THE BIOENGINEERING OF NERVE CONDUITS

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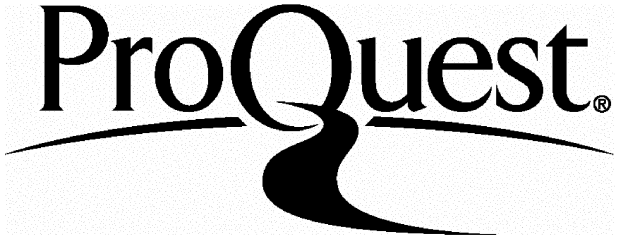

To my family

That knowledge which stops at what it does not know,

is the highest knowledge.

Chuang Tzu

Learning without thought is labour lost;

thought without learning is perilous.

Confucius

The difference between what the most and the least learned people

know is inexpressibly trivial in relation to that which is unknown.

Albert Einstein

Every great advance in science has issued from a

new audacity of imagination.

John Dewey

ABSTRACT

Poly-3-hydroxybutyrate (PHB) conduits are an alternative to nerve autografting and support regeneration across long nerve gaps, although to suboptimal levels. The aim of this study was to improve these results by combining PHB with glial growth factor (GGF), enhancing nerve regeneration by contact guidance and an improved trophic microenvironment. Two and 4cm gaps in the rabbit common peroneal nerve were bridged using PHB-GGF conduits. The rate and quantity of axonal and Schwann cell (SC) regeneration were assessed by quantitative immunohistochemistry at 21, 42 and 63 days, and compared to empty and alginate filled conduits. Addition of GGF improved axonal and SC regeneration, which was sustained up to 63 days independent of gap length. The distance and quantity of axonal regeneration were increased by up to 53% and 4317% respectively. At 120 days axonal and SC regeneration within the PHB-GGF grafts remained superior to the controls resulting in enhanced motor organ reinnervation, as was demonstrated by an improved recovery of muscle mass compared to the controls.

In both the short and long term studies the alginate filled conduits resulted in regeneration inferior to both the GGF and empty tubes. As a result alginate fibres were assessed *in vitro* and *in vivo* as an alternative to alginate hydrogel with a potential to deliver GGF. However, regeneration *in vivo* in alginate fibre filled conduits was inferior to conduits filled with alginate hydrogel.

Polyhydroxyalkatone (PHA) was also evaluated as a conduit material, as GGF linkage and release from its walls is a feasible option. Four different PHA configurations were used to bridge a 1cm rat sciatic nerve gap. All 4 PHA configurations, accelerated axonal regeneration rate to 1mm/day versus 0.7mm/day with PHB conduits and resulted in a quantity of axonal regeneration superior to that seen in the autograft repairs.

In conclusion, GGF improves axonal and SC regeneration across short and long gaps through PHB conduits, but alginate hydrogel appears to limit the trophic effect of GGF. Alginate fibres provide no improvement, however alginate's limitations may be overcome and regeneration further improved by using PHA as a bioconstruct releasing GGF into the conduit microenvironment.

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ABBREVIATIONS

AG	Autograft
ANOVA	Analysis of variance
B	Band of Büngner
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
°C	Celsius
CNTF	Ciliary neurotrophic factor
cm	Centimetre
D	Distal
EDL	Extensor digitorum longus muscle
G	Gauge
GDNF	Glial-derived neurotrophic factor
GGF	Glial growth factor
g	Gram
i.m.	Intramuscular
Kda	Kilodalton
kg	Kilograms
l	Litre
LIF	Leukaemia inhibitory factor
M	Myelinated nerve fibre
mg	Milligram

ml	Millilitre
µl	Microlitre
mm	Millimetre
µm	Micrometer
NGF	Nerve growth factor
NT-3/4/5	Neurotrophin-3/4/5
o.d	Once a day
P	Proximal
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PHA	Polyhydroxyalkatone
PHB	Poly-3-hydroxybutyrate
s.c.	Subcutaneously
SC	Schwann cell
TA	Tibialis anterior muscle
UM	Unmyelinated nerve fibre
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

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1.1 MORPHOLOGY OF THE PERIPHERAL NERVE

Hippocrates provided the first written description of the peripheral nervous system as early as the 4th century B.C., and later Herophilus identified nerves as such, distinguishing them from tendons and tracing them to the spinal cord. The peripheral nervous system is comprised of the peripheral nerve fibres and their associated tissues, which include glial cells, sensory organs, muscle motor end plates, intraneural vasculature and related extracellular matrix (Landon 1976; Dyck & Thomas 1984).

1.1.1 Neurons

The functional unit of the nervous system upon which all structures are dependent is the neuron. The neuronal cell body is the site of all metabolic activities, which are then transported along the axon. Motoneurons innervate striated muscles, and their cell bodies are located in the anterior horn of the spinal cord, the axons pass through the anterior root to join the peripheral nerve bundle. These axons are myelinated except at their origins and terminations, where they divide into numerous branches to form neuromuscular synapses. Motoneurons innervating smooth muscles and glands belong to the autonomic nervous system. Their cell bodies are found in the peripheral autonomic ganglia and their axons are unmyelinated. Sensory neurons are located outside, but close to the spinal cord in the dorsal root ganglia of the spinal nerves. They carry impulses from the skin, muscle spindles, viscera and blood vessels back to the

spinal cord. The sensory fibres may be myelinated or unmyelinated according to the sensory modality.

A nerve fibre is generally described as any process of the nerve cell, either dendrite or axon. It is a cylindrical extension of the cell soma, surrounded by a specialised membrane, called the axolemma that maintains a resting action potential. The nerve fibres can be divided into motor, sensory, sympathetic and parasympathetic, and are classified according to their fibre diameter and conduction velocity into A, B and C fibres (Gasser & Grundfest 1939). The principle content of the fibres are axoplasm, microfilaments, neurofilaments and neurotubules. Microfilaments are made up of actin proteins and may play a role in intra-axonal transport (Varon & Bunge 1978). Neurofilaments are larger and can be distinguished by immunohistology (Lee *et al.* 1982) and disappear following damage. Neurotubules give cytoskeletal support.

Axons are unable to synthesise proteins and are dependent on the neuronal cell body or the glial cells for their maintenance. Macromolecules are transported along both directions of the axon concurrently at various speeds (Tytell *et al.* 1981). The slow anterograde transport (0.1-3mm/day) is for the bulk movement of neurofilaments and is equivalent to the rate of axonal outgrowth sprouting (Di Giamberardino *et al.* 1973). An important function of fast retrograde transport (100-400mm/day) is the uptake of exogenous growth factors produced by the end organs and transport back to the neuronal cell body (Ebendal *et al.* 1980).

Nerve fibres with their supporting Schwann cells (SC), form the bulk of the peripheral nerves. These are surrounded by an outer epineurium, a perineurium which surrounds the nerve fascicles and an inner endoneurium. The endoneurium contains the supporting elements of the nerve fibres, which includes SC, fibroblasts, occasional mast and fat cells and extracellular matrix (ECM) (Thomas *et al.* 1993). Although the fascicular pattern of the nerve fibre is variable, there is a high degree of segregation of afferent and efferent fascicles of pure motor or sensory nerves within each fascicle and nerve type (Terzis & Smith 1990a).

1.1.2 Schwann cells

These are the principal glial cells of the peripheral nerve, and they were first described by Theodor Schwann (1810-1882), who thought that they developed from the axons. However, it is now clear that SC originate form the neural crest and to a lesser extent from the neural tube and they grow with or slightly ahead of the neurites, which arise from the primitive dorsal root ganglia and ventral horn cell bodies (Jessen & Mirsky 1998). The irreversible step from neural crest cells to SC precursors is observed in cells that express high levels of mRNA for P0, which is a major myelin protein (Mirsky & Jessen 1999). SC precursors do not express S100 marker but within days and early during gestation the typical S100 antigen is expressed in the cells (Jessen & Mirsky 1991). The precursors of SC undergo a proliferative phase that might partly be regulated by the developing embryonic neurons as they express high levels of neuregulins, in particular glial growth factor (GGF) (Shah *et al.* 1994). GGF is a potent SC mitogen

(Marchionni *et al.* 1993) and it interacts with SC heterodiameric receptors composed of c-erbB2, c-erbB3 and c-erbB4 (Grinspan *et al.* 1996). Deficiency of SC is observed in mice with the deletion of neuregulin-1 (Meyer & Birchmeier 1995). Further evidence on the role of the neurons is evident when SC isolated from axons become quiescent *in vitro*. SC mitosis continues until there is a 1:1 ratio between axons and myelinating SC, and the segment of axon that is myelinated by a single SC is termed the node of Ranvier. As myelination proceeds, there is a down regulation of GGF receptors, erbB2 and erbB3 (Mirsky & Jessen 1999). Therefore SC proliferate in three distinct circumstances: during development, following loss of contact with axons as a result of axonal degeneration and during the re-establishment of axonal contact that follows axonal regeneration. In all three cases, axon-SC contact, or its loss, has a central role in the induction of the SC proliferative phase (Reynolds & Woolf 1993).

There are two distinct categories of SC, myelinating and non-myelinating. Axons, whether myelinated or unmyelinated are ensheathed by SC along their entire length. A feature of SC is the deposition of a basal lamina, a component of the extracellular matrix, which surrounds the outer surface of the SC and isolates them from the surrounding matrix. Axonal contact has been shown to be a factor for differentiation of SC to myelin forming phenotype (Jessen *et al.* 1987). Axonal diameter is also important, as only axons with a diameter larger than 0.7μm undergo myelination (Windebank *et al.* 1985). On loss of axon contact the SC dedifferentiate and will redifferentiate to express the appropriate ensheathment phenotype on contact with small or large axons. However, the identity of the myelination signal remains unclear. Recently, it has been shown that

SC synthesise progesterone, which stimulates myelin formation (Chan *et al.* 2000) and blocking of progesterone receptors also inhibits myelination (Koenig *et al.* 2000).

The node of Ranvier is the site of interruption of the myelin sheath, where the axon is covered only by microvilli of SC and sodium channels are accumulated at the node of Ranvier. Cajal named it the "germinative territory" because following trauma to the nerve axonal regeneration appeared at the node In contrast unmyelinated fibres have continuous SC sheaths with no gap or node between adjacent SC. The basal lamina however, is continuous for the length of both myelinated and unmyelinated axons (Thanos *et al.* 1998).

The phenotypic markers expressed by SC seem to be dependent on their related axon rather than an inherent difference in SC. This is evident as after loss of axonal contact, SC readily revert back to the expression of markers characteristic of immature non-myelinating SC (Jessen & Richardson 1996) and this phenomenon is irrespective of SC type. Some of the phenotypic markers are common to all mature SC, such as S100, vimentin and laminin (Scherer 1997). Other markers like P0, myelin basic protein and myelin associated glycoprotein are restricted to myelin forming SC (Scherer 1997). Some markers are exclusive to non-myelinating SC such as intermediate glial fibrillary acid protein (GFAP), p75 (low affinity nerve growth factor receptor), neural cell adhesion molecule (NCAM) and L1 (Jessen & Mirsky 1992). Thus it is possible to ascertain the SC phenotype using immunohistochemical stains for the distinctive phenotypic markers.

1.1.3 Extracellular matrix

Neuronal axons and SC are enmeshed in a complex extracellular matrix (ECM), whose two major components are basal lamina and fibrillar matrix. The fibrillar matrix is made up mostly of type I and III collagen, while the basal lamina contains types IV and V collagen, the glycoproteins laminin, fibronectin and tenascin, as well as heparin sulphate (Chernousov & Carey 2000). The ECM components act together to provide structural support for the cellular elements and affect their behaviour during development and maturity.

