, led to the use of video recording of rat gait and computerized analysis. Videos are played frame by

frame and parameters like stance/swing duration, toe spread during the stance phase, exorotation of the foot have been evaluated for different gap repair approaches. Using two-dimensional digital video ankle motion analysis, the ankle angle at different phases of the gait have been recorded for assessing function in the rat sciatic nerve repair model.

A disadvantage of the rat animal model is that only relatively short gaps of 5- 25 mm can be easily created in the hindlimb nerves. The majority of studies have created gaps of 10 mm or less, which is short compared to the target human lesions. For successful employment in clinical trials, the nerve gap models should be larger than 40 mm, and this is not feasible using smaller animals. As studies progress, it will be essential to study the long distances or long gaps reported in human nerve injuries using larger animals (Hems, Clutton et al. 1994; Hess, Brenner et al. 2007). Thus, in order to create a long gap injury, we chose the rabbit for our animal model.

#### 1.9.2 Rabbits

In order for a nerve guide/regeneration technique to be translated to clinic, it would need to be tested in a preclinically in a larger animal model. It has been observed that the nerve regeneration process in large animals is similar to that in humans (Lawson and Glasby 1995; Fullarton, Lenihan et al. 2000). Various large animal models, such as rabbits, dog, cats, goat, sheep, pig and primates, have been employed for nerve regeneration studies. The ethical concerns about the use of domestic animals, such as dogs and cats have restricted their use over the last few years. Rabbits, non-specialised Lagamorpha belonging to the Leporidae family, are known for their speed and agility. The scientific name of the rabbit is *Oryctolagus cuniculus*. They are nocturnal and herbivorous animals and are related to hares and pikas. Over the past years, various bridging strategies have been used to bridge gap lengths from 5mm to 50mm in the rabbit hindlimb nerve model. Different nerve models which include the facial, sciatic, peroneal, tibial, alveolar, saphenous, radial and optic nerves have been studied in the rabbit model (Table 1.4). To date, histological analysis, neuromorphometric analysis and

electrophysiological tests have been most commonly used to evaluate nerve repair in the rabbit model. For electrophysiology tests, parameters of nerve conduction velocity and peak amplitude of muscle action potential have been evaluated distal to the site of injury using recording electrodes.

Table 1.4: Different functional tests used to evaluate nerve regeneration in rabbit animal model

<b><i>Gap length studied in rabbit hindlimb nerve model: 15- 60mm</i></b>			
<b>Nerve</b>	<b>Method</b>	<b>Functional parameter measured</b>	<b>No. of papers</b>
sciatic	Video recording of hopping gait	Motion angle of lower limb	1
sciatic, peroneal	Measurement of weight of dependent muscles	Muscle weight	6
peroneal	Sudden lowering of the animals without touching the ground	Toe spread reflex	4
peroneal	Measurement of isometric contractile force using a force transducer	Tetanic muscle force	3
sciatic, peroneal	Electromyography	Muscle activity	3

Measurement of muscle weight has been used to assess the reinnervation of target muscles and compare between the operated limb and control. Beer et al. reported muscle weight to be a relevant functional parameter in assessing peroneal nerve regeneration in the rabbit model (Beer, Seifert et al. 2008). Schmitz et al. reported toe-spreading reflex in rabbits to

be a sensitive indicator of the onset of motor recovery after peroneal injury (Schmitz and Beer 2001). Using a voluntary or reflex movement, such as toe-spread reflex to evaluate functional recovery would be more accurate as errors due to trick movements can be avoided. Many groups have used isometric twitch, and tetanic muscle force to evaluate motor function in the rabbit model and this technique has been validated by Giusti et al (Giusti, Kremer et al. 2012). Both the need to study longer gaps and the ability to create longer gaps in the rabbit has made the next most popular animal model after the rat.

Lack of adequate behavioural and functional assessment methods along with the high cost of maintenance have limited the use of the rabbit model in peripheral nerve regeneration studies. Our lab has conducted previous studies using the rabbit animal model, and this has helped to gain knowledge and expertise on handling them. Specifically, we demonstrated successful bridging of a 3cm gap in rabbit peroneal nerve model using neurotrophic support provided by pleiotrophin in the biosynthetic nerve implant (Nguyen 2010). Motor and sensory function was evaluated using toe- spread and formalin tests.

#### 1.10 Common Peroneal Nerve Model

Noble et al. has reported that the most frequently injured nerve in the upper limb is the radial nerve and peroneal nerve in the lower limb (Noble, Munro et al. 1998). Peroneal nerve injuries occur due to leg fractures, gunshot wounds and traumatic neuropathies. Also, 0.3% to 0.4% of patients have reported to suffer from peroneal nerve palsy after total knee arthroplasty (Zywiell, Mont et al.). For the purpose of actual clinical translation, it was more reasonable to use the common peroneal nerve as the nerve model for our study. Also, a sciatic nerve injury in a large animal model such as the rabbit would lead to a considerable amount of pain and loss of function in the hindlimb. For the purpose of limiting the type of injury, we chose the common peroneal nerve as the site of lesion.

The common peroneal nerve is a branch of the sciatic nerve arising from the lumbar and sacral nerves (L4-S2). After the division of sciatic nerve, the peroneal nerve begins from the



popliteal fossa, behind the knee. It then supplies the biceps femoris, passes through the lateral head of gastrocnemius and reaches the head of the fibula. At the neck of the fibula, the common peroneal nerve divides into two branches. The superficial and peroneal nerves supply the lateral (peroneus brevis and peroneus longus) and anterior (tibialis anterior, extensor hallucis longus, extensor digitorum longus and peroneus tertius) compartments of the leg respectively. The muscles in the anterior compartment are involved in dorsiflexion of the ankle joint and extension of the toes, while the lateral compartment muscles weakly plantarflex and evert the foot. Peroneal Nerve injury or peroneal nerve palsy leads to the loss of movement and sensation in the foot and leg. The loss of dorsiflexion (foot drop), eversion and spreading reflex of the toes are the effects of motor damage to the peroneal nerve. The effect of foot drop on leg movements and gait is discussed in detail in Chapter 3. The sensory symptoms are pain and parasthesia along the course of the peroneal nerve, dorsum of the foot and space between the toes.

#### 1.11 Specific aims of the project

Meaningful peripheral nerve regeneration has not been reported in studies using artificial conduits as nerve guides to bridge a gap longer than 3 cm. However, by using a combinatorial approach of different individual growth promoting factors, it may be possible to overcome the current barriers in long gap repair. The design for an ideal conduit for long gap peripheral nerve repair should consider various factors including biocompatibility, flexibility and strength of the tube, sufficient surface area for cell attachment, permeability for sufficient diffusion of oxygen and nutrients, biodegradability and the presence of guidance cues to the regenerating axons. The development of an off-the-shelf artificial nerve guide for the repair of long gap nerve defects (>3 cm) would be an advantageous tool to neurosurgeons. In the peripheral system, injured nerves have been shown to respond to stimulatory and inhibitory gradients which act like guidance cues for targeted reinnervation. By exploiting the mechanisms of axonal chemotaxis utilized in the developing nervous system, enticing regenerating axons to cross a longer gap may be possible. The overall goal of this project was to characterize various

parameters of long gap injury and repair in a rabbit animal model. We first fabricated a 5cm BNI using biodegradable CUPE to evaluate its capacity as a candidate for nerve conduit material for long gap repair studies. To study long gap repair in rabbits, we used a 5 cm biosynthetic nerve implant combined with delivery of pleiotrophin inside the conduit. We hypothesized that this approach would enhance axonal regeneration as compared to a conduit lacking growth factors. In order to evaluate this hypothesis, we established methods to assess functional outcome in rabbits using the common peroneal nerve model.

Aim 1: Determine the capacity of cross linked doped polyester elastomer to serve as a conduit material for a multiluminal nerve conduit in maintaining the integrity of luminal filler across a 5cm gap.

Aim 2: Examine the effect of pleiotrophin as a growth factor in enticing axons to cross a 5 cm nerve gap.

Sub-aim 2a) Evaluate the efficacy of using pleiotrophin (PTN) microparticles in a synthetic nerve conduit to bridge a 5cm gap.

Sub-aim 2b) Investigate the effect of gradient delivery of pleiotrophin in a synthetic nerve conduit to bridge a 5cm gap.

Aim 3: Develop methods of assessing functional outcome and deficits in a rabbit model after long gap peripheral nerve injury.

## CHAPTER 2

### BRIDGING A 5 cm GAP IN A RABBIT COMMON PERONEAL NERVE INJURY MODEL

#### 2.1 Introduction

In cases precluded from primary nerve repair, conduits may be used to bridge gaps of  $\leq 3$ cm, while autografts remain the benchmark for larger, motor or mixed nerve defects. The commonly used animal model in nerve repair studies, the rat, is capable of spontaneous regeneration. Therefore clinical application of a synthetic nerve conduit should be preceded only if the results are superior to an autograft or demonstrate successful outcome across very long distances. The use of nerve conduits made of synthetic biodegradable materials is more preferred in replacing the autologous nerve graft. An ideal nerve conduit should be biocompatible, have sufficient mechanical stability during regeneration, be flexible and porous to ensure nutrient supply and degrade into non-toxic products to prevent long-term irritation.

The capability of tissue engineered constructs to effectively support and promote the developing tissue depends on the similarity in their physical properties. The biomaterial, fabrication techniques, internal framework, and rate of degradation all affect the physical properties of the nerve tube. A decisive factor that affects the regenerative outcome in long nerve gaps is the 'distance to the distal stump' (Sinis, Schaller et al. 2007). As the gap gets longer, it becomes difficult for the regenerating axons to sense the growth factors at the distal stump. Studies have shown that exogenous growth factor support to the proximally regenerating axons, promotes axonal outgrowth and effective bridging of nerve gaps. Various growth factor delivery systems have been used including absorption to fibronectin mats, polymer matrices, hydrogels and microspheres. Gradients of different growth factors are used as signaling cues by developing axons to reach their target. This part of the study had two aims.

The first was to evaluate the scope of using CUPE as a nerve conduit material across a 5 cm gap in a rabbit common peroneal nerve model. The second was to evaluate the effect of pleiotrophin released in a uniform or gradient manner in bridging a 5 cm gap.

## 2.2 Methods

### *2.2.1 PTN Microparticles*

#### 2.2.1.1 Fabrication, Characterization and release studies

The PTN microparticles used in this study were prepared and characterized in our lab previously (Lotfi 2011). Briefly, polylactic-co-glycolic acid (PLGA) microparticles were synthesized using the double emulsion (water-in-oil-in-water) evaporation method. The drug solution was prepared using PTN (PeproTech Inc., Rocky Hill, NJ) at concentration of 10 µg/ml (water phase –w1). The oil phase of the emulsion was prepared by dissolving PLGA (Lakeshore Biomaterials, Birmingham, AL) in dichloromethane (DCM; Sigma-Aldrich, St.Louis, MO) at a concentration of 200 mg/ml (phase-o). Finally 0.2g of PVA (Sigma) was dissolved in 20ml DI water to make an aqueous PVA solution (water phase- w2). The drug solution was added to the PLGA solution and vortexed carefully for 30 seconds (water-in-oil). The above prepared solution was then added to the aqueous PVA solution (water-in-oil-in water) and emulsified by vortexing for 2 minutes. The mixture was stirred for an hour to evaporate DCM (solvent evaporation). The solution was then centrifuged at 4000 rpm for 15 min to pellet the particles. The particles were resuspended in 10ml PBS and freeze dried for storage. The overall shape of the particles were analyzed using the scanning electron microscope. Images of microparticles sputter coated with a thin layer of Ag were taken in high pressure using a variable SEM machine (Hitachi S- 3000N; Hitachi High Technology America Inc., Pleasanton, CA).

A release profile of the PTN particles was obtained using an ELISA kit obtained from Invitrogen. The lyophilized microparticles were weighed and 1mg of it was taken and dissolved in 1ml of PBS in a 1.5ml tube. The tubes kept in a shaker incubator at 37 °C for 28 days. At predetermined time points (1, 2, 4, 8, 24, 38, 96 hrs, and weekly thereafter for 4 weeks), the

supernatants were collected and the particles were resuspended in fresh PBS solution. The collected supernatants were kept frozen in -20°C upon usage. PLGA microparticles loaded with BSA were used as controls and their release measured by standard protein assay methods (BCA Assay, Thermo Scientific, Rockford, IL) and 562 nm spectrophotometry.