Tissue culture studies have defined the ECM products that are produced by SC, how their production is controlled, and how endoneurial and perineural fibroblasts co-operate and are regulated by SC in organising ECM. Fibroblasts can also induce synthesis of ECM via SC (Obremski *et al.* 1993), and in turn the SC signal to the surrounding connective tissue, possibly via Desert Hedgehog molecules, to organise perineural fibroblasts (Mirsky & Jessen 1999). ECM is not produced and organised by neurons cultured alone, whereas cultured SC can express basal lamina as identifiable by electron microscopy (Baron-Van Evercooren *et al.* 1986). It is in co-cultures of neurons and SC that a basal lamina is formed on the axonal surface of SC, and a continuous basal lamina production depends on direct contact with axons (Bunge *et al.* 1990). Thus SC have a critical role in the production of ECM, which is essential for the ensheathing of the axons. The importance of ECM was demonstrated in tissue culture studies where collagen production and subsequent myelination was prevented in medium without

ascorbic acid (Eldridge *et al.* 1989). An important effect of the basal lamina is to polarise the myelinating SC, as the surface free of the basal lamina is responsible for axonal adhesion (Chernousov & Carey 2000). Laminin and fibronectin are potent promoters of cell adhesion, while migration, adhesion and signalling functions of ECM are mediated by integrin receptors on SC (Chernousov & Carey 2000). Antibodies to β1 integrin block migration on laminin1 and 2, while αv integrin may be involved in migration of SC on fibronectin (Mirsky & Jessen 1999).

1.2 AXONAL INJURY

Axonal degeneration is the biological response which follows axonopathy. Although there are many causes of axonopathy, either hereditary or acquired, the focus of this section is on traumatic axotomy.

1.2.1 Classification of nerve injury

A scheme for classification of traumatic nerve injuries was first developed by Seddon (Seddon 1954) and subsequently modified by Sunderland (Sunderland 1978). Sunderland classified nerve injuries into five degrees of increasingly severe injury patterns. In first degree injuries (neuropraxia) the gross nerve structure is intact and the decreased function is reversible. In second degree injuries (axonotmesis) the epineurium, perineurium and basal lamina are intact but there is complete axonal disruption which recovers in months. In third degree injuries the basal lamina is also

interrupted but the epineurium and perineurium are intact. In fourth degree injuries only elements of the epineurium are left intact. Finally in fifth degree injuries (neurotmesis) the nerve is completely transected with all the elements of the nerve being separated. It is this latter type of injury that has been experimentally studied in this thesis.

1.2.2 Wallerian degeneration

Following nerve injury, complex changes occur throughout the neuron, both in the proximal and distal nerve segments, extending to the muscle motor end plates and sensory receptors distally, and the cell bodies proximally. Dorsal root ganglia cell death after peripheral nerve injury ranges from 7-50%, with the diversity of experimental models and techniques involved accounting for the wide variation in estimates (Hart *et al.* 2002). However, motor neurones survive unless the lesion is very proximal at the level of the nerve root (Ranson 1909; Himes & Tessler 1989; Liuzzi & Tedeschi 1991). Neuronal cell death occurs as a result of the insult produced by nerve injuries, loss of neurotrophic support from the target organ, abnormal retrograde electrical activity and release of toxic cytokines such as interlukin-6 and tumour necrosis factor α produced by inflammatory cells at the site of injury (Liuzzi & Tedeschi 1991). The closer the injury to the cell body the more likely cell death may occur.

The sequence of events occurring in the distal and the proximal stumps following nerve injury were described by Waller (Waller 1850), these being known under the term Wallerian degeneration. During the first week after injury, remodelling occurs along the

whole of the distal segment and up to the first node of Ranvier in the proximal stump. In the myelinated fibres, the axons and the myelin sheaths in the distal stump break down. The debris is phagocytosed partly by SC but predominantly by macrophages. An influx of macrophages and activation of resident macrophages (Griffin *et al.* 1993) results in the removal of axonal and myelin debris to leave the basal lamina tubes and SC of the distal nerve, these cordons of SC are termed 'bands of Büngner' (Cajal 1928; Perry & Brown 1992). In addition to their phagocytic role the macrophages also secrete cytokines that promote axonal regeneration directly or via their interaction with SC (Brown *et al.* 1991).

Loss of the intimate contact between axons and glial cells causes SC to de-differentiate and to alter their phenotypic marker expression (Nikam *et al.* 1995), thereby becoming mitotic and starting to proliferate. The glial growth factor (GGF) subfamily of axon-associated neuregulins and their putative erbB-tyrosine kinase receptors on SC (Carraway & Burden 1995) play an important role in signalling this event (cf 1.4.2). Carroll *et al.* demonstrated that both GGF and erbB2 and erbB3 receptors were coordinately upregulated and expressed by SC in the distal stump, suggesting an autocrine mechanism in addition to paracrine stimulation of SC proliferation (Carroll *et al.* 1997). Following proliferation, the SC align themselves longitudinally within the basal lamina tubes forming bands of Büngner, ready to receive regenerating fibres that have grown out of the proximal stump and to guide them to their target (Fawcett & Keynes 1990; Hall 1997). The changes in unmyelinated fibres are similar, but there is no formation of bands of Büngner (Graham & Lantos 1997).

In the proximal stump the SC also de-differentiate, proliferate and start to migrate along the internal surface of the basal lamina tube from the initial regeneration zone located at the first node proximal to the site of injury (Thanos *et al.* 1998).

1.3 AXONAL REGENERATION

1.3.1 Factors influencing axonal growth

Axonal regeneration is governed by three main factors; contact guidance, neurotropism and neurotrophism. Contact guidance describes the topographical and microgeometric cues and physical support provided for the regenerating and maturing axons as well as their interaction with cell surface adhesion molecules, which are present along the appropriate pathways. Neurotropism refers to the process by which the distal stump and end organ positively influence and direct regeneration of the proximal stump by chemical signals. Lastly neurotrophism is the support provided to the regenerating axons by the growth factors produced by the surrounding cells and end organs, allowing the neurons to survive the injury and mature following regeneration (cf 1.4).

After nerve gap injury, the process of successful regeneration begins with the formation of a fibrin matrix containing fibroblasts, fibronectin, macrophages, leukocytes and erythrocytes, which bridges the proximal and distal stumps (Lundborg *et al.* 1982a; Lundborg *et al.* 1982b; Lundborg *et al.* 1982c; Lundborg *et al.* 1982d; Longo *et al.* 1984). Within a contained environment this forms a primary scaffold, which orientates

the migration of fibroblasts, SC and axons (Weiss 1944). SC migrate out from the proximal and distal stumps and meet approximately two-thirds of the way along the nerve gap to form a continuous scaffold.

A "latent period" exists between nerve injury and the commencement of axonal regeneration (Gutmann *et al.* 1942). Regeneration rate varies as a function of injury type, age, and species especially during the early stage of regeneration (Gutmann *et al.* 1942; Black & Lasek 1979; Pestronk *et al.* 1980). Outgrowth of axonal sprouts is more rapid after crush injury in which the continuity of nerve sheaths and basement membrane is preserved than after nerve section (Thomas 1964). Regeneration is also more rapid in young mammals and varies across species (Gutmann *et al.* 1942; Black & Lasek 1979). In the rat sciatic nerve model the rate of regeneration through a PHB conduit is 0.15mm/day for the first 7 days, increasing to 0.36mm/day between 7 and 14 days (Hazari *et al.* 1999a). Using the same conduit in the rabbit common peroneal nerve model the rate of regeneration is 0.26mm/day in the first 21 days, which then increases to 1.6mm/day between 21 and 42 days (Young *et al.* 2002). Other researchers have estimated the rate of regeneration to vary between 1 and 3mm/day (Gutmann *et al.* 1942; Black & Lasek 1979).

Within 3 hours of injury, axonal sprouting begins at the node of Ranvier immediately proximal to the site of the injury (Torigoe *et al.* 1996). This happens before any change in the cell body becomes apparent; thus the ability to grow sprouts may be intrinsic to the axon. As many as 50-100 sprouts may stem from a single axon, possibly to

maximise the chances for each neuronal cell reaching it's target organ. Some of these sprouts will 'die back' through axonal pruning (Brushart 1993) because of insufficient survival signal from the target organ, most probably in the form of growth factors.

The terminal tips of the regrowing axons, or growth cones, respond to contact guidance cues and extend along the inner surface of SC regenerating ahead of them, or through the bands of Büngner (Martini *et al.* 1990). Diffusible tropic factors from the distal stump generate a concentration gradient along which the growth cone moves (Lundborg *et al.* 1994a). Therefore through contact guidance and neurotropism the regrowing axons extend into the distal nerve segment. In addition, neurons have been shown to demonstrate tissue specificity, by recognising a distal nerve stump rather than other tissues (Lundborg *et al.* 1986; Mackinnon *et al.* 1986), motor and sensory specificity, separating motor and sensory nerve fibres (Brunelli *et al.* 1987; Brushart & Seiler 1987; Rath & Green 1991; Brushart 1993) and topographic specificity, which manifests in the return of axons to the topographic area they previously served (Zhao *et al.* 1992; Hefti 1994). Preferential motor reinnervation has been demonstrated, its mechanism is still obscure although it has been suggested that it is due to the presence of recognition molecules in motor SC tubes as opposed to sensory SC tubes (Brunelli *et al.* 1987; Brushart 1987; Brushart 1988; Brushart 1993). Other hypotheses include the chemical coding of individual neurons for their target, the regeneration of axons to appropriate SC tubes by diffusible factors and the random regrowth of regenerating axons with survival of only specific and appropriate axons (Rende *et al.* 1991). Longo and coworkers demonstrated that the presence of the distal nerve stump in the silicone chamber was

essential if nerve regeneration was to occur beyond the first few mm (Longo *et al.* 1983). However, there is debate about the distance over which the distal stump can influence the proximal regenerating stump, and this may be as little as 5-10mm in the rat (Lundborg *et al.* 1982b; Politis *et al.* 1982; Longo *et al.* 1983; Brunelli *et al.* 1994; Hefti 1994; Frey *et al.* 1996; Kiyotani *et al.* 1996; Strauch *et al.* 1996).

Lundborg *et al.* demonstrated that fluid taken from a silicone or mesothelial chamber bridging a 1cm gap in the rat sciatic nerve contained trophic factors, which ensured the *in vitro* survival and growth of sensory neurons from rodent dorsal root ganglia (Lundborg *et al.* 1982a; Lundborg *et al.* 1982c). In their *in vivo* rat sciatic nerve studies, they observed that the growth-promoting influences from the distal stump on the regenerating proximal stump acted over a limited distance of 6-10mm and increasing the gap length to 15mm resulted in no regeneration (Lundborg *et al.* 1982b). Brushart *et al.* demonstrated that this influence of the distal stump on proximal regeneration also has a high degree of specificity and when the distance between the proximal and distal stumps is too short the benefits of the distal stump directing appropriate regeneration may be lost (Brushart & Seiler 1987). They observed that when a 2mm gap existed between the ends of the peripheral nerve, selective motor reinnervation was not demonstrated, yet when the gap distance was increased to 5mm selective motor reinnervation occurred (Brushart & Seiler 1987). This has resulted in the concept that entubulation with the deliberate creation of a small nerve defect of 3-5mm might be superior to "state of the art" microsurgical fascicular nerve repair even when the nerve ends can be brought together without tension (Dellon 1994; Weber *et al.* 2000).