#### 2.2.1.2 Biological activity in vitro

Cortical neuron culture using deeply anaesthetized neonate mice (postnatal day 3) (Charles Rivers, Wilmington, MA) were collected under sterile conditions and kept in cold Hank's balanced solution (HBSS; Hyclone, Waltham, MA) supplemented with 1% penicillin/streptomycin. Then the tissues were transferred to a solution (0.25 % trypsin diluted in L-15), added to the pellet and kept at 37°C for 30 minutes. Afterwards, a flamed tip glass was used to fragment the tissue. After dissociation, cells are precipitated by centrifuging at 1200 gs (rcf) for 5 minutes. Later, cells were seeded in 8 chamber slide plates (Lab-Tek™, Thermo Scientific, Rochester, NY) and kept in Neurobasal medium complemented with 1% B-27 and L-glutamine and penicillin/streptomycin (Gibco, Carlsbad, CA). All the cell cultures were kept in normal condition at 37°C and 5% CO<sub>2</sub>. PLGA-PTN microparticles (3 mg) or BSA encapsulated PLGA microparticles (control; 4 mg) were added to cell culture media and kept for 3 days. Cells were fixed after 3 days and immunohistochemically stained with  $\beta$ -tubulin (mouse anti-  $\beta$ -tub, 1:500; Sigma, St.Louis, MO) to be visualized using confocal fluorescent microscopy.

### 2.2.2 PTN Fibers

#### 2.2.2.1 Fabrication and release studies

Poly-lactic-co-glycolic acid (50:50 DLGA 4A; Lakeshore Biomaterials) coiled fibers were fabricated using wet-spinning. Briefly, a solution 20 wt. % PLGA was dissolved in dichloromethane (DCM; Sigma-Aldrich, St. Louis, MO) and was allowed for complete dissolution. The polymer solution was loaded into a 5ml glass syringe (gas-tight syringe; Hamilton) and placed in a programmable syringe pump (New Era Pump Systems, Farmingdale, NY) as is described in other publications (Nelson, Romero et al. 2003). The syringe was

connected to a bent needle inserted through a gas-tight cork into a glass tube. The polymer solution was injected through the glass tube into the coagulation bath containing isopropanol to form the fiber. The spinning solution injection rate was kept constant. The coagulated fibers were collected using a pair of forceps and dried around a steel rod over night to let the DCM dry out. PLGA solution formulations containing the nerve growth factor (NGF; 5 µg/ml; Invitrogen, Carlsbad, CA) or BSA (20 mg/ml; Sigma-Aldrich, St. Louis, MO) or pleiotrophin (PTN; 4 µg/ml; Peprotech) were prepared by adding the required amount of growth factor. The PLGA fibers were wrapped around slides and stored in freezer till usage.

PTN release from the fibers was estimated using release profile of NGF loaded fibers. Briefly, NGF release was obtained using an ELISA kit obtained from Invitrogen. The fibers were cut into length of 5, 10 and 20 cm and dissolved in 1ml of PBS in a 1.5ml tube. The tubes kept in a shaker incubator at 37 °C for 28 days. At predetermined time points (1, 2, 4, 8, 24, 38, 96 hrs, and weekly thereafter for 4 weeks), the supernatants were collected and the particles were resuspended in fresh PBS solution. The collected supernatants were kept frozen in -20°C upon usage. PLGA fibers loaded with BSA were used as controls and their release measured by standard protein assay methods (BCA Assay, Thermo Scientific, Rockford, IL) and 562 nm spectrophotometry.

#### 2.2.2.2 Bioactivity evaluation of PTN

Postnatal (P3) mice were anesthetized hypothermically. Each pup was dissected and their dorsal root ganglions (DRG) were collected and placed on 35 ml plate with 1.5ml of L-15 and then transferred to 15ml centrifuge tube. To the tube 400µl of 1% type IV collagenase and 10µl/ml of DNase was added and placed in an incubator set at 37°C for 40 min. Later, the tube was centrifuged for 5 minutes, the supernatant removed and 2ml of 0.25% trypsin (diluted in L-15) was added. The tube was then placed in an incubator set at 37°C for 15min. To this 2ml of L-15 and SBTI trypsin inhibitor was added, dissociated the DRG and centrifuged for 5 minutes. One ml of resuspended cells were added in 2ml of Neurobasal medium, placed on an 8 well

plate and cell counted. The cells were allowed to grow for 24hrs. After 24 hours, complete media was removed and replaced with serum-free media for 2hours to starve the cells. After 2 hours of starvation, pleiotrophin (100 ng/ml; Peprotech) or PTN loaded fibers (5cm) was added to 3 wells. In two wells, no treatment was added as they were used as controls. Cells were fixed after 3 days and immunohistochemically stained with RT97 (mouse anti- RT97, 1:200; Sigma, St.Louis, MO) and GFAP (rabbit anti-GFAP, 1:1000) to be visualized using fluorescent microscopy.

### *2.2.3 Preparation of Conduits*

In order to mimic the multifascicular anatomy and increase the surface area of the nerve conduit, the BNI previously designed in our lab was used in this study. The design of the BNI helps to linearly restrict the path of regenerating axons through multiple agarose microchannels filled with collagen. With the help of a casting device, CUPE or microrenathane tubes (Braintree Scientific, Inc., MA; OD 2.4mm, ID 1.7mm, and length 50mm) were used to fabricate the BNIs. The tubes were manually perforated to increase nutrient and gas exchange. The tubes were then sectioned into 50 mm sections, rinsed with DI water, soaked in 70 % ethanol and left inside a sterile laminar flow hood under UV light overnight.

The casting device comprised of a loading well, end plate and a brush made of metal fibers (350µm and 500µm). The brush was inserted through the tube guided by holes made on the well and endplate. Using a 30G needle, 1.5 wt.% agarose was injected into the tube then allowed to polymerize. After the agarose cooled and solidified, a suspension of PTN microparticles mixed with collagen was added to the loading well. The subsequent removal of the metal fibers generated negative pressure, drawing the mixture into the BNI having a volume of 44µl. After all the embedded fibers were removed, the end plate and well was removed and the BNI was placed in a petridish with a small amount of 1X PBS. The time between completion of BNI and implantation inside the animal was kept under less than half an hour.

Three groups of BNIs were fabricated: pure collagen, 2.5 mg/ml PTN microparticles, and a 5 cm PTN fiber. The fabrication process of the BNI group having a PTN loaded fiber was a bit different. In this group, one of the 350µm metal fiber had a 5cm PTN loaded PLGA fiber wrapped in a gradient manner at the distal end before it was inserted into the tube. This was performed under sterile conditions.

#### 2.2.4 Animals

Nine female New Zealand white rabbits (2-2.9 kg) (Myrtle Rabbitry, Thompson, TN) were chosen for these experiments. The animals were divided into three treatment groups with a sample size of three in each group (Table 2.1)

Table 2.1: Design of different groups in the pilot study

Group 1(n=3)	BNI made of CUPE filled with collagen
Group 2(n=3)	BNI with PTN Microparticles
Group 3(n=3)	BNI with PTN fiber(gradient) + soluble PTN

The animals were maintained under conditions of controlled light and temperature. Food and water were available ad libitum. Institutional Animal Care and Research Advisory Committee regulations were observed for surgical, behavioral, and care procedures.

#### 2.2.5 Surgical Implantation

For all the surgical procedures, the animals were anesthetized with an intramuscular injection of 35 mg/kg ketamine and 5 mg/kg xylazine. Then the left thigh was shaved and cleaned with 70 % ethanol and betadine. Anesthesia was maintained with 1-3% isoflourane and oxygen. Through a muscle-sparing incision, the left peroneal nerve was exposed along the sciatic vein between the semitendinosus and the bicep muscles. Using a muscle retractor, the



muscles were gently spread to expose the proximal part of the undivided peroneal nerve. The nerve was then bisected and a small segment of the nerve was cut to accommodate the BNI. The proximal and distal nerve stumps were placed inside the BNI and secured to the underlying muscle using resorbable sutures. After the BNI was implanted, the muscles were then closed over the nerve and sutured together with 3-0 chromic gut sutures, the skin was stapled together and triple antibiotic ointment was applied to the wound. Using gauze and adhesive bandages, the wound was wrapped. The animal was taken off the 3% isoflurane and allowed to wake before it was returned to its cage.

#### *2.2.6 Post-operative Treatment*

For the first two weeks after surgery, the animals were given 1.5ml trimethoprim sulfamethoxazole orally and 1 mg of buprenorphine subcutaneously once a day. If the animals exhibited additional pain symptoms, pain medication was continued. After the first week, the bandages were changed and wounds were checked for any infection. At the end of the second week, the bandages and staples were removed. Three animals in the study showed signs of automutilation on the dorsum of the left foot. The wounds were cleaned and covered with topical antibiotic treatment before being rebandaged. Once the animal's outer wounds had healed, they were allowed to hop on a treadmill for 20 minutes and were given approximately one hour of exercise once every three days.

#### *2.2.7 Behavioural Testing: Toe spread*

Motor functional recovery was assessed by measuring toe spread reflex of the injured and uninjured hindlimbs. Here the animals were held by the loose skin of the back and suddenly lowered in the air without letting them touch the surface. The distance between the animal's first and fourth toes was measured using a ruler. If functional reinnervation occurred, the animals spread their second and fourth toes reflexively in an attempt, to enlarge the surface area for safer landing. Six animals exhibited severe biting behavior on their injured hindlimbs. In order to

allow sufficient time for healing, toe spread was obtained in all the animals only at the 12<sup>th</sup> week.

#### *2.2.8 Assessment of Sensory Function Using Formalin*

The formalin test was used to assess the return of sensory function in the area innervated by the peroneal nerve. This test has been previously used in mice models, where formalin (irritant) is injected into the skin and pain/sensation is evaluated based on the licking response at the injection site. In the formalin test, 0.2 ml of 2 wt.% of paraformaldehyde in 1X PBS was subcutaneously injected on the dorsal side of the animal's injured foot between the two inner most toes. In each animal, the number of licks in the injured and uninjured feet was counted separately in a span of 10 minutes. Due to autotomy, four animals had to be excluded from this test.

#### *2.2.9 Measuring Isometric Contractile Force of Tibialis Anterior (TA) Muscle*

The measurement of maximal output force of a muscle innervated by the specific nerve after nerve repair is clinically pertinent. In this study, at the terminal stage we measured the isometric tetanic muscle force of the tibialis anterior muscle innervated by the deep peroneal branch of the common peroneal nerve (Figure 2.1). The animals were anesthetized using a mixture of ketamine and xylazine in the ratio of 5:1 according to their body weight. The site of the initial surgery was shaved again as needed. Keeping the medial side of the leg facing down, the distal femur and ankle of the animal were fixed to a wooden platform using stainless steel pins. Through a posterolateral incision in the thigh, the biceps femoris was separated from the gluteus medius and vastus lateralis. The left peroneal nerve was exposed, a bipolar hook electrode was clamped proximal to the implant. At the anterior aspect of the ankle, the skin was excised to expose the TA tendon. The distal TA tendon was secured with a hemostat and connected to a rod end (RE -125; Load Star Sensors) attached to the force transducer (MFM-010-050-A, Load Star Sensors, CA).

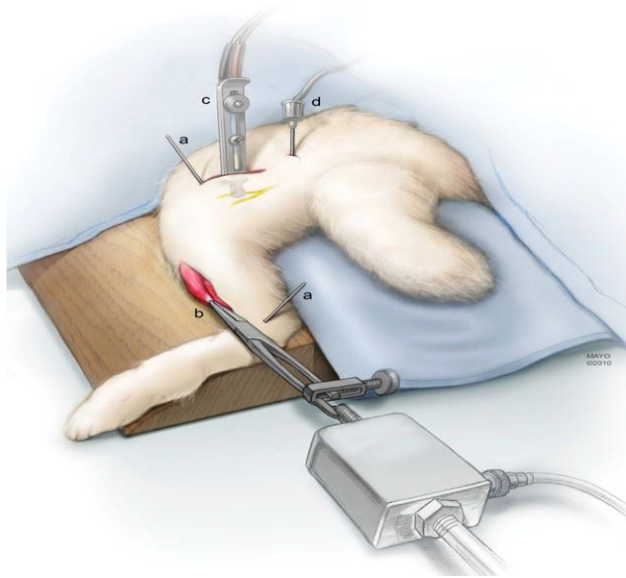


Figure 2.1 Schematic of the experimental setup for TA isometric twitch force testing. (a) The rabbit's femur and ankle were attached to the wooden block using metal pins. (b) The distal tendon was attached to the force transducer using a custom clamp. (c) A bipolar hook electrode was connected proximal to the conduit and (d) the rabbit was electrically grounded. Figure adapted from (Giusti, Kremer et al. 2012)

The anatomical position of the TA muscle was maintained throughout the test. Using an USB interface (DQ 1000U) the force transducer was connected to a laptop. The peroneal nerve was stimulated proximal to the conduit (Isolated Pulse Modulator, Model 2100, AM Systems) with increasing voltage until the maximum isometric single twitch force was obtained. For each muscle contraction, the frequency signal generated by the force was digitized and processed on a laptop using LoadVUE software (Load Star Sensors). Using the 'TARE' option in the software, any preload force on the cell was cleared before each measurement. The surgical procedure and testing process was repeated in the uninjured (right) side.