1.3.2 Role of Schwann cells and basal lamina

SC migration into the site of injury from the proximal and distal stumps is independent of accompanying axons (Andeson *et al.* 1991), but SC are pivotal to peripheral nerve regeneration as inhibition of SC proliferation has shown to severely retard this process (Millesi 1984; Hall 1986a; Hall 1997). Indeed intraneural injection of mitomycin C proximal to the site of peripheral nerve transection inhibited SC proliferation, resulting in absence of axonal regeneration when the proximal stump was anastomosed to an acellular graft. However, axonal outgrowth occurred when the proximal stump was sutured to a viable nerve graft rich in SC (Hall 1986a).

SC have multiple roles in peripheral nerve regeneration. During Wallerian degeneration the ensheathing SC assist in the removal of the debris of both axons and myelin sheaths. Following injury, SC upregulate the synthesis of many known growth factors, including nerve growth factor (NGF) (Heumann *et al.* 1987), brain-derived neurotrophic factor (BDNF) (Acheson *et al.* 1991), platelet-derived growth factor (PDGF) (Eccleston *et al.* 1993), insulin-like growth factor (Cheng *et al.* 1996), ciliary neurotrophic factor (CNTF) (Friedman *et al.* 1992) and leukaemia inhibitory factor (LIF) (Matsuoka *et al.* 1997), all of which play a role in nerve regeneration (Terenghi 1999). SC express a number of cell adhesion molecules on their surface which are known to influence neurite growth (Jessen & Mirsky 1992; Jessen & Richardson 1996). They are responsible for the ensheathment of unmyelinated nerve fibres and the sequential myelin

segments that characterize myelinated nerve fibres (Bunge 1993). They also produce basal lamina and fibrillar matrix (Bunge 1993).

The basal laminae associated to SC also play a major role in supporting nerve regeneration (Thanos *et al.* 1998), forming the main scaffold for regenerating axons and surrounding the external aspect of the SC-axon unit of both myelinated and unmyelinated nerves. The basal lamina contains types IV and V collagen, the glycoproteins laminin, fibronectin and tenascin, as well as heparin sulphate (Chernousov & Carey 2000). The basal lamina has been demonstrated to be a suitable conduit for peripheral nerve regeneration on its own (Ide *et al.* 1983; Hall 1986b). In addition anti-laminin antibodies have been shown to reduce the rate of regeneration (Wang *et al.* 1992; Bryan *et al.* 1993).

Both neuronal and glial elements express a number of cell adhesion molecules such as NCAM, L1 and laminin (Jessen & Mirsky 1992). These are upregulated following denervation, and by two weeks after injury SC display L1 and NCAM phenotype (Martini *et al.* 1994). The presence of adhesion molecules on SC and basal lamina are essential for adequate axonal regrowth as they mediate interaction with counterpart molecules present on the growth cone providing structural guidance (Jessen & Richardson 1996).

1.4 GROWTH FACTORS

1.4.1 Neurotrophic factors

Neurotrophic factors are endogenous soluble proteins synthesised by the neurons, glial cells, muscles and glands and delivered to the neuronal soma via retrograde transport. They regulate survival, growth, morphological plasticity and synthesis of proteins for the differential functions of neurons (Purves 1986; Oppenheim 1991; Hefti 1994). They are present in the mature peripheral nervous system at low concentrations, but after nerve injury altered gene expression of trophic factors occurs resulting in their upregulation in a specific order.

Their existence was first proposed by Forssman (Forssman 1898) and confirmed in 1953 when nerve growth factor (NGF) was discovered (Levi-Montalcini & Hamburger 1953). NGF is part of the neurotrophin family of which other members include brain derived neurotrophic factor (BDNF) (Barde *et al.* 1978), neurotrophin-3 (NT-3) (Ernfors *et al.* 1990) and neurotrophin 4/5 (NT-4/5) (Berkemeier *et al.* 1991). The neurotrophin family share a low-affinity receptor p75 (Chao *et al.* 1986), which interacts with high affinity receptors belonging to the tyrosine kinase (trk) receptor family (Barbacid 1994). Three trk receptors have been identified, each specific for a different neurotrophin: trkA binds NGF (Kaplan *et al.* 1991), trkB is specific for BDNF and NT-4/5 (Klein *et al.* 1989) while NT-3 binds preferentially to trkC (Lamballe *et al.* 1991). All 3 trk receptors are distributed to discrete but overlapping subpopulations of primary sensory neurons

(McMahon *et al.* 1994; Bennett *et al.* 1996), while trkB and trkC are also present in spinal motorneurons (Ernfors *et al.* 1993).

NGF is specific for a subset of primary sensory neurons and sympathetic neurons. It is produced by the target organs (Bandtlow *et al.* 1987), from where it is delivered to the neuronal cell bodies by retrograde axonal transport following binding to trkA receptors and internalisation by the nerve terminals (Lewin & Barde 1996). Following crush or transection of the peripheral sciatic nerve, there is dramatic increase in NGF, which is sustained over the period of axonal regeneration (Hengerer *et al.* 1990; Seniuk 1992). The high affinity trkA receptors are down regulated whilst there is a massive upregulation of low-affinity p75 receptors and NGF mRNA in SC (Taniuchi *et al.* 1986; Seniuk 1992). This is induced by the lack of normal axonal contact with SC and is later suppressed by contact with regenerating axons (Taniuchi *et al.* 1988). It is postulated that normal, intact, sensory and sympathetic nerves are the usual targets of NGF, but denervated SC may be the primary target of NGF following nerve injury (Raivich *et al.* 1991; Seniuk 1992). After axotomy exogenous administration of NGF has resulted in sustained axonal regeneration (Terenghi 1999).

BDNF is synthesised in adult primary sensory neurons (Ernfors *et al.* 1990; Thompson *et al.* 1999). Axotomy increases the synthesis and anterograde transport of BDNF from sensory neurons (Tonra *et al.* 1998) acting as an anterograde trophic messenger. Skeletal muscle also expresses BDNF mRNA and the released BDNF binds to a trk receptor expressed in the periphery by alpha motor neurones. BDNF has been shown to reduce

motorneuron death after axotomy in neonatal animals (Yan *et al.* 1992) and in adults after ventral root avulsion (Novikov *et al.* 1995). Administration of exogenous BDNF to the cut sciatic nerve has been shown to improve functional recovery (Lewin *et al.* 1997).

NT-3 is functionally distinguished from NGF and BDNF by its weak activity on sympathetic neurones. NT-3 induces survival and differentiation in sensory and parasympathetic neurones (Henderson *et al.* 1993). It is present in significant amounts in adult skeletal muscle (Griesbeck *et al.* 1995), exerting a trophic role for sensory neurons innervating muscle spindles and for motorneurons (Ernfors *et al.* 1994). Following axotomy targeted administration of NT-3 to the cut sciatic nerve using fibronectin mats improves the rate and amount of axonal regeneration (Sterne *et al.* 1997a).

NT-4/5 binds to the trkB receptor expressed by most rat retinal ganglion cells and motorneurons (Escandon *et al.* 1994) and following nerve injury SC from the distal stump upregulate the synthesis of NT4/5. NT-4/5 enhances the survival of injured retinal ganglion cells (Sawai *et al.* 1996), acts as an extremely potent survival factor for motorneurons (Henderson *et al.* 1993) and increases the ability of motorneurons to innervate skeletal muscle fibres in co-cultures of rat spinal cord and human muscle (Braun *et al.* 1996).

In addition to the neurotrophin family a growing number of neurotrophic factors have been identified including ciliary neurotrophic factor (CNTF), glial derived neurotrophic factor (GDNF) insulin-like growth factors, fibroblast growth factors, neuropoietic

cytokines and the neuregulins (Longo *et al.* 1984; Glasby *et al.* 1986a; Liuzzi & Tedeschi 1991; Seniuk 1992; Tham *et al.* 1997; Thanos *et al.* 1998; Yin *et al.* 1998; Bryan *et al.* 2000).

1.4.2 Glial growth factor

Glial growth factor (GGF) is a trophic factor specific for SC rather than neurons, but it has a significant role in the reciprocal neuron-glia interaction (Terenghi 1999). GGF was originally described in brain and pituitary extracts (Raff *et al.* 1978; Brockes 1983) and was later identified as a basic, heat-resistant 31-kDa protein (Lemke & Brockes 1984). Three forms of GGF have been isolated; GGF-I, a 34-kDa species with properties similar to the previously identified molecule, GGF-II, and GGF-III, of 59kDa and 45kDa, respectively (Goodearl *et al.* 1993). Subsequent purification showed that GGF is a group of proteins encoded by differentially spliced transcripts of a single gene (Goodearl *et al.* 1993; Marchionni *et al.* 1993), which belong to a larger family including the structurally related heregulins (Holmes *et al.* 1992), the neu differentiation factor (Wen *et al.* 1992) and the acetylcholine receptor inducing activity protein (ARIA) (Falls *et al.* 1993). These proteins are known collectively as neuregulins and they stimulate phosphorylation of the heterodimers of erbB2, erbB3 and erbB4 receptors (Carraway & Burden 1995). Neuregulin mRNA is expressed by primary sensory neurons, motorneurons and sympathetic neurons (Marchionni *et al.* 1993).

During development GGF is trophic for SC precursors, stimulates SC proliferation (Dong *et al.* 1995) and is critical for the survival of SC in developing neuromuscular junctions (Trachtenberg & Thompson 1996). All three isoforms of GGF are potent mitogens for rat SC *in vitro* at nanomolar concentrations, whereas at lower concentrations they promote SC survival, in the absence of cAMP elevating agents (Glasby *et al.* 1986a).

The continued expression of neuregulins in adults suggests an additional role in peripheral nerve maintenance and/or regeneration (Chen *et al.* 1994). Mahanthappa *et al.* demonstrated that rhGGF-II exerted multiple effects on mature SC *in vitro.* At doses submaximal for proliferation (2.5ng/ml), rhGGF-II solely promoted SC migration. At higher doses (500ng/ml) rhGGF-II drove SC proliferation. At concentrations greater than necessary to saturate the mitotic response, there was induction of neurotrophic factor secretion by SC (Mahanthappa *et al.* 1996). Hence GGF may promote neuronal survival and proliferation indirectly, by promoting glial cell-neuron interaction (Reynolds & Woolf 1993). Indeed, following rat sciatic nerve crush, treatment with rhGGF-II promoted nerve regeneration, as measured by functional recovery and by qualitative assessment of nerve morphology (Chen *et al.* 1998).

After axotomy the expression of erbB2 and erbB3 receptors on SC in the distal stump is rapidly upregulated and correlates with the induction of mRNAs encoding GGF in the nerve and the onset of SC DNA synthesis (Li *et al.* 1997; Bryan *et al.* 2000). However, chronic denervation of SC *in vivo* results in downregulation of c-erbB receptors and

poor regeneration when an acutely transected proximal stump is anastomosed to the denervated distal stump (Li *et al.* 1997). Hence these results underline the axonal-glial interdependence after nerve injury, as the persistence of responsive SC in the distal stump is dependent on axonally derived trophic signalling and in turn the responsive SC provide trophic support for the regrowing axons.

1.5 PERIPHERAL NERVE REPAIR

Peripheral nerve injuries are common and the injury may be traumatic, iatrogenic, inflammatory, metabolic or infectious. These differing aetiological factors lead to a common pathway involving disruption in the continuity or function of axons and hence the loss of conduction of nerve impulses. In traumatic injury this problem maybe further compounded by the actual loss of axonal tissue resulting in the presence of an irreducible gap between the ends of the proximal and distal stumps. The clinical outcome from nerve repair and reconstruction today is not much different from 25 years ago, with complete recovery of sensory and motor function being a rarity.

Functional recovery following nerve injury depends upon the number of new nerve fibres reaching the periphery and undergoing maturation; the speed by which the new fibres reach the periphery, thereby limiting the amount of end organ atrophy; and the cortical reorganizational processes in the somatosensory and motor brain cortex. The aim of nerve repair is to optimise the first two conditions, hence minimising the permanent disability suffered by the patient. The type of nerve repair performed is

dependent on whether the two ends of the severed nerve can be brought together in a tension free manner. Nerve gaps are generally divided into short gaps of up to 2cm and long gaps greater than 2cm (Terzis & Smith 1990b). With short nerve gaps the proximal and distal stumps can be mobilised and a tension free end-to-end coaptation can be performed. Long nerve gaps require the use of additional material to bridge the gap between the proximal and distal stumps. As discussed previously three main factors are required for nerve regeneration. The first requirement is a channel to grow down, the second is the presence of appropriate cellular elements such as SC and macrophages and the third is the provision of neurotrophic and neurotropic factors.

1.5.1 Historical perspective

For many centuries surgeons avoided touching the nerve stumps of divided nerves, for the fear of causing convulsions and some tried to achieve indirect closure by approximating the surrounding soft tissue (Omer *et al.* 1997). The first recorded nerve repair took place in the thirteenth century (Browne 1951; McCarthy 1998). The initial results were very variable but some degree of success was claimed in isolated cases. The subsequent five centuries saw a steady increase in the knowledge and understanding of the structure and function of the peripheral nervous system. During the nineteenth century researchers published accurate descriptions of peripheral nerves and their response to injury that are still valid today (Waller 1850; Büngner 1891; Cajal 1928; Snyder 1980). As knowledge increased the repair of divided nerves became more commonplace, but surgical outcome was still poor and the limiting factor was the

available materials in terms of sutures and instrumentation (Huber 1895). Many of the suture materials produced a foreign body reaction that is now known to be detrimental to nerve regeneration (Sunderland 1984; Glasby *et al.* 1990). Also the lack of operating microscopes and accompanying microinstruments meant that the accuracy of coaptation and the placement of sutures were not optimal (Orgel 1984).

In the nineteenth century as surgeons became more confident they went to great lengths to achieve end-to-end coaptation, perhaps unaware of the consequences. Mobilisation of the proximal and distal stumps followed by stretching of the nerve and hence suturing under tension was common. When the proximal and distal nerve stumps could not be brought together by mobilisation alone various methods, some very drastic, were developed to allow the surgeon to repair the nerve directly. These included flexion of adjacent joints, bulb suture, nerve re-routing and even bone shortening (Richardson 1886; Fields *et al.* 1989; Huber 1895; Seddon 1947; Sunderland 1978). Post-operative immobilisation of adjacent joints in flexion followed by gradual extension with concomitant elongation of the nerve failed because scars formed at the site of the repair (Highet & Sanders 1943; Seddon 1963). This precluded good axonal regeneration and hence a useful functional outcome (Millesi 1984; Zhao *et al.* 1992). Even if a direct repair could not be performed by these early methods, the attempts to regain axonal continuity were conceptually similar to those utilised by surgeons today. These fall into two broad categories. The first is the use of a nerve graft to bridge the gap. The second is the use of a nerve conduit to guide the regenerating axons from the proximal to the distal stump.

1.5.2 Nerve grafting

Nerve grafts may either be *full-thickness grafts*, where a nerve of equal or larger diameter than the host nerve is used, or *cable grafts*, where a graft of equal diameter to the host nerve is attained by the use of a bundle of small diameter nerve pieces Nerve autografts are currently used for clinical applications whereas allografts and xenografts have been explored experimentally.

The first successful nerve graft was performed in 1870 by Philipeaux and Vulpain when they grafted the hypoglossal nerve of a dog (Huber 1895; Millesi *et al.* 1972). Albert in 1878 carried out the first human nerve graft, when he used an allograft to bridge a defect created by tumour resection (Huber 1895; Browne 1951). Over the next century more surgeons began to use nerve grafting for the management of long gaps although the results varied greatly (Huber 1895; Huber 1919). The outcome depended on whether xenografts, allografts or autografts were used (Sanders 1942; Seddon 1947). Sanders in 1942 concluded that autografts were the most successful type of nerve graft, after observing an acute tissue reaction around allografts (Sanders 1942; Sanders & Young 1942). Although autografts produced better results than allografts and xenografts the outcome was still considered to be poor and the benefits did not outweigh the loss of functioning donor nerve, which resulted in some strong opposition (Platt 1919; Stopford 1920; Sanders 1942; Seddon 1947; Millesi 1981; Millesi 1984).

However, as more reports were received of consistent and beneficial outcomes after peripheral nerve autografting, the technique began to gain favour after the publication of a successful series (Huber 1919). Soon after, Bunnell reported success in digital nerve grafting (Bunnell 1927), shortly followed by the description of successful facial nerve grafting (Ballance & Duel 1932). During World War II nerve autografting was increasingly used (Seddon 1954).

In 1947, Seddon published a report on a series of 58 peripheral nerve autografts and concluded that nerve autografting was the best option for the management of long gaps and that the recovery was as good as direct end-to-end suture in about half of his cases (Seddon 1947). After introduction of microsurgery techniques in the 1960s and the improvement of suture materials, the results of nerve autografting improved, indicating that suboptimal coaptation and suture line foreign body reaction and scarring contributed to the poor results. (Kurze 1964; Millesi *et al.* 1972; Millesi 1973; Orgel 1984).

Pioneering work by Millesi developed the concept of interfascicular and group fascicular nerve grafting which are in routine clinical practice today (Millesi *et al.* 1972; Millesi 1981; Millesi 1984). As well as these free non-vascularised grafting techniques, pedicled nerve grafts and free vascularised nerve grafts have been described and developed for particular situations (Strange 1947; Taylor & Ham 1976). Vascularised nerve grafts have been used when the vascular bed is poor and when long gaps have to be bridged. There is still conflicting clinical evidence as to whether an epineural suture

is better than a fascicular repair and a non-vascularised graft is better than a vascularised graft (Orgel 1984; Mani *et al.* 1992; Best & Mackinnon 1994).

Independent of the repair technique the recovery following nerve grafting depends on a number of factors: the type of nerve involved, the level of the injury, the delay at the time of repair, a healthy soft tissue bed, an adequate trimming of the proximal and distal stumps, a graft longer than the defect, a graft with a cross-sectional area at least as large as the recipient nerve, an accurate and tension free coaptation, avoidance of sepsis, associated morbidities and the age of the patient (Seddon 1947; Vanderhooft 2000). Generally the results of nerve grafts in the upper limb are better than those in the lower limb (Donzelli *et al.* 1998).

There has been increasing interest in developing an alternative method to nerve autografting in order to overcome the associated problems. The harvesting of a nerve graft results in co-morbidity for the patient, including a further wound site, loss of sensation and the possibility of painful neuronal formation. There are limited cutaneous sensory nerves available for harvesting and often they are of a smaller calibre requiring the autograft to be cabled. Despite advances in microsurgical techniques, instrumentation and sutures, full functional recovery is never achieved (Mackinnon & Dellon 1988a). Surveys of the clinical literature have shown that in patients undergoing either a direct end-to-end coaptation or a nerve autograft for the repair of the median nerve at the level of the wrist, less than 25% recovered complete voluntary motor function and less than 3% full normal sensation, 5 years after repair. (Young *et al.* 1980;

Dellon & Jabaley 1982; Beazley *et al.* 1984; Mackinnon & Dellon 1988a). These results are significantly worse the more proximal the injury.

1.5.3 Nerve Conduits

The concept of nerve conduits and tubulisation is an alternative to nerve autografting. The materials used are either naturally tubular or can be fashioned into the form of a tube. They can be divided into biological and non-biological, degradable and non-degradable materials. Theoretically tubulisation techniques offer several advantages, such as reducing invasion by connective tissue and scarring of the nerve, reducing adhesions, aiding guidance of growing fibres along appropriate paths by mechanical orientation, discouraging the formation of neuromas, confining trophic factors to the site of regeneration, directing vascularizaiton longitudinally from intraneural vessels, preventing displacement or rotation of nerve stumps, providing a means of administering adjuncts to promote regeneration, providing a quicker surgical procedure and the possibility to have "off-the-shelf" prostheses (Fields *et al.* 1989). Currently however, only a few of these can be achieved in practice.

The experimental use of nerve conduits was first reported by Gluck in 1880 followed by Vanlair in 1882 (Fields *et al.* 1989). Both were unsuccessful and used decalcified bone grafts as conduits for axonal regrowth. A decade later Büngner used a segment of artery to repair the hypoglossal nerve of a dog with some success (Büngner 1891; Huber 1895; Fields *et al.* 1989). Kirk & Lewis described the use of a tube constructed from fascia for

grafting a 1cm gap in the sciatic nerve of a dog (Kirk & Lewis 1915). The interest in conduits waned until a report by Weiss re-kindled interest in the field (Weiss & Taylor 1946). In 1948 Mattson bridged nerve gaps of 5-14mm in the monkey posterior tibial nerve with tantalum and nylon fibres and an outer vein ensheathment. Nerve regeneration and functional recovery were documented histologically and electrophysiologically (Matson *et al.* 1948). For structural reasons blood vessels were an obvious choice (Walton *et al.* 1989) and were used with a number of modifications (Benito-Ruiz *et al.* 1994; Ferrari *et al.* 1999). The results were still inferior to nerve grafts and the technique has been mainly restricted for the repair of short gaps (Chiu 1999). Muscle grafts attracted interest as the basal lamina of the myofibril is similar to that of the nerve and can guide regenerating axons (Fawcett & Keynes 1986). It was shown that pre-degeneration of the muscle graft (Glasby *et al.* 1986b; Hall 1997) was necessary and that the method of pre-degeneration was important (Whitworth *et al.* 1995a). Further improvements on regeneration were seen when the nerve and muscle were combined as a sandwich graft (Calder & Green 1995; Whitworth *et al.* 1995b). Encouraging results have also been reported using the combination of veins filled with muscle grafts (Battiston *et al.* 2000a; Battiston *et al.* 2000b). However, the overall results of the previous methods are still not satisfactory especially for long nerve defects (Hems & Glasby 1993).

The first report of the use of a non-biological material for gap repair was by Bassett in 1959, when a millpore tube was used to bridge nerve gaps of 1-2.5cm in the cat sciatic nerve (Bassett *et al.* 1959). Other non-biological materials used for making conduits

included magnesium, formalin treated casein, gelatin, rubber, and cellulose (Fields *et al.* 1989). More recently the silicone tube model was introduced which proved to be an important tool for studying the basic biological mechanism of nerve regeneration. Silicone conduits support regeneration across a 1cm gap in the rat (Lundborg *et al.* 1982b) but when used clinically, they require a second surgical procedure for removal after regeneration, because of the chronic nerve compression and irritation caused by the rigidity of the material (Lundborg *et al.* 1994b; Lundborg *et al.* 1997a). Empty silicone tubes are unable to support regeneration across gaps longer than 1cm (Lundborg *et al.* 1982b) but the addition of fibronectin-laminin significantly enhanced regeneration across a 1.8cm gap in the rodent model (Woolley *et al.* 1990; Bailey *et al.* 1993). Interposing nerve segments in a silicone conduit also supported nerve regeneration across a 1.5cm gap (Francel *et al.* 1997).