#### 2.2.10 Harvesting BNI and Muscle Tissue

Following the isometric force testing, the animals were overdosed with Euthasol (87 mg/kg). Through transcardial perfusion, approximately 500 ml of 0.9% of sodium chloride mixed with heparin was pumped into the body through the left ventricle and out the right atrium. This was followed by similar amount of 4% paraformaldehyde. After the animal was thoroughly fixed,

the BNI and peroneal nerve was harvested along with the TA muscles from both sides, and all tissue samples were immersed in a cold fixative solution containing 4% paraformaldehyde overnight. The samples were removed from the fixative the next day, placed in 1X PBS and stored at 4°C.

#### *2.2.11 Immunohistochemistry*

The harvested BNIs were cleaned and the outer tubing was cut to isolate the tissue. The tissue was divided into proximal, mid and distal sections which were then embedded in paraffin wax. Cross sections of 10µm were sliced from the mid sections using a Leica microtome, mounted on positively charged slides and labeled. The paraffin sections were heated in 60°C for 10 minutes, deparaffinized with two changes of xylene, rehydrated in descending grade alcohol, then washed in distilled water. Blocking solution made with 5% normal donkey serum mixed in 1% Triton X-100 (Sigma) in 1X PBS was added to the slides for 1 hour at room temperature. The sections were then stained with the following primary antibodies overnight at 4°C: mouse antineurofilament protein and rabbit S-100. The following day the sections were washed three times with washing solution for 10 minutes in between and incubated with secondary antibodies, Cy-2 conjugated anti rabbit and Cy-3 conjugated anti-mouse (Jackson Immunoresearch) for one hour at room temperature. DAPI was diluted in the ratio of 1:4000 and used as counter-stain. The slides were then washed, coated with mounting media and coverslipped for storage in 4°C.

#### *2.2.12 Statistical Analysis*

All the statistical analyses were performed with GraphPad Prism (Version 5, GraphPad Software, Inc.). The results are represented as the average ± standard error of the mean unless otherwise stated. Statistical differences were identified between two groups using paired two-tailed t-test and between two or more groups using One-way ANOVA followed by post-hoc Neumann Kohl's test. Differences were considered significant when  $p \leq 0.05$ , very

significant when  $0.001 \geq p \geq 0.01$  and extremely significant when  $p < 0.001$  denoted by \*, \*\*, \*\*\* respectively.

## 2.3 Results

### 2.3.1 PLGA-PTN microparticles

Figure 2.2 (a) shows the SEM picture of the PTN encapsulated PLGA microparticles prepared by the double-emulsion solvent evaporation method. The image shows that the particles are spherical and have a smooth surface. The mean diameter of the particle was 947nm (Figure 2.2 b).

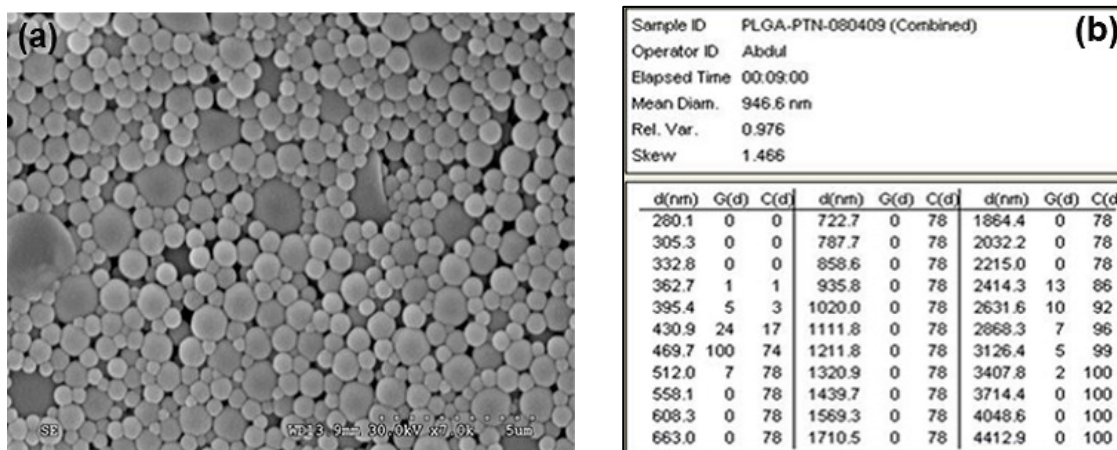


Figure 2.2 Characterization of PTN microparticles. (a) SEM image of the microparticles showing the spherical morphology (b) Size analysis of the microparticles showed that 74% of the particles had diameter 469nm or smaller (Lotfi 2011).

The release of the PTN-loaded microparticles is illustrated in Figure 2.3. PTN was released in two phases: an initial burst phase in which 40-55% of the PTN was released by day 5 followed by a plateau at the end of the study where 75% of the total PTN loaded was released.

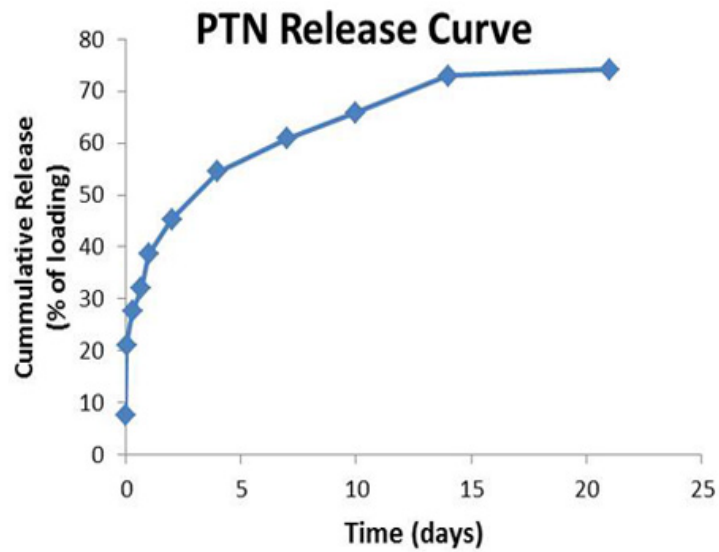


Figure 2.3 Release profile of PTN loaded microparticles (Lotfi 2011)

### 2.3.2 PTN loaded microparticles stimulated axonal growth

The biological activity of BSA and PTN loaded microparticles was tested using cortical neuron cell culture. It was observed that the culture supplemented with PTN microparticles had extensive growth. The average length of axons and average surface of the soma was significantly larger in the PTN group, as compared to BSA (control) (Figure 2.4).

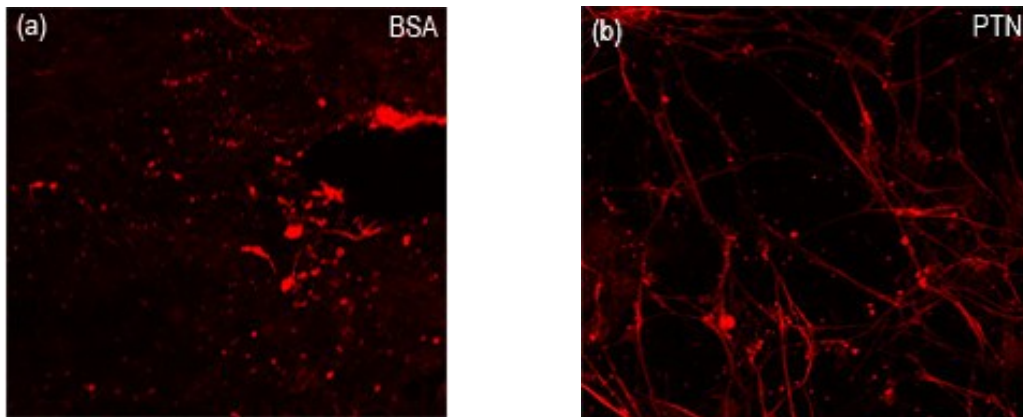


Figure 2.4 Dissociated cortical axons growing in the presence of PLGA-PTN microparticles had longer axons compared to PLGA-BSA microparticles (Lotfi 2011)

### 2.3.3 *In vitro* release kinetics of PLGA-PTN fibers.

The release kinetics of PTN loaded fibers was estimated from NGF fibers made in our lab using the same technique. The release profile *in vitro* for the NGF and BSA loaded fibers are illustrated in Figure 2.5. The results of the ELISA shows a burst release of NGF protein and by day 5, about 40% of the growth factor had been released. A plateau was reached later, and by day 14, 50% of the total NGF loaded had been released.

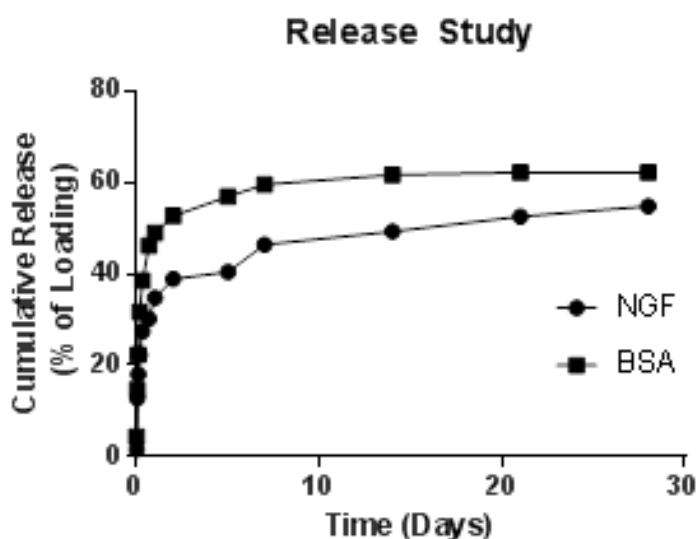


Figure 2.5 Release profile of NGF and BSA loaded PLGA fibers. The release shows that 54% is released in 28 days

### 2.3.4 *PTN stimulated longer axons compared to control*

The biological activity of PTN loaded PLGA fibers were tested using dissociated DRG cell culture. The number of neurons and average axonal length was quantified in each group. The neurons which had axons twice their cell body length was only used for quantifying axonal length. As shown in figure 2.6, the average length of axons was significantly larger in the PTN group compared to control. None of the neurons in the control group had any axonal processes. However, there was no significant difference in the number of neurons observed between the PTN fibers and control group (Figure 2.7).