The most promising conduits have been biodegradable, manufactured from naturally occurring or synthetic substances (Fields *et al.* 1989; Doolabh *et al.* 1996; Strauch 2000). Advances in material engineering and improved production techniques have also enabled surface texture modifications which have shown to be important in guiding cellular migration (Buehler *et al.* 1990; Curtis & Wilkinson 1997; Curtis & Wilkinson 2001) and regenerating axons (Guenard *et al.* 1991; Miller *et al.* 2001). Biodegradable conduits shown to be useful in nerve gap repairs are fibronectin, precipitated from blood and shaped into mats (Whitworth *et al.* 1995c), collagen (Archibald *et al.* 1991; Archibald *et al.* 1995; Madorsky *et al.* 1998; Kitahara *et al.* 2000), polyglactin (Molander *et al.* 1982), polylactic acid (Evans *et al.* 1999), polyglycolic acid (Lee *et al.*

1995; Matsumoto *et al.* 2000), their co-polymer (Hadlock *et al.* 1999), polyester urethane (Borkenhagen *et al.* 1998), polytetrafluoroethylene (Pogrel *et al.* 1998; Miloro & Macy 2000; Vasconcelos & Gay-Escoda 2000), as well as inorganic materials (Den Dunnen *et al.* 2000). Clinically polyglycolic acid conduits have been used for digital nerve repair (Weber *et al.* 2000). When conduits were used for digital nerve gaps of less than 4mm or more than 8mm, there was improved sensory recovery compared with primary or autograft repairs (Weber *et al.* 2000).

The ideal conduit has as yet not been identified, but the desirable material should be inert, biocompatible, bioabsorbable, flexible, easy to handle with a good tensile strength. Regardless of the material used the dimensions of the tube affect nerve regeneration significantly, as optimal regeneration occurs when the internal cross-sectional area is 2.5-3 times that of the nerve (Ducker & Hayes 1968). If the diameter is to small, chronic nerve compression may occur resulting in nerve damage, while if the fit is too loose there may not be sufficient support for the regenerating nerve, as vital neurotropic and neurotrophic factors will leak out and infiltration of fibroblasts may occur. The thickness of the conduit wall also affects regeneration, a wall thickness between 0.13-1.65mm promotes regeneration and thinner tubes are associated with less likelihood of neuroma formation proximally because of greater elasticity of the thin wall (Ducker & Hayes 1968).

1.5.4 Polyhydroxyalkatone conduits

Polyhydroxyalkatones (PHAs) comprise a large class of polyesters that are synthesized by many bacteria as an intracellular carbon and energy compound. They gained particular interest since they were shown to be biodegradable and biocompatible (Brandl *et al.* 1990). Both properties are best achieved by production in bacteria, thus, guaranteeing complete stereospecificity, which is essential for their biodegrability and biocompatibility. The type of bacterium and growth conditions determine the chemical composition of PHAs and the molecular weight, which typically ranges from 2 x 10^5 to 3 x 10^6 Da (Byrom 1987; Lee 1996). Poly-3-hydroxybutyrate (PHB) is the most widely spread PHA and only in 1983 PHAs with longer side chains were first reported (de Smet *et al.* 1983). Today PHAs are separated into three classes: short chain length PHAs (C3-C5), medium chain length PHAs (C6-C14) and long chain length PHAs (>C14). Of special interest are functionalised groups in the side chain that allow further chemical modifications (Kessler *et al.* 2001; Kim & Lenz 2001). The physical and chemical properties of PHAs depend on the monomeric composition that is determined by the producing microorganism and their nutrition, the length of the chain and its functional group.

The degradation of short chain length PHAs *in vitro* and *in vivo* is very slow probably due to the high crystallinity. Thus, it is reasonable that the less crystalline medium chain length PHA is a better candidate for medical applications that require rate-controlled

degradation. However, limited research has been undertaken on the medium chain length PHAs due to their limited availability on the market (Zinn *et al.* 2001).

Potential medical applications of PHAs include: 1) Wound management – sutures, skin substitutes, nerve cuffs, surgical meshes, staples, swabs 2) Vascular system – heart valves, pericardial patches, vascular grafts 3) Orthopaedics – scaffolds for cartilage engineering, bone graft substitutes, internal fixation devices 4) Drug delivery – micro and nanospheres for anticancer therapy and 5) Urology – urological stents.

A feature of PHAs which makes them an attractive candidate for use as scaffold materials in tissue engineering is their surface structure (Zinn *et al.* 2001). Porous surfaces can be produced by the leaching technique, which is done by blending of PHA with a salt that can be washed out with water. Also the surface of PHA materials can be rendered more hydrophilic by treatment with allyl alcohol gas plasma that results in an increase of wetability (Mas *et al.* 1997). Also further modifications may be obtained by blending with other polymers.

One of the key physical properties of the conduit wall is its porosity, which affects the diffusion of soluble factors into and out of the guidance channel. Aebischer *et al.* demonstrated that semipermeable channels with a molecular weight cut off of 50kD demonstrated improved regeneration compared with impermeable silicone tubes, whereas semipermeable channels with a molecular weight cut off of 100kD resulted in impaired nerve regeneration (Aebischer *et al.* 1989). These results suggest that

semipermeable channels permit the influx of necessary nutrients and growth factors from the external environment, but at high porosities (ie 100kD cut off), this influx includes inhibitory molecules that are detrimental to regeneration. Thus, using PHA a semipermeable guidance channel could be engineered with an optimal porosity to permit the diffusion of beneficial growth factors into the guidance channel while simultaneously excluding inhibitory molecules.

An interesting aspect of PHA scaffolds is the fact that the tissue-engineered cells can be implanted together with the supporting scaffold (Williams *et al.* 1999). Sodian and coworkers used PHA for the fabrication of a tri-leaflet heart valve scaffold (Sodian *et al.* 2000). A porous structure was produced and the scaffold was seeded with vascular cells, which formed a confluent layer and the scaffold was moulded completely and fixed to a model conduit.

PHAs have also been used as drug carriers due to their biodegrability and biocompatibility. Researchers demonstrated that microspheres of PHA loaded with rifampicin resulted in the release of 90% of the drug within 24 hours. However, by altering the drug loading and particle size the drug release rate could be controlled. (Kassab *et al.* 1997). Yagmurlu *et al.* implanted PHA rods loaded with antibiotics into a rabbit tibia to achieve successful treatment of chronic osteomyelitis (Yagmurlu *et al.* 1999). It is possible by altering the length of the PHA chain and its porosity that the drug release rate can be adjusted. Therefore these tailor made PHA micro-, nanospheres

and rods provide an alternative method for loading the conduit lumen with neurotrophic factors, which are then released at a predefined rate.

1.5.5 Poly-3-hydroxybutyrate conduits

In 1926 poly-3-hydroxybutyrate (PHB) was discovered in *Bacillus megaterium*, making it the first member of the PHA family to be identified. It is a biodegradable polymer that can be easily produced *in vitro*, as PHB granules are a natural storage product of bacteria and algae being found in their cytoplasm. The polymer is manufactured by a fermentation process followed by solvent extraction to produce a pure polymer, which can be extruded and moulded into the desired shape (Holmes 1988). PHB can also be produced from carbon substrates as diverse as glucose, ethanol, acetone, methane and gaseous mixtures of carbon dioxide and oxygen (Anderson & Dawes 1990). *In vivo* PHB is degraded by hydrolysis, Lysosomal enzymes and non-specific esterases to (R)-3-hydroxybutanoic acid, which is a normal metabolite in human blood (Adams *et al.* 1987) and it is completely resorbed within 24 to 30 months (Holmes 1988; Malm *et al.* 1994; Malm *et al.* 1999). It is also found in the cell envelope of eukaryotes e.g. yeast, peanut, spinach (Reusch 2000). *In vivo* testing has shown that PHB is biocompatible, non-antigenic, non-cytotoxic, bioabsorbable, easy to handle with a good tensile strength (Holmes 1988; Anderson & Dawes 1990; Malm *et al.* 1994; Malm *et al.* 1999). PHB sheets (Astra Tech®, Gothenberg, Sweden) are manufactured from individual PHB fibres with a diameter ranging from 2-20μm and within each sheet the fibres are orientated unidirectionally. Previous studies in our laboratory have demonstrated that

longitudinal alignment of these fibres with the direction of regenerating axons aids neuronal and glial growth by contact guidance and mechanical orientation (Hazari *et al.* 1999a; Hazari *et al.* 1999b; Young *et al.* 2002). SC align themselves along the longitudinal fibres, preferentially propagating in the longitudinal axis and thereby facilitating the regrowth of axons through the conduit (Hazari *et al.* 1999b). PHB has been used successfully as a wrap around repair technique for sharply divided nerves, resulting in neuronal survival, nerve regeneration and an inflammatory response comparable to the standard method of epineural suturing used for primary nerve repair (Hazari *et al.* 1999b). This provides an effective alternative to epineural repair, which is easier, simpler and quicker, allowing the nerve to repair by its intrinsic capacity, rather than attempting to surgically align the traumatized fascicles correctly with the risk of further damage (Hazari *et al.* 1999b). A multi-centre clinical randomised control trial has been started using PHB as a wrap around repair for sharply divided peripheral nerves. PHB conduits have also been used for the repair of a 10mm gap in the rat sciatic nerve model where good regeneration and biocompatibility was again demonstrated (Hazari *et al.* 1999a). More recently long nerve gaps in the rabbit common peroneal nerve have been repaired with empty PHB conduits (Young *et al.* 2002). These studies demonstrated that the PHB conduits became vascularised at an early stage after implantation, which should aid regeneration by delivering the relevant cells and nutrients to the regenerating nerve (Young *et al.* 2002). PHB does not produce excessive fibrosis or inflammatory response (Seddon 1963; Hazari *et al.* 1999b) and it has already been used clinically in cardiovascular surgery, to provide a scaffold for regenerating pericardial tissue (Duvernoy *et al.* 1995).

1.5.6 Nerve conduits for long gap repair

A nerve gap is classified as long when it exceeds 2cm (Terzis & Smith 1990b). The majority of published research on peripheral nerve regeneration has used the rat sciatic nerve model to investigate direct repair and short gap grafting (Den Dunnen *et al.* 1993; Longo *et al.* 1984; Whitworth *et al.* 1995a; Whitworth *et al.* 1995c). Although this is a useful experimental model to test a given hypothesis, the intrinsic disadvantages are the prodigious nerve regeneration capacity of rats and the relatively short length of nerve that is available to create a nerve gap (Grabb *et al.* 1970; Mackinnon *et al.* 1985; Millesi 1985; Hems & Glasby 1992; Amillo *et al.* 1995).

One of the more commonly investigated biological conduits for the repair of long nerve gaps is the freeze-thawed-muscle autograft. Glasby *et al.* used freeze-thawed-muscle autografts to repair a 3cm gap in the marmosets ulnar nerve. By six months functional, electrophysiological and morphological examination demonstrated successful axonal regeneration (Glasby *et al.* 1986b). Freeze-thawed-muscle autografts have been shown to be effective conduits across nerve gaps of upto 4cm in the rabbit common peroneal nerve (Fawcett & Keynes 1986; Whitworth *et al.* 1995b; Lenihan *et al.* 1998b), but various studies would suggest that the upper limit for spontaneous regeneration through an empty biological conduit is 4cm (Glasby *et al.* 1986b; Glasby *et al.* 1990; Hems & Glasby 1992; Hems & Glasby 1993; Lawson & Glasby 1998).