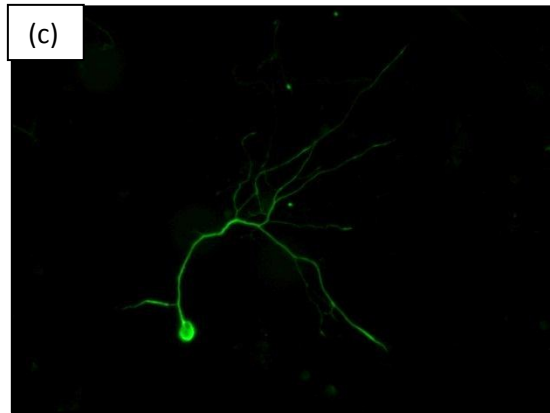
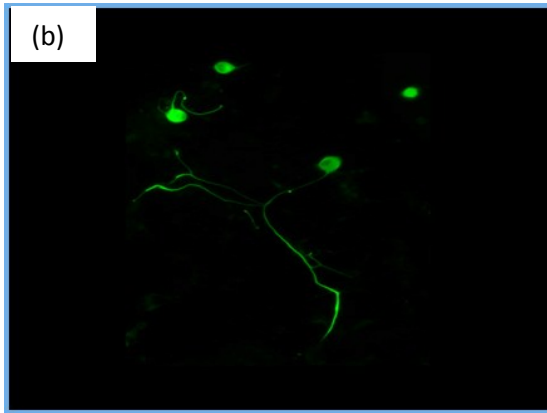
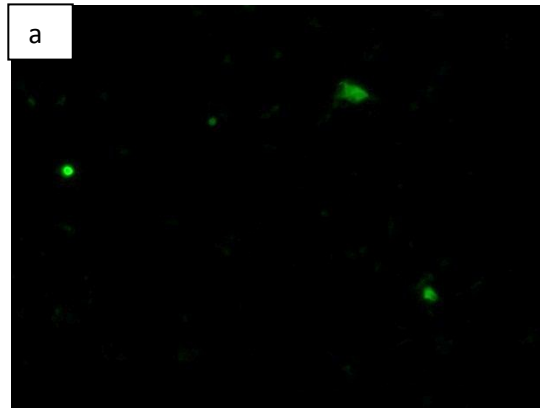


Figure 2.6 Dissociated DRG growing in media containing no PTN (control), PLGA-PTN fibers and soluble PTN. (a) Neurons growing in medium containing no PTN had no visualization of axons. (b and c) In contrast, those growing PLGA- PTN fibers and soluble PTN had long axons.



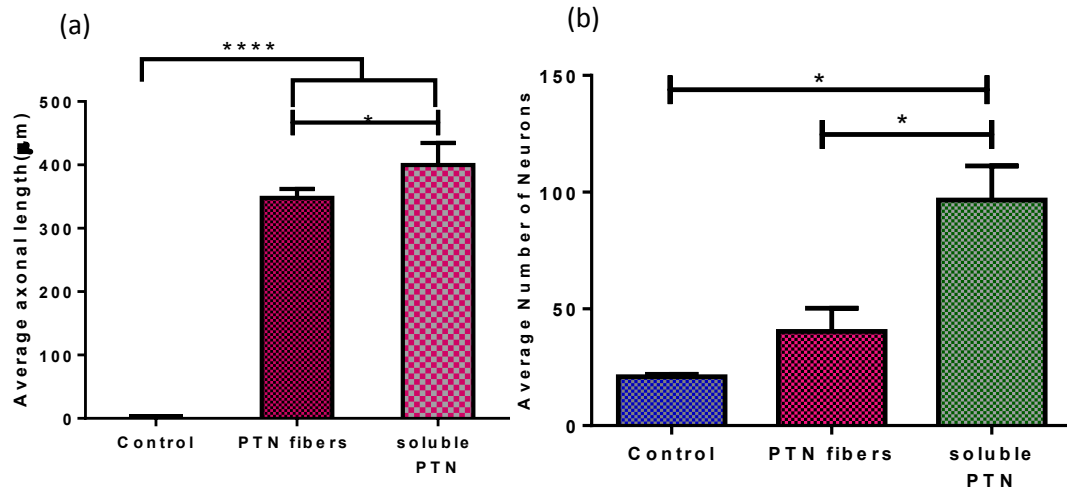


Figure 2.7 Dissociated DRG growth in response to PLGA-PTN fibers. (a) Neurons had longer axons in cultures containing PTN loaded fibers compared to control (b) Cultures containing PTN had significantly more number of neurons compared to control ( $p \leq 0.05$ ).

### 2.3.5 Architecture of luminal agarose maintained in the 5 cm CUPE BNIs

The tissue inside the three BNIs in the CUPE group was examined. Figure 2.8 (A) shows the CUPE BNI harvested at 12 weeks. Figure 2.8 shows the fluorescent microscope images of the mid sections stained with NFP-200 and DAPI. No positive staining for the neurofilament proteins or DAPI was observed. In all three of the CUPE BNIs, the structure of the agarose luminal filler was maintained better than in the microrenathane BNIs. Though some of the microchannels were disrupted, the overall structure of agarose was maintained.



Figure 2.8 Picture of a 5cm CUPE BNI harvested at 12 weeks

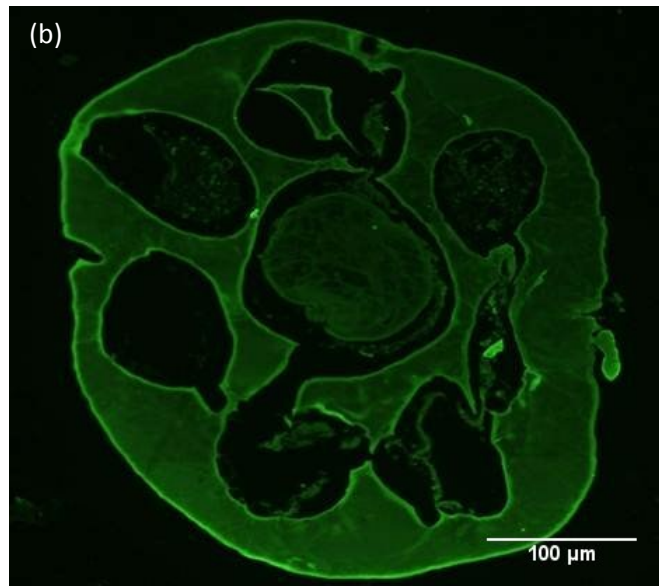
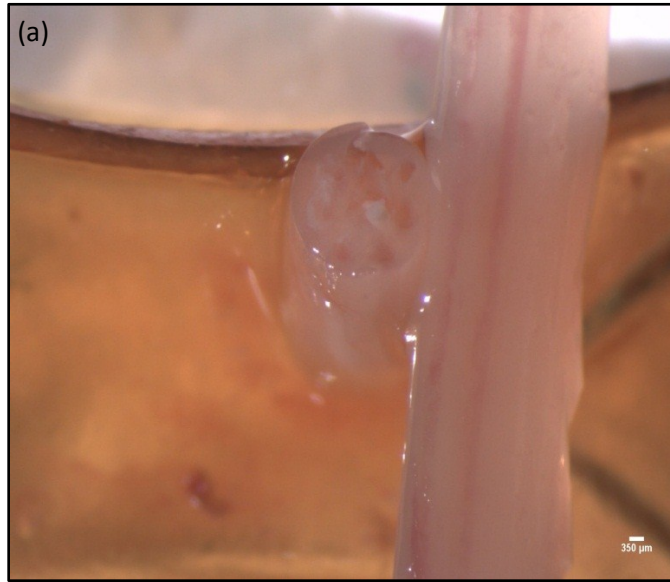


Figure 2.9 CUPE as a nerve conduit material in a BNI. (a) Luminal structure of agarose preserved inside the CUPE BNI. Mid-section from the CUPE BNI stained with NFP-200. No positive stain was observed

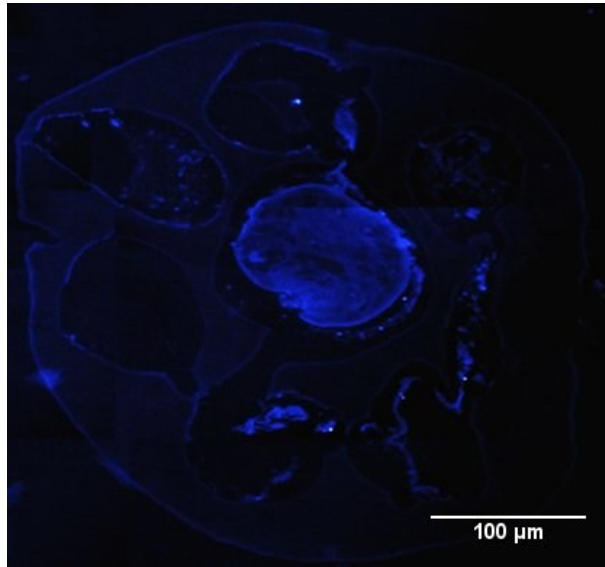


Figure 2.10 Mid-section from the CUPE BNI stained with DAPI. No positive stain was observed.

#### 2.3.6 Evaluation of nerve regeneration in animals treated with pleiotrophin-supported BNIs

Images taken twelve weeks post implantation showed no multifascicular like nerve tissue connected from the proximal stump to the distal stump in any of the injured animals treated with pleiotrophin-supported 5 cm BNIs. This was confirmed by histological and immunohistochemical characterization. Fluorescent images of the mid cross sections of the pleiotrophin-supported BNIs is illustrated in Figure 2.11 and Figure 2.12. No positive staining for the neuronal markers was observed in any of the BNIs; although very few positive DAPI stains were seen in 2 of the BNIs.

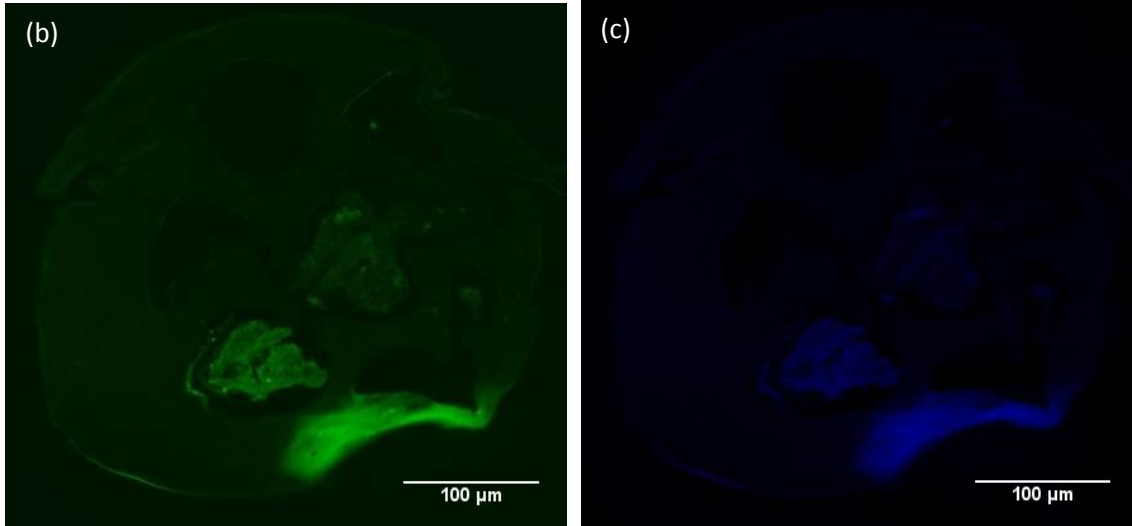
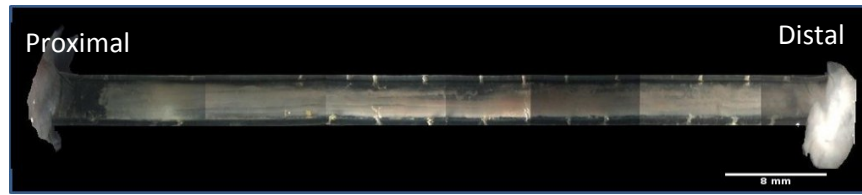


Figure 2.11 BNIs loaded with PTN microparticles implanted in the rabbit peroneal nerve model. (a) PTN microparticle loaded 5 cm BNI harvested at the end of twelve weeks post implantation. (b) Mid-section stained with NFP-200, showed no positive axonal signs of regeneration. (c) Mid-section stained with DAPI, showed no positive nuclei staining

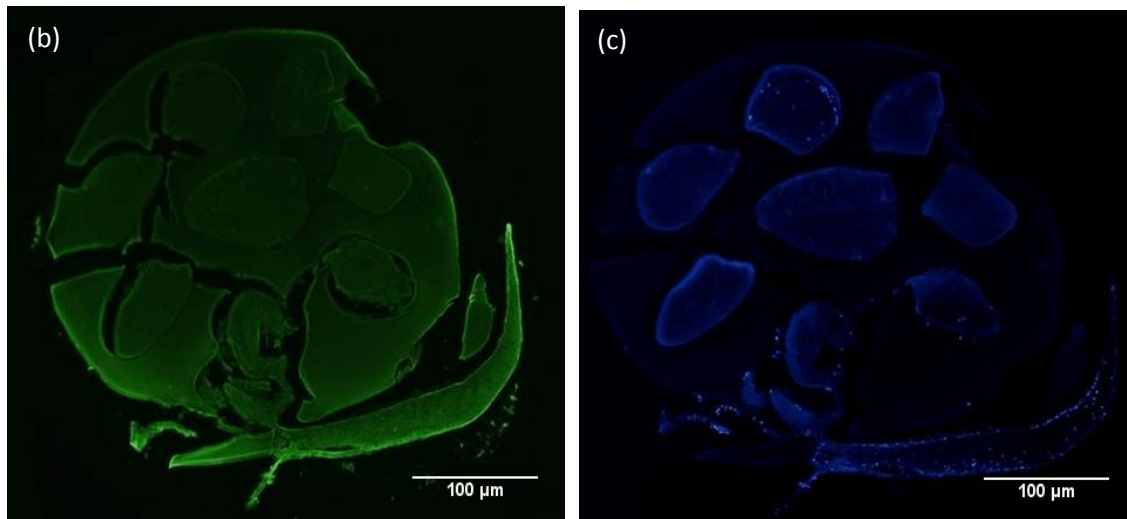


Figure 2.12 BNI loaded with 5cm PTN loaded finer and soluble PTN. implanted in the rabbit peroneal nerve model. (a) PTN fiber and soluble PTN loaded BNI harvested at the end of twelve weeks post implantation. (b) Mid-section stained with NFP-200, showed no positive axonal signs of regeneration. (c) Mid-section stained with DAPI, showed no positive nuclei staining in the microchannels, except for few stains in an outside layer.