In the primate model both pseudosynovial sheaths and polyglycolic acid tubes have supported regeneration across a 3cm gap in the ulnar nerve (Dellon & Mackinnon 1988; Mackinnon *et al.* 1985). Glycolide trimethylene carbonate conduits (Maxon®) have resulted in successful regeneration across a 2cm gap in the monkey ulnar nerve (Mackinnon & Dellon 1990a). However, on increasing the nerve gap to 5cm the regeneration seen in the Maxon® conduits was very poor (Mackinnon & Dellon 1990a). This research would again support the view that regeneration in empty tubes is limited to less than 5cm in the primate model.

In the rabbit common peroneal model vein conduits support functional axonal regeneration across gaps of up to 3cm (Strauch *et al.* 1996). Using the same model PHB conduits support axonal regeneration across 4cm nerve gaps, but do not result in successful target organ reinnervation (Young *et al.* 2002). A clinical study carried out by Stanec & Stanec used polytetrafluoroethylene tubes for the repair of median and ulnar nerve gaps of 1.5 to 6cm (Stanec & Stanec 1998). Patients with nerve gaps of 1.5 to 4cm had a 79% recovery of motor and sensory function, whilst those with nerve gaps of 4.1 to 6cm only had a 13% recovery.

These empty biological and synthetic nerve guides provide contact guidance through topographical and microgeometric cues, which direct and orientate the growth of the migrating SC and regenerating axons. This may be a property of the conduit which is due to the manufacturing process (Whitworth *et al.* 1995c; Dubey *et al.* 1999; Hazari *et al.* 1999a; Hadlock *et al.* 2000) or to post-manufacturing modification such as the

addition of microgrooves to the surface. Alternatively guidance fibres can be added to the lumen of the conduit. Matsumoto *et al.* used a conduit made of polyglycolic acid mesh coated with collagen and filled with laminin-coated collagen fibres to bridge an 80mm gap in the peroneal nerve of a dog. Electrophysiological and histological restoration was obtained by 12 months (Matsumoto *et al.* 2000). This is the only report of successful functional regeneration through an artificial conduit greater than 5cm in length. Suzuki *et al.* used a polyglycolic acid conduit filled with freeze-dried alginate gel to bridge a 5cm gap in the cat sciatic nerve model. Functional reinnervation of motor and sensory nerves had occurred 13 weeks after implantation and by 7 months the material had completely disappeared (Suzuki *et al.* 1999).

Sandwich grafts have also been used to enhance long gap peripheral nerve regeneration, where segments of nerve autograft are interspersed within the conduit. Initial work demonstrated beneficial effects of using muscle/nerve sandwich grafts to bridge 2.4cm gaps in the rat sciatic nerve (Maeda *et al.* 1993). A freeze-thawed muscle/nerve sandwich graft also allowed regeneration across a 5cm gap in the common peroneal nerve of the rabbit, giving similar results to the nerve autograft controls (Whitworth *et al.* 1995b). The advantage of this technique is the presence of native basement membrane and Schwann cells to facilitate regeneration, but the drawback is the multiplicity of suture lines, which axons must negotiate.

It is commonly acknowledged that further improvement of surgical technique alone will not improve the results of nerve repair. Instead a complete understanding of the

pathophysiological events of degeneration and regeneration are required, in order to manipulate correctly the microenvironment of the nerve injury and to obtain optimal nerve regeneration.

1.6 DELIVERY OF NEUROTROPHIC FACTORS

The role of neurotrophic factors in peripheral nerve regeneration is becoming more apparent (Seniuk 1992; Rush *et al.* 1995; Yin *et al.* 1998). Manipulation of the local environment has met with success in improving regeneration over short gaps (Whitworth *et al.* 1996; Sterne *et al.* 1997a; Sterne *et al.* 1997b; Tham *et al.* 1997; Bryan *et al.* 2000). Despite the interest shown by investigators in the concept of nerve guidance, little attention has been given to finding a suitable system for the delivery of neurotrophic factors to the site of injury. Ideally delivery needs to be over a protracted time course that promotes maximal and directed growth using a system, which will ultimately be clinically applicable.

Neurotrophic factors have commonly been delivered to the regenerating nerve in physiological solutions loaded into the lumen of a hollow conduit (Rich *et al.* 1989; Hollowell *et al.* 1990; He *et al.* 1992; Derby *et al.* 1993; Bradshaw *et al.* 1993; Vejsada *et al.* 1994; Spector *et al.* 1995). Whilst this method allows initial concentrations to be high, the concentration falls dramatically over a period of days as diffusion into the surrounding tissues and breakdown by local enzymes occurs. Another approach has been to impregnate the factors into the wall of a biodegradable conduit such as

fibronectin and allow them to diffuse into the regenerative milieu over time in a bioactive form (Whitworth *et al.* 1995d; Whitworth *et al.* 1996; Sterne *et al.* 1997a; Simon *et al.* 2000). However, this is unsuitable for long gap repairs as the fibronectin is degraded before adequate axonal regeneration and maturation has taken place. An alternative method has been the use of mini-osmotic pumps to deliver the neurotrophic factors (Seckel *et al.* 1995). However, these are cumbersome to implant, are easily dislodged, may evoke a foreign body response and require additional surgery to remove after complete regeneration. Systemic administration (Verge *et al.* 1990) either oral, intravenous, intramuscular, subcutaneous or intraperitoneal is not practical, as it requires high concentrations of neurotrophic factors to be used and may result in systemic side effects

Matrigel has been used as a matrix for the slow release of growth factors *in vivo* (Hobson 2002) as well as for SC transplantation. However, it is a basement membrane extracted from sarcoma cell lines thereby making it unsuitable for clinical application. Collagen, fibrin and hyaluronic acid have all been used as matrices for the transplantation of SC (Guenard *et al.* 1992; Kim *et al.* 1994; Silverman *et al.* 1999; Hu *et al.* 2000; Rodriguez *et al.* 2000) and could also be potentially used for the delivery of neurotrophic factors. However, collagen, fibrin and hyaluronic acid are of animal origin again making them unsuitable for clinical use.

1.6.1 Alginate

Alginate hydrogel is a naturally derived water soluble polymer extracted from brown seaweed algae (Figure 1.1). Alginates constitute a family of unbranched binary co-polymers of 1,4-linked β-D-mannuronate (M) and 1,4-linked α-L-guluronate (G), of widely varying composition and sequence depending on the algae of origin (Figure 1.2). It is the proportion and distribution of these blocks (MM, GG, MG), which determines the various chemical and physical properties of the alginate such as viscosity, intrinsic inflexibility, swelling capacity, gelation and pore size (Smidsrod & Skjak-Braek 1990; Draget *et al.* 1997). The ability to adjust the chemical and physical characteristics of alginate by altering its composition allows the most ideal functional properties for neurotrophic factor delivery to be selected. The viscosity and gelation can be altered such that the alginate does not pour out of the conduit but also does not form a solid mass. Increasing the length of the polymer increases the viscosity and exposure to divalent cations such as Ca^{2+}, bridges the negatively charged guluronic acid residues resulting in gel formation. By increasing the number of G blocks the gel becomes stronger. The links in the G chain are not that flexible therefore the intrinsic inflexibility of the alginate molecules in solution increases in the order MG < MM < GG and the less flexible the alginate the less it swells. The pore size and distribution can be influenced by the alginate composition with high G content alginates having the most open pore structure, owing to their long G-blocks and short elastic segments. The correct pore size is essential to allow both the outward diffusion of growth factors as well as the passage of regenerating axons through the alginate.

Alginates contain pyrogens and antigenic materials such as proteins and complex carbohydrates, but for biomedical applications ultra-pure, endotoxin free forms are available. Mammalian cells lack enzymes to digest alginates but as calcium ions dissolve out of alginate hydrogel, the gel structure is lost and it is slowly excreted renally. By altering the alginate composition and or the availability of non-gel-inducing ions, such as Na^{2+} the degradation profile can be altered (Smidsrod & Skjak-Braek 1990; Draget *et al.* 1997). Previous studies have shown that they retain their approximate configuration and dimensions for at least 90 days (Rivard *et al.* 1995). However, by varying the proportion and distribution of the M and G blocks the degradation profile can be adjusted.

In vitro studies have suggested alginate as a suitable cell carrier matrix as it is porous (Eiselt *et al.* 2000) and retains extracellular matrix (Burgi-Saville *et al.* 1998) both of which are conducive to regeneration. Low viscosity mannuronate (LVM) alginate has been demonstrated to be more amenable to molecular diffusion (Amsden & Turner 1999) and allows for better alignment of individual molecules along the more flexible polymer chain (Moe *et al.* 1994) which are important for potential use as a neurotrophic factor delivery vehicle in addition to providing a scaffold to aid neuronal and glial growth. High mannuronic acid content alginate has been used *in vitro* as a SC matrix. The alginate supported both SC viability and function as well as neurite growth from chick embryo dorsal root ganglia (Mosahebi *et al.* 2001). The biocompatibility of high mannuronate residue alginate has been demonstrated both *in vitro* and *in vivo* (Klöck *et al.* 1997) and shown to produce a lower inflammatory reaction. Alginate sponges have

been used for *in vitro* cell cultures (Shapiro & Cohen 1997). They possessed structural and morphological properties appropriate for cell growth, proliferation and neovascularization. Alginate beads (Smidsrod & Skjak-Braek 1990) have been used *in vitro* as a vehicle for sustained release of endothelial cell growth factor (Ko *et al.* 1995).

Alginate has been successfully used *in vivo* for the controlled-release of various neurotrophic factors in the central nervous system (Maysinger *et al.* 1996). Beads, rods and tubes made from alginate hydrogel and loaded with leukaemic inhibitory factor (LIF) have been reported to release the growth factor at a rate of less than 1% per day for a period of several months (Austin *et al.* 1997). After serious muscle injury the insertion of these LIF containing rods within or close to the site of injury accelerated regeneration of the injured muscle (Austin *et al.* 1997). Suzuki *et al.* demonstrated that a conduit composed of freeze-dried alginate gel covered by a polyglycolic acid mesh, supported functional axonal regeneration across a 50mm gap in the cat sciatic nerve. By 7 months the material had completely disappeared (Suzuki *et al.* 1999).

Alginate is clinically applicable having been extensively used for the microencapsulation and transplantation of pancreatic islet cells in the treatment of diabetes mellitus, as well as for the transplantation of chondrocytes and hepatocytes.

The success of alginate as a growth factor delivery vehicle will rely on an appropriate chemical composition of alginate, which provides adequate sites for the attachment and sustained release of growth factors. As well as structural and morphological properties

that are appropriate for cell growth, proliferation, regeneration as well as neovascularization. The use of alginate fibres may possibly provide further enhancement for nerve regeneration by providing contact guidance. The mechanical properties should be such that the matrix can withstand the surgical procedure (Shapiro & Cohen 1997). With these factors in mind ultra pure, endotoxin free, low viscosity alginate with a high mannuronic content would be a suitable growth factor delivery matrix.