In terms of regeneration, none of the explanted BNIs revealed regenerated nerve tissue across the 5 cm gap in the peroneal nerve; therefore, the injured limbs in all nine of the animals had been denervated for a period of twelve weeks. From this point, we went ahead and grouped the denervated (left) limbs in all the animals as injured and the unaffected (right) limbs as uninjured. The parameters of toe spread, sensory function, force and muscle weight were grouped and then quantified in this manner.

### 2.3.7 Toe Spread Analysis

The toe spread reflex was assessed in the injured and uninjured hindlimbs during the 12<sup>th</sup> week to measure motor function. The extent of toe spread is approximately the same in both hindlimbs in case of uninjured animals. At the end of twelve weeks, none of the animals regained their toe spreading function in the injured (denervated) hindlimb as indicated by the significantly lower toe spread values as compared to the uninjured hindlimb values (Figure 2.10).

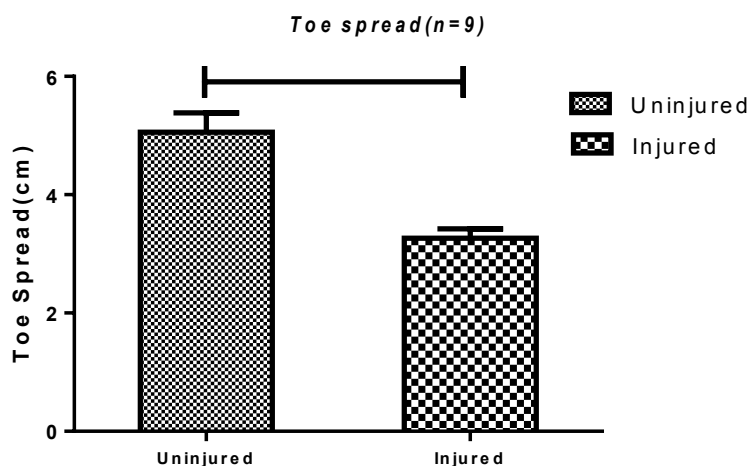


Figure 2.13 Comparison of the toe spread between uninjured and injured hindlimb at week 12. There is a significant reduction in toe spread in the denervated hindlimb ( $p=0.0002$ )

### 2.3.8 Sensory Function Analysis

The formalin test was used to assess the animal's ability to sense and respond to an irritant on the dorsal surface of the foot. On evaluating the licking response between the injured and uninjured hindlimb, significantly lower number of licks was observed in the injured hindlimb (Figure 2.11). The lack of innervation in the tested areas indicated a loss of sensory function in the injured hindlimb. A two-tailed paired t-test of the number of licks between injured and uninjured hindlimbs gave a p-value of 0.0108.

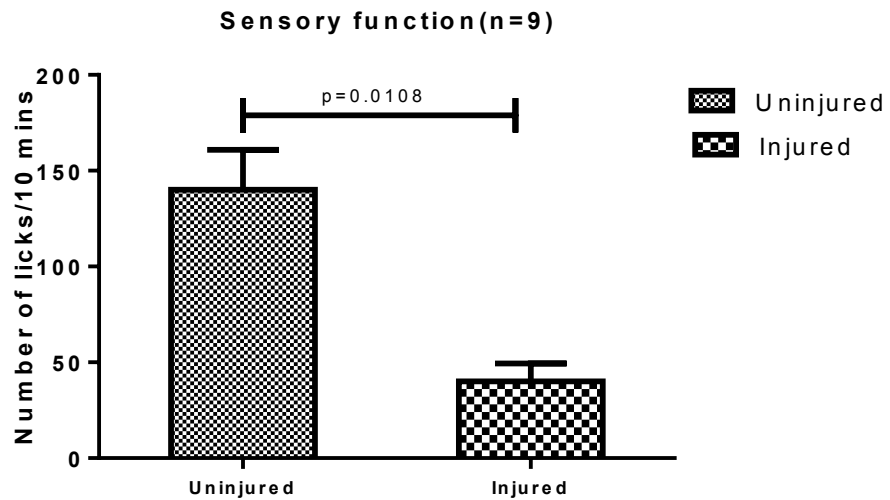


Figure 2.14 Comparison of animal's toe lick response between injured and uninjured hindlimb. The animal's response to stimulus was significantly reduced in the injured hindlimb ( $p=0.0108$ )

### 2.3.9 Isometric Contractile Force Analysis

The maximum output muscle force of the TA muscles in both the hindlimb was assessed to measure effective functional outcome. In the injured hindlimb, the force of TA muscles was significantly lower as compared to the uninjured hindlimb for all animals (Figure 2.12). Due to prolonged denervation over 3 months (12 weeks), the muscle fibers atrophied, and the motor potential of the TA muscles was considerably reduced

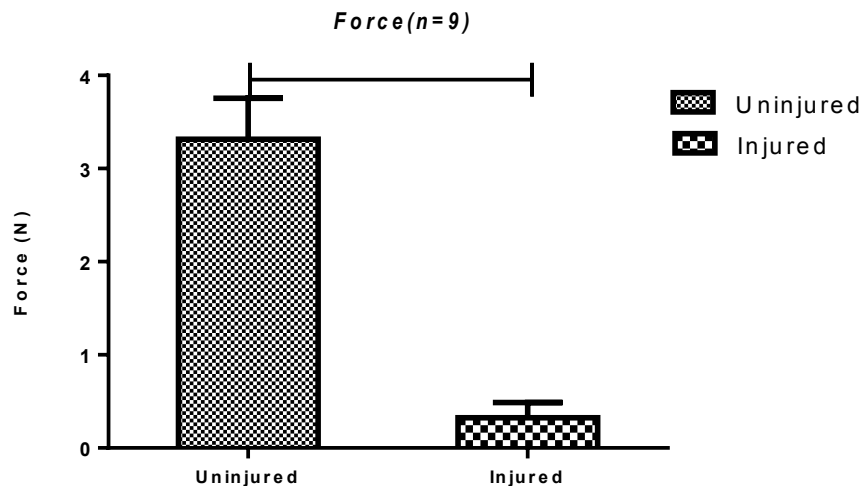


Figure 2.15 Comparison of Twitch muscle of TA muscle in uninjured and injured hindlimb. The force generated in the injured hindlimb was significantly reduced ( $p=0.0002$ ).

### 2.3.10 Muscle weight Analysis

Measurement of muscle weight was done at the end stage of the study. Figure 2.13 shows graphically the difference in muscle weights harvested from the injured limb (left) and its contralateral uninjured side(right) in the TA muscles from all treatment groups. The lack of functional innervation for a period of 12 weeks resulted in significant atrophy in the injured hindlimb. This loss in muscle mass of the TA muscle could be observed peripherally over the skin in the injured hindlimb in all the animals.

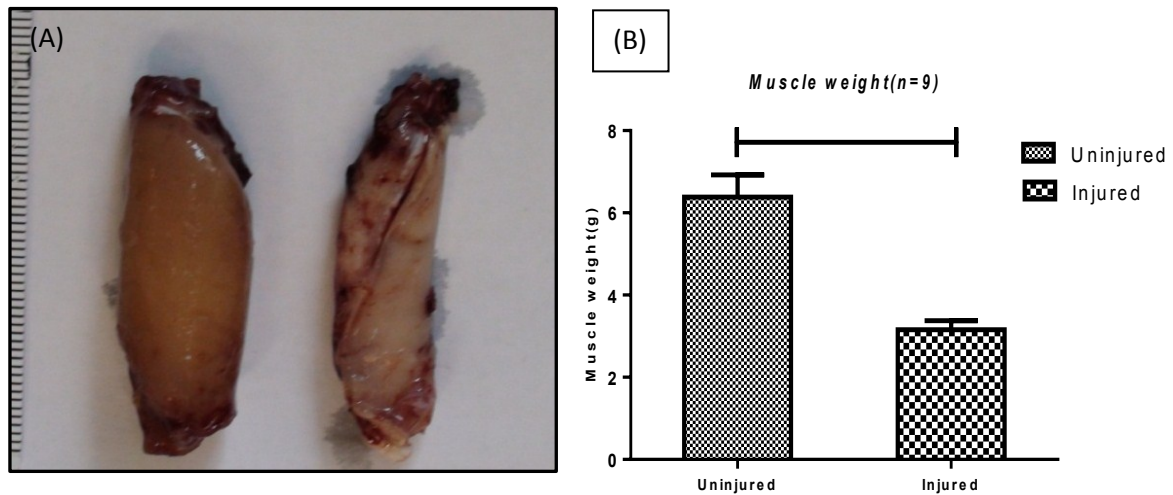


Figure 2.16 Comparison of the difference in TA muscle weights between uninjured and injured hindlimb. (A) Picture of the uninjured and injured TA muscle. (B) The muscle weight in the injured hindlimb had significantly reduced ( $p < 0.0001$ )

## 2.4 Discussion

Research in the field of nerve regeneration and biomaterials have led to improvements in managing peripheral nerve lesions. The choice of biomaterial is an important step in the development of an ideal nerve conduit. Different biodegradable synthetic materials such as poly(L-lactic acid) (PLLA) (Wang, Mullins et al. 2009), poly(glycolic acid) (PGA) (Waitayawinyu, Parisi et al. 2007), polycaprolactone (PCL) (Mligiliche, Tabata et al. 2003), and their copolymers have been extensively used as biomaterials for neural scaffolds. All the currently FDA approved nerve tubes, have the basic design of a hollow tube. The natural



regenerative processes occurring within a hollow nerve guidance conduit has been observed up to a critical nerve gap of around 4 cm in humans and around 1.5cm in a rat sciatic nerve model, after which regeneration is restricted (Daly, Yao et al. 2012). Previous studies in our lab have shown successful regenerative outcomes using a multiluminal nerve conduit (biosynthetic nerve implant) as compared to hollow tubes (Tansey, Seifert et al. 2011) across a 10 mm gap in a rat sciatic nerve model.

This study evaluated the capability of CUPE to serve as a nerve conduit material in a BNI across a 5 cm gap in the rabbit common peroneal nerve model. The results showed that the CUPE BNIs were able to maintain the overall framework of the agarose channels, though few channels were slightly disrupted. Agarose microchannels in the BNI help in restricting axonal dispersion and provide linear topographic cues to support unidirectional axonal regeneration. The BNIs made of biodegradable and translucent CUPE, a soft elastomer, were able to support the hydrogel microchannels and withstand the mechanical tension across a 5 cm gap, without collapse or losing their shape. The unique mechanical properties of CUPE include tensile strength of  $41.07 \pm 6.85$  MPa with corresponding elongation at break of  $222.66 \pm 27.84\%$  (Dey, Xu et al. 2008). This likely helped the CUPE BNIs withstand the constant longitudinal and perpendicular stresses along the nerve bed. Future nerve repair studies need to further inspect the mechanical strength of the conduits pre and post implantation. However, no signs of axonal regeneration were observed across the CUPE BNIs. This could be attributed to the absence of essential neurotrophic support across the 5 cm gap.