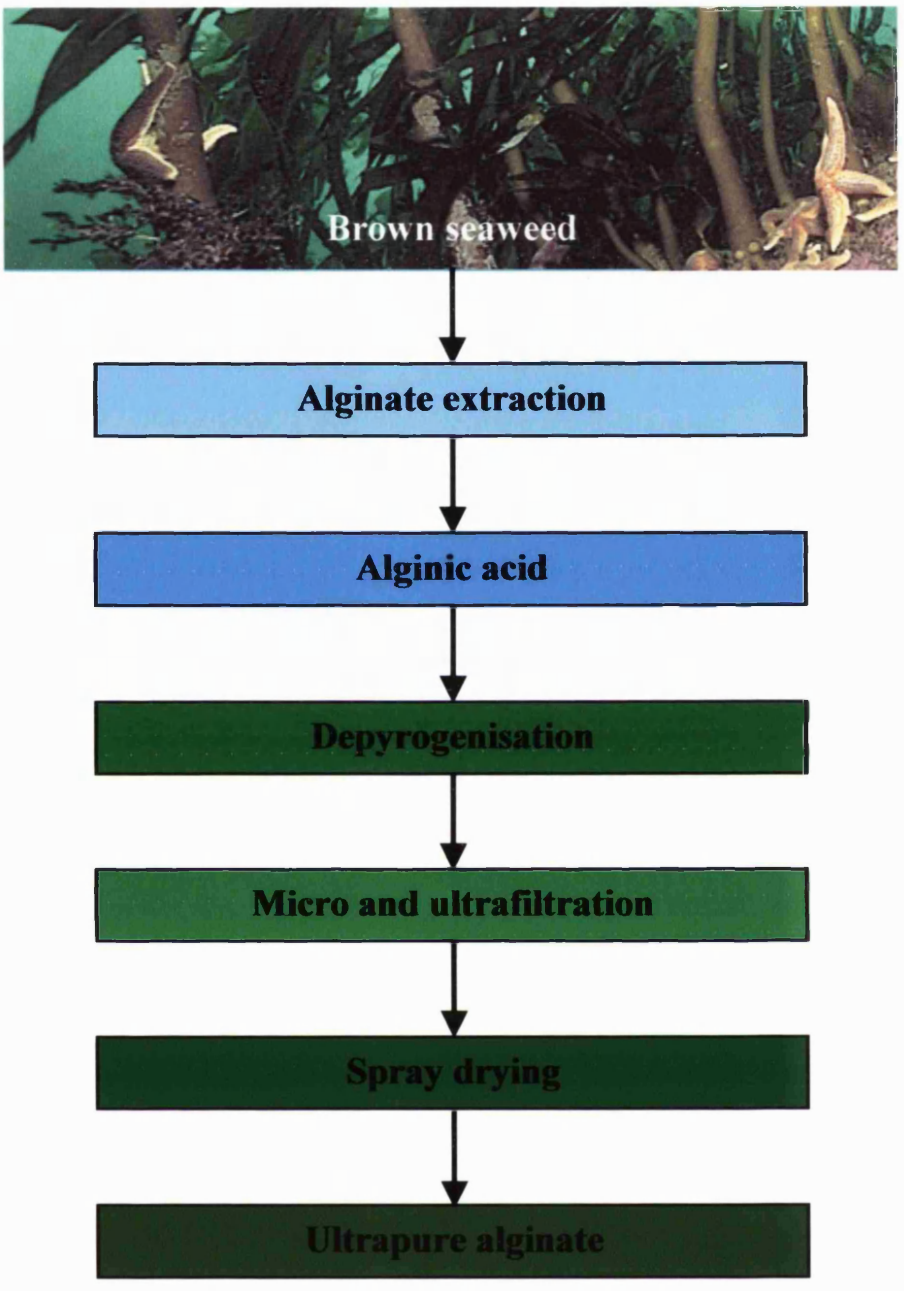

Figure 1.1 Extraction process of alginate from seaweed. Blue: crude production, green: purification process. (Adapted from Pronova literature, Norway).

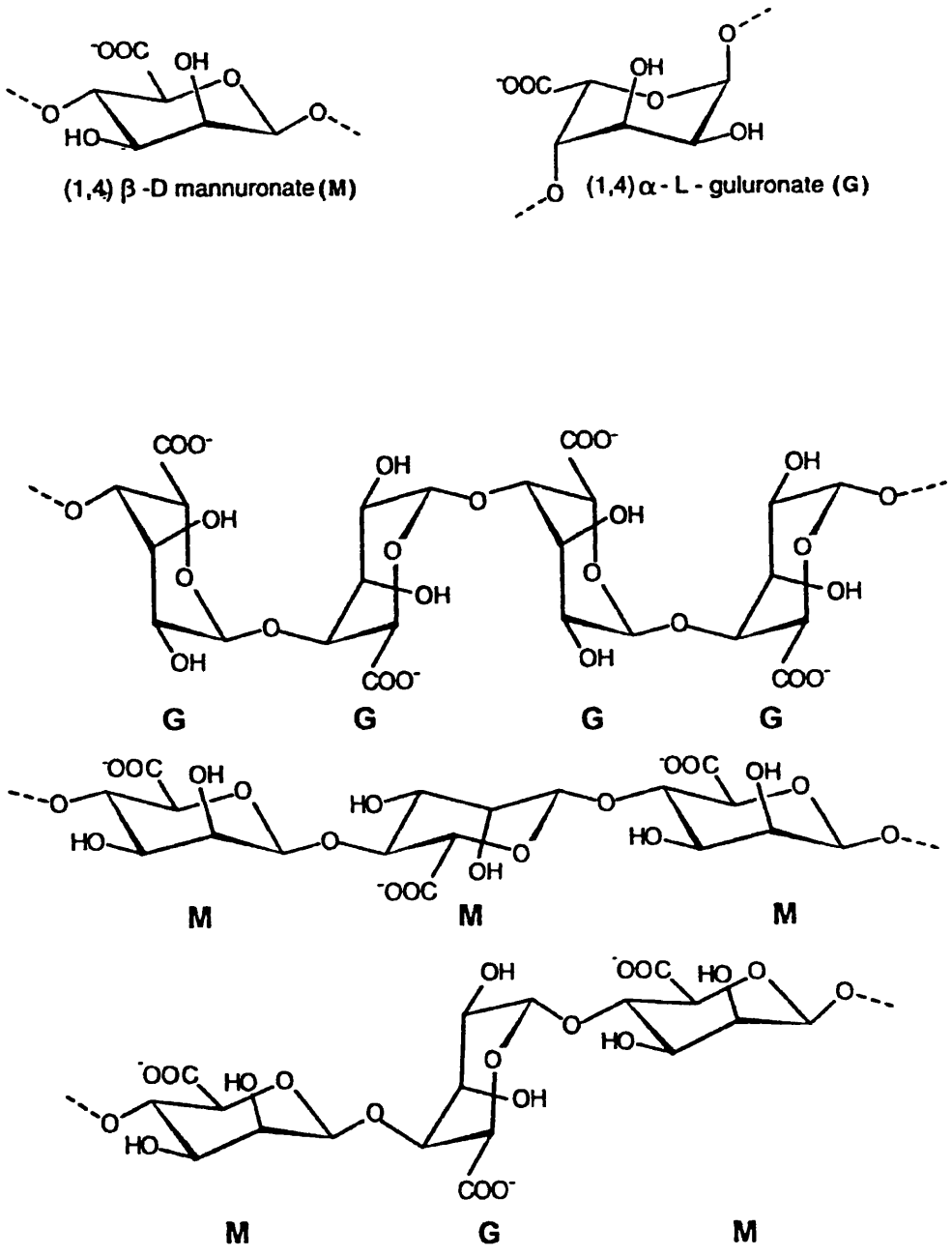

Figure 1.2 Molecular structure of alginate G blocks, guluronate and M blocks, mannuronate. (Adapted from Pronova literature, Norway).

1.7 HYPOTHESIS

The use of empty conduits to bridge long gaps still results in functional and morphological recovery inferior to that of nerve autografting. Therefore there is a need to search for an ideal bioengineered conduit, where the addition of components to an empty conduit will "mimic" the composition of an autograft, providing the model environment to promote, enhance and support nerve regeneration.

It is known that PHB with its coaxially aligned fibres aids neuronal and glial growth through contact guidance and mechanical orientation. The degradation profile of PHB means that it is present long enough to provide support for both nerve regeneration and maturation across long nerve gaps. However, empty PHB conduits result in suboptimal long-term regeneration across long nerve gaps. The presence of SC are fundamental to nerve regeneration and conduits seeded with cultured SC are known to improve regeneration, but are not practical for clinical use. Alternatively the native SC can be manipulated with trophic factors to improve their proliferation, migration and release of neurotrophic factors. The use of alginate for the local delivery of GGF within the PHB conduit should therefore result in optimisation of the conduit microenvironment, enhancing SC proliferation and regeneration and indirectly promoting axonal regeneration.

In this study, it was proposed that using a bioengineered PHB-GGF composite conduit the morphological and functional outcome of long gap repair may be improved.

1.8 AIMS

It was the aim of this study to determine whether a composite PHB-GGF conduit can enhance and sustain nerve regeneration over long gaps of 2 and 4cm, possibly leading to successful reinnervation of sensory and motor target tissues.

It was also of interest to determine whether a different configuration of the alginate matrix may further improve the regeneration potential of this bioengineered nerve conduit.

Finally, an alternative novel conduit material, PHA, was also tested because of the future possibility of directly loading this material with growth factors. The release of these factors from the conduit wall into the regenerative milieu can then improve nerve regeneration.

CHAPTER 2

MATERIALS & METHODS

2.1 ALGINATE GEL

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2.1.2 Dilution of stock and gel setting

2.2 ALGINATE FIBRES

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2.4 POLY-3-HYDROXYBUTYRATE (PHB)

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2.4.3 Loading of PHB conduit with alginate fibres

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2.6.1 Anaesthesia, pre-operative preparation, recovery and euthanasia

2.6.2 Operative procedure

2.7 SPRAGUE DAWLEY RAT MODEL

2.7.1 Anaesthesia, pre-operative preparation, recovery and euthanasia

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2.8.1.1 Short-term group

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2.10 MORPHOLOGICAL ASSESSMENT

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2.10.2 Histological staining

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2.11.2 Regeneration area

2.12 STATISTICAL ANALYSIS

2.12.1 Regeneration distance

2.12.2 Regeneration area

2.12.2.1 Short-term group

2.12.2.2 Long-term group

2.12.3 Motor reinnervation

2.1 ALGINATE GEL

2.1.1 Preparation of alginate gel

Low viscosity mannuronic acid rich (LVM) alginate powder was supplied by Pronova. (Oslo, Norway). The guluronic to mannuronic acid residue was 30:70. The powder was mixed in 150mM sodium chloride solution (3g in 75 ml) to make a 4% w/v alginate solution with pH 7.5. This was autoclaved at 121°C for 24 minutes producing a sterile homogenous solution, which was kept at 4°C ready to use and opened in the vented hood to minimise contamination.

2.1.2 Dilution of stock and gel setting

Equal volumes of the 4% w/v alginate stock solution and 0.1% fibronectin solution (Sigma), were mixed giving an alginate working dilution of 2% w/v. The resulting solution was viscous enough to keep it within the conduit prior to gelling.

The alginate solution sets as a gel on cross linkage with calcium solution, however the exposure time and the concentration of the calcium solution determine the final consistency of the alginate gel. Therefore a standardised gel setting technique was used. The alginate/fibronectin solution once loaded into the PHB conduits (cf 2.4.2) was exposed for 2 minutes to 0.1M solution of calcium chloride at room temperature prior to

implantation producing a hydrogel that was soft yet could be handled without loosing its integrity.