The apparent disadvantages of using autografts have led to renewed interest in developing peripheral nerve conduits for long gaps. Studies have shown a lack of trophic support or chemotactic gradient as one of the limiting factors for regeneration across a long gap. Exploiting the principle of neurotropism, exogenous growth factors applied at the site of nerve lesion have shown potential for improving nerve repair across critical gaps (Novikov, Novikova et al. 1997; Mi, Chen et al. 2007). Previously, our lab has shown BNIs supplemented

with PTN microparticles to be effective in bridging a long (3 cm) gap in the rabbit peroneal model (Nguyen 2010). Here, we designed two groups with the aim of evaluating the effect of pleiotrophin (PTN), in bridging a 5 cm long gap in a rabbit common peroneal nerve model. In the first group, PLGA microparticles were chosen as the delivery system, as a controlled release of growth factor would be more effective across a long gap than localized placement of them in the lumen of the tube. Each BNI had microchannels filled with collagen and PTN microparticles at a concentration of 2.5mg/ml. This concentration was chosen as it was found that concentration higher than 2.5 mg/ml blocked the channels, restricting regeneration. Quantification of the amount of PTN gave 11.1474 ng of PTN in each BNI which was one tenth the required amount. Based on the in vitro release profile of PTN microparticles, 8.36 ng of PTN released by day 20 with an initial burst release of 6.131 ng by day 5. Assuming an average growth rate of 2 mm/day, the amount of PTN loaded in the BNI channels and the release kinetics did not yield a concentration of 100 ng/ml needed to maintain biological activity across the 5 cm gap for a minimum of 24 days required to bridge the gap.

In the second group, each BNI had microchannels filled with collagen and soluble PTN at concentrations of 100ng/ml, and one outer microchannel had a 5 cm PTN-loaded PLGA fiber wrapped with more coils toward the distal side to create a gradient. Fibers could not be wrapped in the other channels, due to collapse of agarose while removing the needle during BNI fabrication. Since 4.4 ng of soluble PTN gets used up almost immediately, only the PTN-loaded (5 cm) fiber provided supply of PTN in the BNI. The 5cm PLGA-PTN fiber had 133 ng of PTN loaded, from which there was initial burst release of 53 ng by day 5 and 71.82 ng by day 28. The release of the 5 cm PTN fiber in one of the microchannel and the lack of PTN in the other microchannels was insufficient to provide 100 ng/ml across the 5 cm gap for a minimum of 24 days required to bridge the gap. At the end of the 12 weeks, no functional regeneration was observed in any of the rabbits in either groups. The histological results supported this gross observation by the complete absence of fascicular-like tissue in the middle portions of the BNIs

in both groups. Prolonged denervation (12 weeks) led to significant muscle atrophy and reduced toe spread, muscle weight and sensory function in the injured hindlimb, as compared to the uninjured control.

This lack of nerve regeneration across the 5 cm gap in the six animals provided with exogenous pleiotrophin is possibly explained by insufficient chemotactic cues, presence of an intact fibrin cable or growth substrate across the large distance between the proximal and distal stump. Following axonal disruption, macrophages and Schwann cells (SC) at the distal stump secrete cytokines and growth factors which are vital for axonal regeneration and remyelination (Perry, Brown et al. 1987). During Wallerian degeneration, Schwann cells proliferate and play an essential role as a source of neurotrophic factors. These factors diffuse across the injury area and exert a trophic effect on the regenerating axons from the distal stump. Lundborg et al. has shown the influence of gap length on growth promoting influence of distal stump components (Lundborg, Dahlin et al. 1982). They reported regeneration across 6 mm and 10 mm long silicon chambers, while no regeneration occurred in 15 mm chambers in proximal-distal system. It was stated that overdilution or limited diffusion of distally secreted factors within longer gaps, might be the reason for absence of axonal growth. Sinis et al. has shown that the proliferative activities at the proximal stump are highest when located close to the distal stump, due to secretion of growth promoting distal stump factors (Sinis, Haerle et al. 2007). A large distance between the nerve stumps provides ineffective chemotactic gradients across the gap, thereby aborting any regenerative activity across the gap. A number of studies have provided evidence for limited or absent regenerative potential across long gaps (Lundborg, Dahlin et al. 1982; Diaz-Flores, Gutierrez et al. 1995). The silicone chamber model studies across a 10 mm gap in a rat model has shown that it take 5-7 days to form a complete bridge of fibrin matrix across the chamber gap, along which cellular migration into the gap occurs. Across a 5 cm long gap, this process would take longer time, and by the time Schwann cells reach the middle of the gap, the formed fibrin cables could have dissolved. In our study, though exogenous supply of

PTN was provided it was not sufficient to maintain biological activity of PTN for at least 24 days, which is the minimum amount of time needed for the proximally regenerating axons to cross the 5 cm gap. This could be one of the possible reasons for no axonal regeneration. As more microparticles cannot be added to each channel, other methods of supplying PTN should be developed. Alternative solutions include increasing the amount of PTN loaded into the PLGA microparticles or decreasing the size of the particles formed. The difficulty in optimizing the release kinetics of PTN and the potential carcinogenic effect of PTN (Jin, Jianghai et al. 2009) complicates the amount of PTN that can be loaded in each BNI. Use of double-walled microspheres or nanospheres for growth factor delivery would lead to varying delivery times, as the surface area of the polymer matrices will influence the growth factor release. The use of exogenous fibrin matrix precursors inside the BNI might stimulate the matrix formation crucial for cell migration into the gap, hence increasing the temporal progress of regeneration across a long gap.

In the case of larger nerve gap repairs, flexibility of the nerve tube is important as the nerve ends might not be in the same plane or the gap that needs to be bridged may be across a joint (de Ruitter, Malessy et al. 2009). Peripheral nerves which may cross over joints at the hip, knee, ankle, shoulder, elbow and wrist experience mechanical loads during limb movement and motion (Bueno and Shah 2008). Nerves at these joints need to bend and stretch to accommodate the changes during movement. In three of the microreticulated BNIs, the agarose channels were compressed, possibly indicating excessive pressure on the BNI caused by bending and stretching of muscles in such locations. Hence, nerve conduits developed for long gap nerve repairs need to be resistant to a combination of forces and easily fit to the body movements to avoid nerve guide detachment from the implant site.

## CHAPTER 3

### KINEMATIC ANALYSIS IN RABBIT COMMON PERONEAL NERVE INJURY MODEL

#### 3.1 Introduction

In order to evaluate the repair of axonotmesis and neurotmesis injuries in an in vivo animal model, different regeneration parameters have been used. Electrophysiological and histological methods are commonly used to assess nerve repair outcome, but they may not necessarily correlate with function (Schmitz and Beer 2001). The complete restoration of normal function is considered the end point of regeneration. In most peripheral nerve repair studies, functional recovery still remains limited at the end stage of the study, and hence, these results cannot be translated to clinical studies. In an experimental animal model for peripheral nerve injury or spinal cord injury research, choice of the correct functional assay is a question of profound research interest.

After a period of long-term denervation, the number of axons that can successfully regenerate through the deteriorating intramuscular nerve sheaths is significantly reduced, which lead to decreased muscle force capacity. Gradual muscle atrophy and changes in the myofibers disconnect the communication between skeletal muscle and fibers. Variance in gait pattern is one of the most significant observations after peripheral nerve injury (Varejao, Cabrita et al. 2003; Luis, Amado et al. 2007). Dysfunctional or reduced sensory endings and neural and mechanical disruption are some of the limitations in studying functional recovery after peripheral nerve repair which have led to the increased use of biomechanical models. In the commonly used rat model, the Sciatic Functional Index (SFI), measured using walking track analysis, is a standard technique used to evaluate the combination of motor and sensory recovery (de Medinaceli, Freed et al. 1982). Though this technique measures the overall function of the

affected foot, it does not consider the dynamic limb function (gait). Computerized gait analysis is more accurate and consistent in evaluating the changes after nerve injury or repair.

The overall gait requires coordinated function involving sensory input, cortical integration and motor response (Dellon and Mackinnon 1989; Walker, Evans et al. 1994; Varejao, Meek et al. 2001). Biomechanical research methods can be used to understand adaptive or compensatory changes in motor activity following peripheral nerve injury and repair. Different approaches have been used to analyze gait, one of them being: subdividing the gait cycle according to variations in reciprocal floor contact by the two feet. Here the cycle is divided into stance and swing phase, where stance begins at initial contact and ends at toe-off, while in swing phase, the foot is off the ground. A number of gait parameters have been analyzed using ankle joint motion analysis in nerve repair studies using a rat model. In 1995, Santos et al., first used ankle joint kinematics to evaluate ankle angle during the swing phase after a crush injury of the peroneal nerve in a rat model (Santos, Williams et al. 1995). This was measured by using two intersecting lines joining the knee and ankle and the fifth metatarsal head to the ankle. Recently, Varejao et al. measured ankle motion during stance at predefined events of the gait cycle such as initial toe contact, opposite toe-off, heel rise and at toe-off (Varejao, Cabrita et al. 2002). Using digital 2D video analysis of rat gait different gait parameters which include ankle angle at different phase, walking speed stance factor, step length, tail height, midline deviation and tail deviation have been assessed to evaluate dynamic limb function (Yu, Matloub et al. 2001). Angle at mid swing and midline deviation contributed significantly to peroneal functional index in a rat model after peroneal injury (Yu, Matloub et al. 2001).

In Chapter 1, we illustrated the need to study the effects of a longer nerve graft substitute, for which a larger animal model than a rat must be used. Here, we used a rabbit animal model to study the effects of CUPE and pleiotrophin in bridging a 5 cm gap in the peroneal nerve. Toe spread reflex, formalin test, muscle force and muscle weight were used to evaluate motor and sensory parameters in these rabbits. To the best of our knowledge,

changes in rabbit gait after peripheral nerve injury or repair has not yet been studied. There is clearly a need to develop proper functional assessment methods in the rabbit animal model. As none of the BNIs showed positive sign of regeneration across the 5 cm gap (shown in Chapter 2), the injured limb remained denervated at the end of study phase. Here, we studied ankle angle and gait parameters in the injured and uninjured rabbit hindlimb during different phases of the rabbit hop. The mode of locomotion in rabbits is saltatorial (hopping), while their gait is quadrupedal.

## 3.2 Methods

### *3.2.1 Animals*

New Zealand white rabbits, weighing 2- 2.9 kg were used for this study. A total of 5 rabbits were taken from the previous study (Chapter 2). The animals were maintained under conditions of controlled light and temperature. Food and water were available ad libitum. Institutional Animal Care and Research Advisory Committee regulations were observed for surgical, behavioral, and care procedures.

### *3.2.2 Peroneal nerve injury*

As explained, in Chapter 2 Section 2.2.5 the animals had undergone a peroneal nerve transection and a 5cm BNI was placed between the two nerve ends and sutured. At the end of the study period (12 weeks), all the BNIs were analyzed for nerve tissue. The staining results using NFP-200 and S100 were negative in every group confirming permanent nerve injury in the operated limbs. As the left limbs of rabbits in all the groups remained denervated, we grouped them together and treated them as injured limb. The uninjured limb prior to BNI implantation was used as a control for baseline values.

### *3.2.3 2D digital video ankle motion analysis*

Ankle kinematics during different phases of the rabbit hopping gait was recorded prior to injury (week-0) and at the end of the study (week-12). In each rabbit, the left hindlimb was shaved prior to the BNI implantation surgery. Different color dots were placed on bony

landmarks with a permanent marker (Figure 3.1). The joints were identified by palpation while moving them. The knee, ankle and fourth metatarsal head were marked to create a 2D biomechanical model of the ankle in the rabbit. Filming was done in a properly lighted room by two observers using the Cine Plex Studio (v3 License: Recording, Tracking and Behaviour Features).

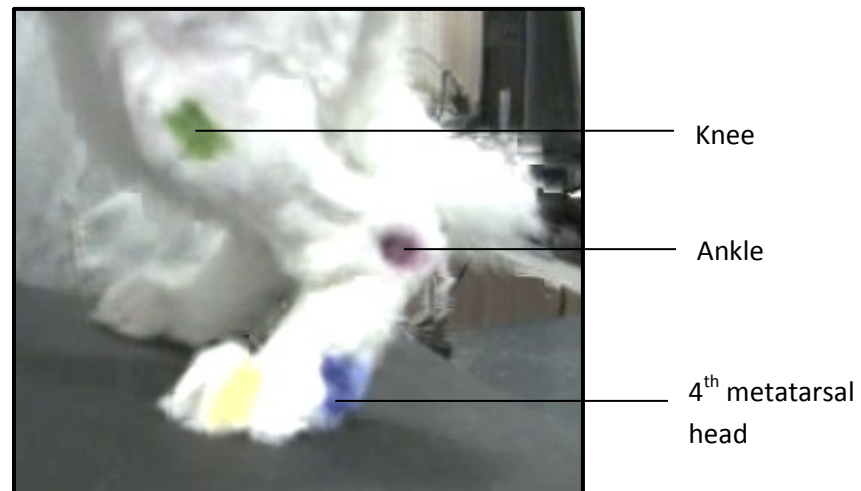


Figure 3.1 Skin landmarks were marked at three points: the knee, the ankle, and the fourth metatarsal head

Animals were placed on a motorized treadmill with a built-in eleven degree slope. Video was recorded using a stingray camera mounted on a tripod at 80 frames/second placed perpendicular to the treadmill. In the saltatory pattern of progression in a rabbit, the forelimbs show alternate rhythmical flexion and extension while the hind limbs retract and extend simultaneously followed by an aerial phase of movement. These phases were used to define a hop. After a few trial hops in each rabbit, they hopped spontaneously towards the upper slope of the treadmill. Hopping trials of approximately 30 hops were recorded for ankle motion analysis. Hops in which the hindlimbs had slipped off the treadmill during hopping and the rabbit had tilted to side of the belt during motion were excluded from the analysis. The recorded video was analysed using a computer on a frame by frame basis. Digital still images were captured and



analyzed using Image J analysis software. Ankle angle and Knee height was recorded at different event times during the gait cycle:

Toe off (TOF): the moment the left limb comes off the treadmill, the moment of maximum plantar flexion.

Mid-swing (MSW): the moment the left limb is in the middle of the aerial phase after limb extension, the moment of maximum dorsiflexion.

Touch-down (TOD): the moment the left limb touches the treadmill after terminal swing.

Ankle angle was measured in degrees using the angle tool in Image J. Foot drop was measured in the mid swing phase, as maximum dorsiflexion occurs at this time. Using Equation.1,

$$\text{Foot Drop} = \text{MSW}_{\text{inj}} - \text{MSW}_{\text{uninj}} \longrightarrow \text{Eq.1}$$

foot drop was calculated after peroneal injury. The time during swing phase, from toe off to touchdown was measured in seconds.

#### 3.2.4 Statistical Analysis

All the statistical analyses were performed with GraphPad Prism (Version 5, Graph Pad Software, Inc.). The results are represented as the average  $\pm$  standard error of the mean unless otherwise stated. Statistical differences were identified using paired two-tailed t-test. Differences were considered significant when  $p \leq 0.05$ .

### 3.3 Results

#### 3.3.1 Gait Analysis

The hopping gait of rabbit hindlimbs before and after peroneal injury was also analyzed (Figure 3.3). The denervated dorsiflexor muscles led to increase in mid swing ankle angle in all animals. Peroneal nerve damage presented significant foot drop symptoms in the injured limb of the rabbit. In contrast to the normal pattern of gait, high steppage gait is seen after injury (Figure 3.5). Specifically, the knee and thigh is lifted in an exaggerated fashion during the swing phase to avoid foot dragging and significant increase in knee height in the

injured hindlimb was observed (Figure 3.5). In one of the rabbits, there was uncontrolled slapping of the injured foot over the treadmill during every hop.

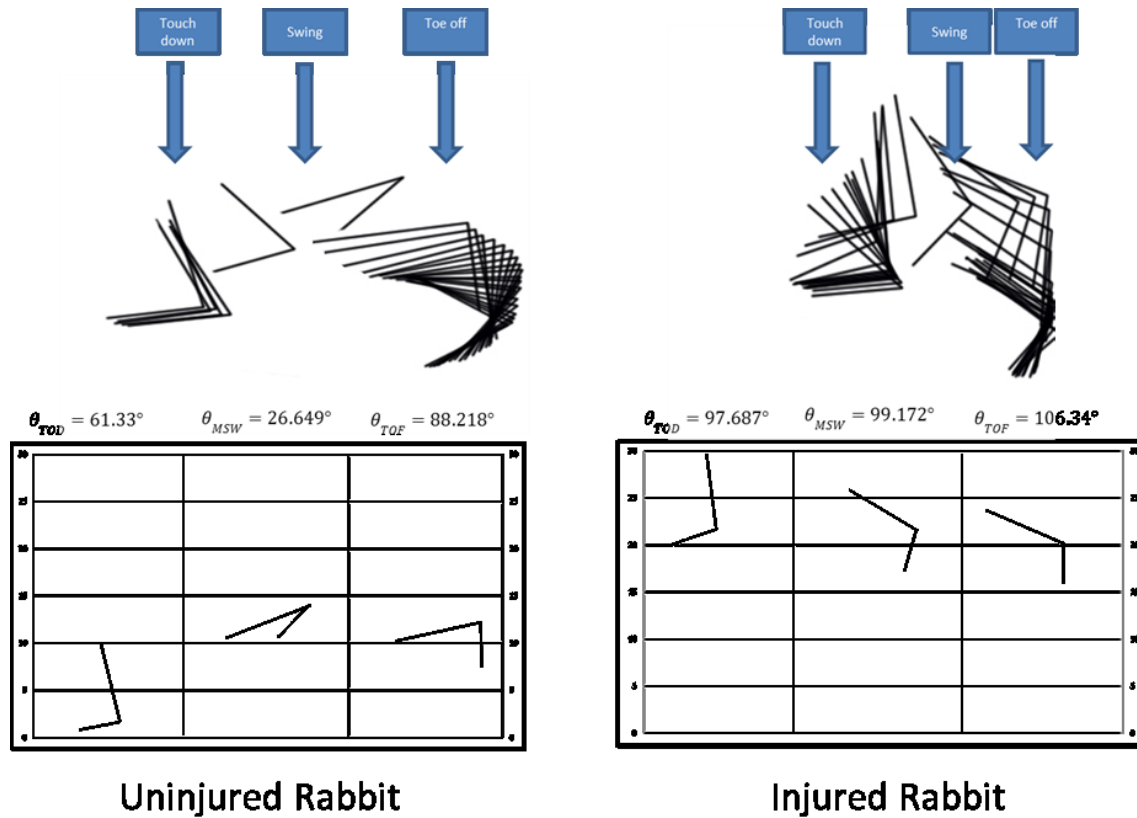


Figure 3.2 Ankle angle and knee height of injured and injured rabbit was evaluated during toe off, mid swing and touch down. Steppage gait symptoms were observed in the injured rabbit.

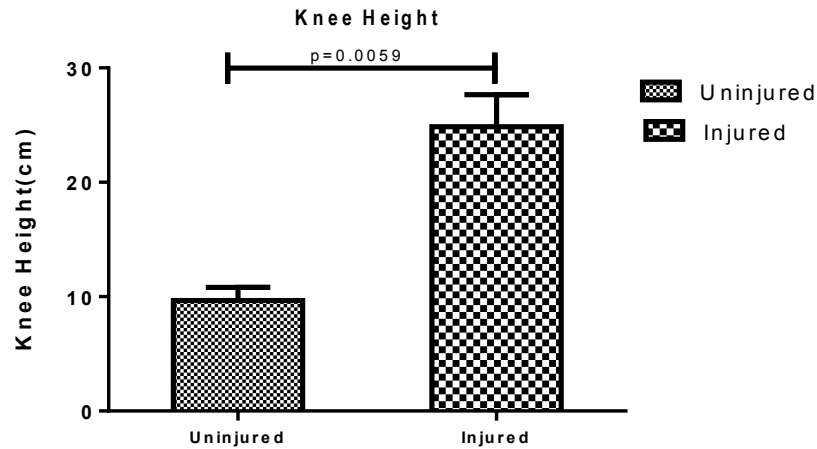


Figure 3.3 Relative knee height was compared between uninjured and injured limb. Significant difference was observed between two groups

### 3.3.2 Ankle Angle Evaluation at Toe off and Mid Swing

Adopting the model developed by Varejao and his collaborators, 2-D biomechanical analysis (sagittal plane) was carried out applying a two segment model of the ankle joint in the rabbit animal model. During toe off, the ankle plantar flexors (gastrocnemius, soleus, plantaris, tibialis posterior, flexor hallucis posterior, flexor digitorum longus) are active, while the ankle dorsiflexors (tibialis anterior, extensor digitorum longus, extensor hallucis Longus, peroneus tertius) are active during mid-swing phase. Hence the mid swing phase was chosen to analyze foot drop. As the plantar flexors were not much affected due to the injury, no significant difference in the toe off ankle angle was observed between uninjured and injured limb ( $p=0.2215$ ) (Figure 3.4). In clear contrast to the normal pattern of ankle motion during mid-swing phase, the injured limbs had a significantly higher ankle angle. ( $p=0.0091$ ) (Figure 3.5) Deficit in ankle motion due to peroneal nerve injury was characterized by foot drop presented by a larger midswing ankle angle. Foot drop was measured and ranged from 49 to 75 degrees in the uninjured hindlimb. Peroneal nerve damage, and, thus, lack of innervation to the dorsiflexor muscles led to foot drop symptoms in the affected limb.

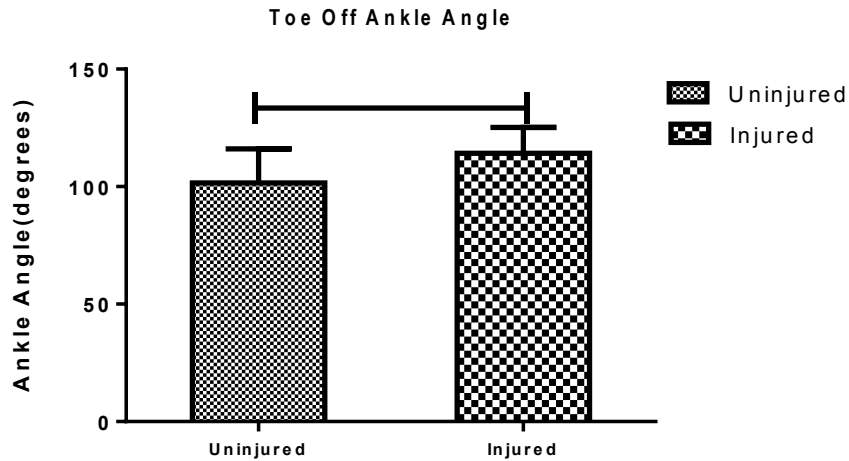


Figure 3.4 Ankle angle during toe off was compared between uninjured and injured rabbits. No significant difference between the two groups ( $p=0.2215$ ).

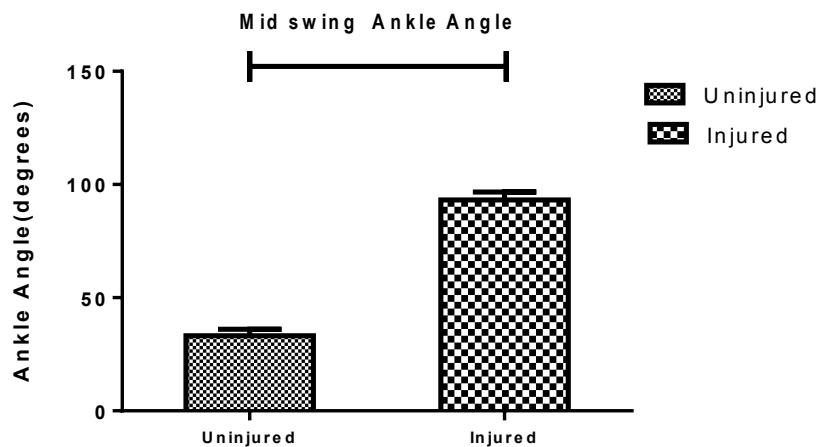


Figure 3.5 Ankle angle during mid swing was compared between uninjured and injured hindlimbs. Significant difference was observed between the two groups ( $p=0.002$ ).

### 3.3.3 Swing Duration Evaluation

At week 12, there is significant foot drop in the injured limb which leads to a lag in the swing phase duration. Due to loss of dorsiflexion, the injured limb enters swing phase earlier than the uninjured hindlimb in contrast to simultaneous retraction and extension of hindlimbs in normal motion. There is significant increase in swing duration in the injured hindlimb, as compared to the uninjured hindlimb ( $p=0.0032$ ) (Figure 3.6)

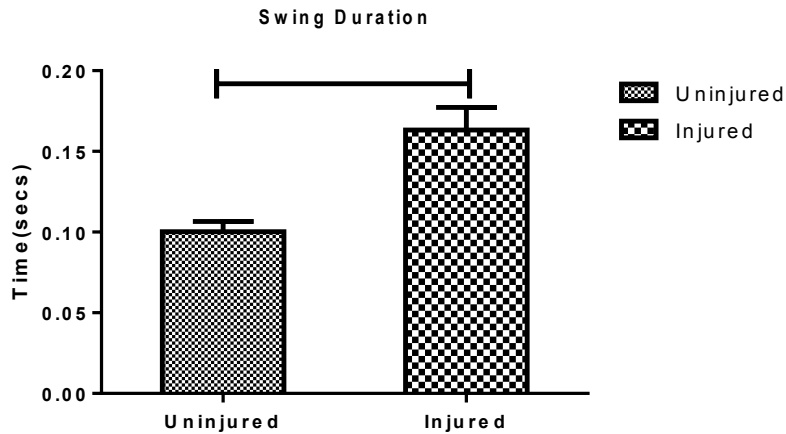


Figure 3.6 Duration of swing phase was compared between uninjured and injured hindlimbs. Significant difference observed between two groups ( $p=0.0032$ )

### 3.4 Discussion

Though a large variety of tests has been developed and applied for evaluating nerve regeneration, many of them refer to completely different aspects of nerve regeneration. Recently, studies have focused on assessing specific functional outcome of the repaired nerve to evaluate the extent of nerve regeneration. Standardized methods to evaluate function are necessary for the clinical translation of experimental treatments. For the purpose of testing repair strategies in clinically relevant gaps (> 3 cm), larger animal models are required, including rabbits, canines and non-human primates. Developing functional assessment methods in animal models is a challenging task, especially in larger animals.

The total of all types of anatomical regeneration plus the imponderable factor of plasticity constitutes functional regeneration, and this might be one of the reasons why functional assessments and structural assessments have shown less correlation (de Medinaceli 1995; Kanaya, Firrell et al. 1996; Wolthers, Moldovan et al. 2005). Functional assessment can denote how well the body is adapting to or compensating for problems that can arise from aselective reinnervation (Dellon and Mackinnon 1989). Normal gait is a complex activity. This requires a command system; specifically, the central nervous system, in combination with peripheral

nervous system, organizes and performs at different levels of coordination (intra-muscular, intrajoint, inter-joint, inter-body segment, etc.) within an animal. Gait is controlled using a neural pathway, a network that connects various parts of the motor system together with feedback loops. Nerve regeneration studies in rats have used gait and ankle motion analysis to study gait abnormalities after different kinds of nerve injury. In sciatic injured rats, ankle motion analysis has shown higher sensitivity in detecting motion deficits and demonstrated a better relationship to the degree of regeneration (Amado, Armada-da-Silva et al.). To the best of our knowledge, no gait analysis studies have been performed in peripheral nerve research using rabbit animal model. Here, we used 2D biomechanical analysis of the ankle joint to study the gait abnormalities in the uninjured and peroneal nerve injured rabbits.

This study showed that motion analysis of ankle joints in a rabbit model can be used to effectively measure foot drop due to peroneal injury. Previous report by Schmitz et al., mentioned that a complete peroneal nerve lesion would not cause significant changes in the stance or gait in a rabbit (Schmitz and Beer 2001). Our results showed that denervated dorsiflexor muscles at 12 weeks produced significantly higher mid swing ankle angle, which can be quantified. Immediately after interruption of the peroneal nerve, foot drop is evident, but diminishes before the muscles are reinnervated due to the sitting posture of the rabbits (Gutmann 1942). Due to autotomy in most of the rabbits, we were unable to use ankle motion analysis before the 12<sup>th</sup> week to study the variations in ankle angle during the denervation phase. Further studies are needed to evaluate the effectiveness of using gait analysis in rabbit models of nerve repair. Rabbits with or without peroneal nerve lesions can hop with the same speed (Schmitz and Beer 2001). In our study, we observed a significant increase in swing duration after nerve injury (at 12 weeks) which reduced the hopping speed in the rabbits. As maximum dorsiflexion happened during swing phase, paralyzed dorsiflexor muscles required more time to complete the swing phase of a hop.

Foot drop is characterized by steppage gait where hip and knee undergoes excessive flexion to lift the foot higher to clear the ground. This is result of a compensatory mechanism that becomes active as a result of peroneal nerve dysfunction. We observed a similar increase in relative knee height in the denervated group compared to the control to prevent the injured limb from hitting the treadmill. The motor cortex is not a static control center, but it has an elastic relationship with the end organs, the muscles (Donoghue, Suner et al. 1990; Sanes, Suner et al. 1990; Sanes and Donoghue 2000) and can be remodeled throughout life (Nudo, Milliken et al. 1996). After peripheral nerve lesions, or motor skill training these motor maps do undergo changes which are reversible (Donoghue, Suner et al. 1990; Sanes, Suner et al. 1990; Huntley 1997) Peripheral nerve injuries induce an expansion of the adjacent connections of the motor cortex, leading to an association between affected areas of the motor cortex and new muscle groups (Donoghue, Suner et al. 1990; Sanes, Suner et al. 1990; Huntley 1997). Using functional compensatory processing, the rabbits use hip and knee motion changes to overcome the dysfunction at the ankle and foot and for allow for locomotion. During toe off, the ankle joint extension increased due to the loss of load -bearing ability on the injured limb, observed hip and knee extensions during hops but these were not quantified. Possible reason for observed higher hip range of motion can be explained by replacement of the ankle joint in its functions of both push off and foot forward displacement during toe off and swing phases, respectively. From initial swing to terminal swing, the hindlimb were protracted, which seems to be assisted by flexion of the trunk. All these were changes that occurred to compensate for the foot drop in the injured limb.

The method of using of joint kinematics during hopping can be useful to evaluate functional recovery after long gap peripheral nerve injury or repair in the rabbit only if its sensitivity and specificity are acceptable. Future studies should include hip, knee and ankle joints for joint kinematic analysis, in order to evaluate its ability to detect functional recovery or loss non-invasively. A reliable method of assessing functional outcome in a rabbit model, in

conjunction with other parameters of nerve function, will help to quantitate nerve regeneration effectively.



## CHAPTER 4

### CONCLUSION AND FUTURE WORK

Although there is potential for the improved repair of longer nerve defects, the development of a nerve tube that meets the challenges of long gap nerve repair requires extensive research. To study long gap nerve repair, larger animal models with standardized functional tests to evaluate the outcome are needed. In this pilot study, a rabbit common peroneal nerve model was used. The capability of CUPE to maintain the structure of a BNI (agarose microchannels filled with collagen) across a 5 cm gap was evaluated. The CUPE BNI results demonstrate that it was strong enough to hold the internal structure across the 5 cm gap in the rabbit model across 12 weeks. Another aim of the study was to attract axons using pleiotrophin in a synthetic BNI and to compare the regeneration between PTN released from microparticles and from a fiber in a gradient form to bridge the 5 cm gap. The results indicated that there was no nerve tissue growth through any of the BNIs, with the main reason possibly being incorporation of insufficient PTN across the 5 cm gap. We used video gait analysis of injured and uninjured rabbits to study ankle angle and other gait parameters during the hopping gait of each rabbit. Foot drop and an increase in knee height and swing duration were observed in the injured hindlimbs. Further studies to assess the sensitivity of the techniques to measure gait abnormalities and recovery at different time points in a rabbit model are needed. Limitations of the present design have been identified, and they are currently being rectified or will be addressed in the near future as discussed below.

In the repair of larger gaps, physical characteristics of the nerve tube become more important. In the case of larger nerve gap repairs, flexibility of the nerve tube is crucial as the nerve ends might not be in the same plane or the gap that needs to be bridged be across a

joint. It has been reported that stiffness of nerve tissue varies, with regions near to the joint being more compliant than those away from the joint (Phillips, Smit et al. 2004). Thus, it is important for nerve conduits that are used to bridge gaps across this region to be flexible and elastic, yet strong enough to accommodate the changes during locomotion without breaking or tearing of the suture from the conduit. Biodegradable synthetic polymers having elastomeric properties, meeting conduit requirements for long nerve gaps, need to be evaluated. In order to find the right balance, polymers having different molecular weights, copolymer ratios and diol content need to be tested in-vitro. Bending studies using a dynamic mechanical analyzer performed on the porous nerve tube, together with the multichannel structure, would enable us to understand the stiffness of the BNI and the force that would displace the tube and its contents. Porosity and structural content also affect the mechanical properties.

In the case of long gap nerve repairs, the distance between the regenerating nerve and target organ is long, thus lack of appropriate growth substrate and trophic support seems to be the main reason for regenerative failure. Though it might seem that addition of exogenous growth factors might solve the issue, this is not the case. The effect of growth factors is often dose dependent and requires release over an extended period of time; hence, optimal delivery systems need to be developed and tested in vitro. Cellular activities such as cell migration, differentiation and proliferation, are typically dependent on the spatio-temporal distribution of specific growth factors. Using specifically designed composite polymer systems, simultaneous delivery of multiple growth factors can be obtained and studied for their efficacy in promoting nerve regeneration.

As the time required for the bridging of a long gap requires more time, requirements for growth factors will change with proceeding regeneration, a delivery system capable of responding to stimuli of the local environment might be more beneficial (Matsumoto, Ohnishi et al. 2000; Fine, Decosterd et al. 2002; Kim, Yoo et al. 2004; Duobles, Lima Tde et al. 2008). A growth factor delivery system that responds to compressive stimulation of the nerve tube matrix

across a joint region might be a novel approach that needs to be evaluated in meeting the demands of long gap nerve regeneration (Lee, Peters et al. 2000). Dose-dependency and release kinetics of individual growth factors need to be established initially using in vitro models.

We estimated the release kinetics of PTN from the fibers based on NGF release from previous fibers and quantified the amount using ELISA. This did not yield a proper measure of the amount of PTN released at different time points, leading to higher or lower than optimal amounts of PTN which would affect the biological activity. A standardized protocol (ELISA for PTN, Western blot analysis using heparin beads) that can determine the exact amount of PTN released at different time points needs to be used. The supernatant of microparticles or fibers suspended in PBS at different time points was used to evaluate the release profile, but this did not mimic the actual environment inside the BNI. It is important to study how the release of the growth factor would be affected when surrounded by collagen, such as in the BNI. Though the structure of nerve conduits help in localizing the growth factors, the bioactivity of the growth factors released over time needs to be studied. In addition, the effect of the degradation products of the PLGA on PTN activity needs to be tested. The permeability of the nerve tubes plays a significant role in case of longer gaps. This is probably due to intermingling of contents of the nerve tube with the extracellular contents, facilitating formation of ECM proteins which enhance regeneration. It is important that the holes made on the BNI are large enough, permeability properties of the nerve conduit material are optimal for nutrient and gas support across a long gap.

Use of noninvasive diagnostic techniques such as Diffusion-weighted imaging (DWI) and diffusion tensor imaging (DTI) that use MRI principles can prove to be a successful tool in tracking the different stages of regeneration, especially in long gap repair studies. Several innovations which include fat suppression, optimized pulse echo times and T2-weighted scans can be used to obtain high fidelity images to track conduit failure, degenerative or regenerative events. Li et al. investigated the correlation between MRI parameters and histology in an acute

crush injury of rabbit sciatic nerve model (Li, Shen et al. 2008). The slope of the treadmill used in the gait analysis might have had some degree of contribution to the variations in gait. So, it is important for further studies to use a straight motorized treadmill for gait analysis. Hip, knee and ankle joints should be considered together to study the changes due to nerve dysfunction and compensatory mechanism. Ground reaction forces can be sensed specifically in injured and uninjured limbs using a force platform giving useful information on the deficit or recovery of force exerted (Howard, Blakeney et al. 2000).

The development of an ideal nerve tube meeting the demands of bridging a long nerve gap requires close collaboration of bioengineers, neuroscientists, and peripheral nerve surgeons. The presence of different signaling cues in the developing nervous system, indicate that the use of combination of growth factors with pre-programmed release kinetics can be expected to enhance nerve regeneration. Delivery systems with integrated growth factor delivery and cellular components(schwann cells) need to be designed and evaluated to meet the trophic support demands across a long gap. Studies have shown successful implementation of concentration gradients of both ECM proteins and growth factors and their positive effect in vitro. Though challenging, nerve conduits incorporated with optimal concentration gradients of different growth factors, with controlled release might possibly lead to development of a bio artificial nerve in the near future.

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