2.2 ALGINATE FIBRES

Alginate fibres were supplied by Pronova in two batches. The first batch consisted of commercially available alginate fibres: Sorbasan, Comfeel and AMS (Advanced Medical Solutions), all of which had a high M content (M:G = 60:40) (cf 1.6.1). Although they were from the same algae of origin, they were of varying molecular weights. The fibres of the second batch were manufactured specifically for our experimental use and consisted of an ultrapure mannuronic acid rich alginate (LVM) alginate (M:G = 67:43) and ultra pure guluronic acid rich alginate (LVG) (M:G = 43:67). These originated from the Macrosystis Pyrifera and Laminaria Hyperborea algae respectively. These were used in the *in vitro* and *in vivo* studies. Both were sterilised using gamma irradiation.

2.3 GLIAL GROWTH FACTOR (GGF)

A stock solution of recombinant human GGF2 (rhGGF2) with a protein concentration of 1.29mg/ml and a molecular weight of 80kDa, was supplied by CeNeS Pharmaceuticals (Norwood, Massachusset, USA). Under sterile conditions, 10µl aliquots of stock solution were prepared and stored at –80°C. 1µl of GGF stock solution was diluted in

1ml of 50:50 alginate fibronectin solution giving a final GGF concentration of 1250ng/ml.

2.4 POLY-3-HYDROXYBUTYRATE (PHB)

2.4.1 PHB conduit preparation

The conduits were formed from PHB sheets (Astra Tech, Gothenberg, Sweden). The sheets consist of two compressed perpendicular layers of PHB fibres (measuring 2-20microns in diameter), with the fibres running in a parallel direction in each layer. The PHB sheets have a soft consistency with a good tensile strength hence they are flexible and easy to shape into conduits.

Under a dissecting microscope in the vented hood, the two layers of the PHB sheet were teased apart using a scalpel and separated producing two sheets, each with unidirectional fibres (Figure 2.1). The sheet which lacked the compressors markings was used to form the conduit with its smoother side forming the inner aspect of the tube wall. The sheet was orientated such that the PHB fibres ran longitudinal to the long axis of the conduit. The latter is known to aid neuronal and glial growth by contact guidance and mechanical orientation (Curtis & Wilkinson 1997). Rectangular sheets of PHB with an 8mm width and a length of either 14mm, 24mm or 44mm were cut to form conduits bridging gap lengths of 1cm, 2cm and 4cm respectively. These were dipped in normal saline and rolled around a 16G intravenous cannula (16G Abbocath®-T, Abbott Ireland,

Sligo, Republic of Ireland), thus standardising the internal diameter of the conduits at 1.6mm, more than 1.5 times the diameter of rabbit common peroneal and rat sciatic nerves, thus allowing space for post-injury swelling (Figure 2.2a). The rolled sheets were heat sealed longitudinally with a fine tipped soldering iron (Figure 2.2b). Scanning electron micrographs of these PHB sheets confirmed that the disruptive effect of welding had remained localised to the outer aspect of the tube wall, and the unidirectional orientation of PHB fibres had been preserved (Figure 2.3). The conduits, still rolled around the cannula were pre-soaked in normal saline to allow expansion of the PHB fibres without a reduction in the internal diameter of the conduit.

2.4.2 Loading of PHB conduit with alginate gel

The conduits were dismounted from the cannula and a Hamilton syringe was used to fill them with either 2% alginate-fibronectin mixture or 2% alginate-fibronectin-GGF mixture (Figure 2.2c). 20µl/cm length of conduit was used to ensure over-filling. Because of the viscous nature of the alginate the filling process had to be done gently to avoid the formation of air bubbles. The alginate was then gelled (cf 2.1.2).

Figure 2.1 Separating PHB sheets.

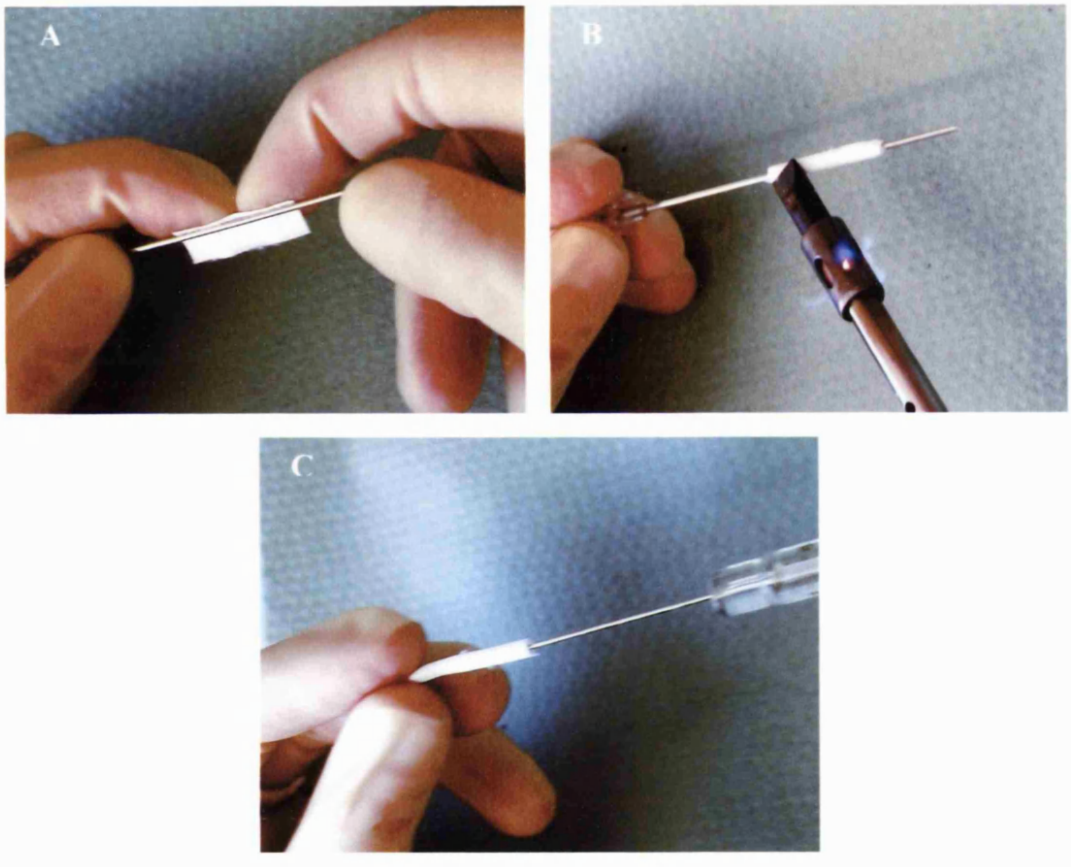

Figure 2.2 PHB conduit preparation. (A) Rolling of a PHB sheet around a 16G cannula. (B) Heat sealing longitudinally. (C) PHB conduit being filled with a mixture of alginate + GGF or alginate alone.

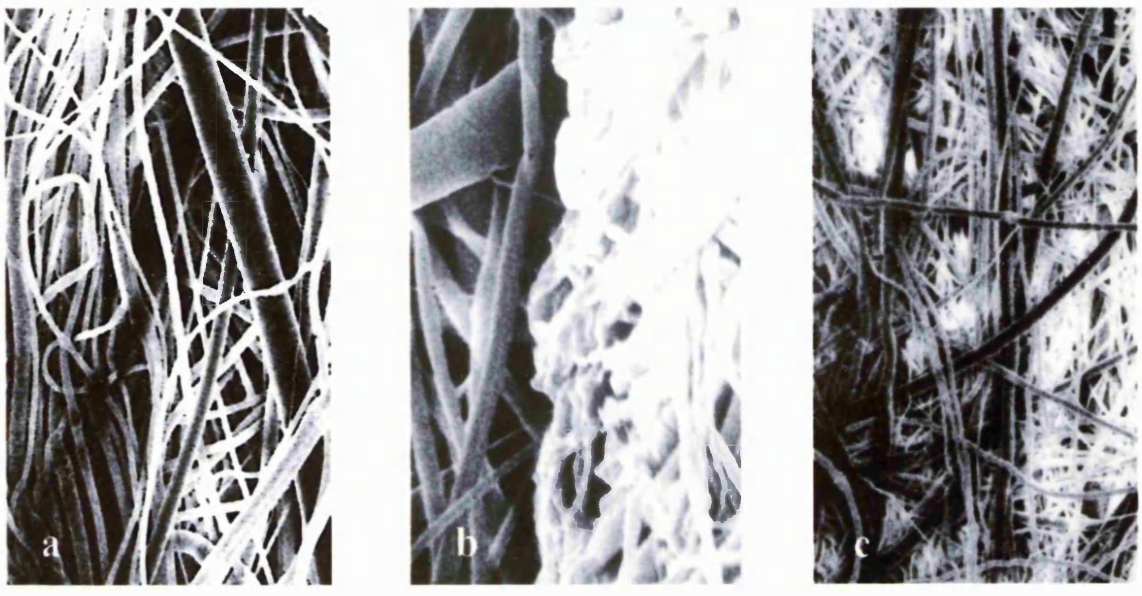

Figure 2.3 Scanning electron micrographs of PHB demonstrating (a) the filamentous structure of the material with fibres measuring 2-20μm in diameter running in a unidirectional orientation. (b) Shows the appearance of a weld site on the outer surface of the PHB conduit with loss of architecture. (c) Shows the inner aspect of the PHB conduit at a weld site where the architecture is maintained.

2.4.3 Loading of PHB conduit with alginate fibres

PHB conduits of 1cm and 2.4cm were prepared (cf 2.4.1) weighed and the alginate fibres were cut to lengths of 3cm. A random quantity of alginate fibres were taken and sterile aluminium foil was wrapped tightly around one end to form a sharp tip (Figure 2.4a). Using the tip the fibres were guided through the PHB conduits (Figure 2.4b). For the *in vitro* studies 1cm long tails were left at either end of the tube (Figure 2.4c). For the *in vivo* work, the alginate tails were trimmed so as to leave a 2mm space at the proximal and distal ends of the conduit to allow for tubulisation of the nerve ends. As it was not possible to count the number of fibres in each conduit the loaded conduits were reweighed and the weight of the empty conduit subtracted to obtain the weight of the alginate fibres in each tube. This allowed all the conduits to be loaded with a similar weight and therefore quantity of alginate fibre, thereby allowing comparisons to be made.

2.4.4 *In vitro* PHB and alginate fibres

Six PHB conduits measuring 1cm in length were prepared (cf 2.4.1) for each of the 5 types of alginate fibres and a similar quantity of the fibres, as measured by weight were loaded into each conduit (cf. 2.4.3). Each alginate fibre loaded conduit was placed in the well of a 6-well tissue culture plate (Falcon, Becton & Dickenson) (Figure 2.4d). The culture medium was prepared using fetal calf serum (FCS) (Imperial Laboratories) and Dulbecco's Modified Eagle's Medium plus Glutamax (DMEM) (Gibco) at the ratio of

1:9. 3mls of the culture medium was added to each well and the culture trays were incubated at 37°C, with the culture medium being changed every 2-3 days.

At days 2,7,and 10 the alginate tails and the cross-sectioned PHB tubes were visualised under the dissecting microscope using various magnifications (x6, 16, 25 and 40 objectives) and photographed through the microscope eyepiece with the digital camera. Qualitative assessment of the maintenance of fibre structure, degree of fibre swelling, tensile strength on handling and degradation profile was carried out.

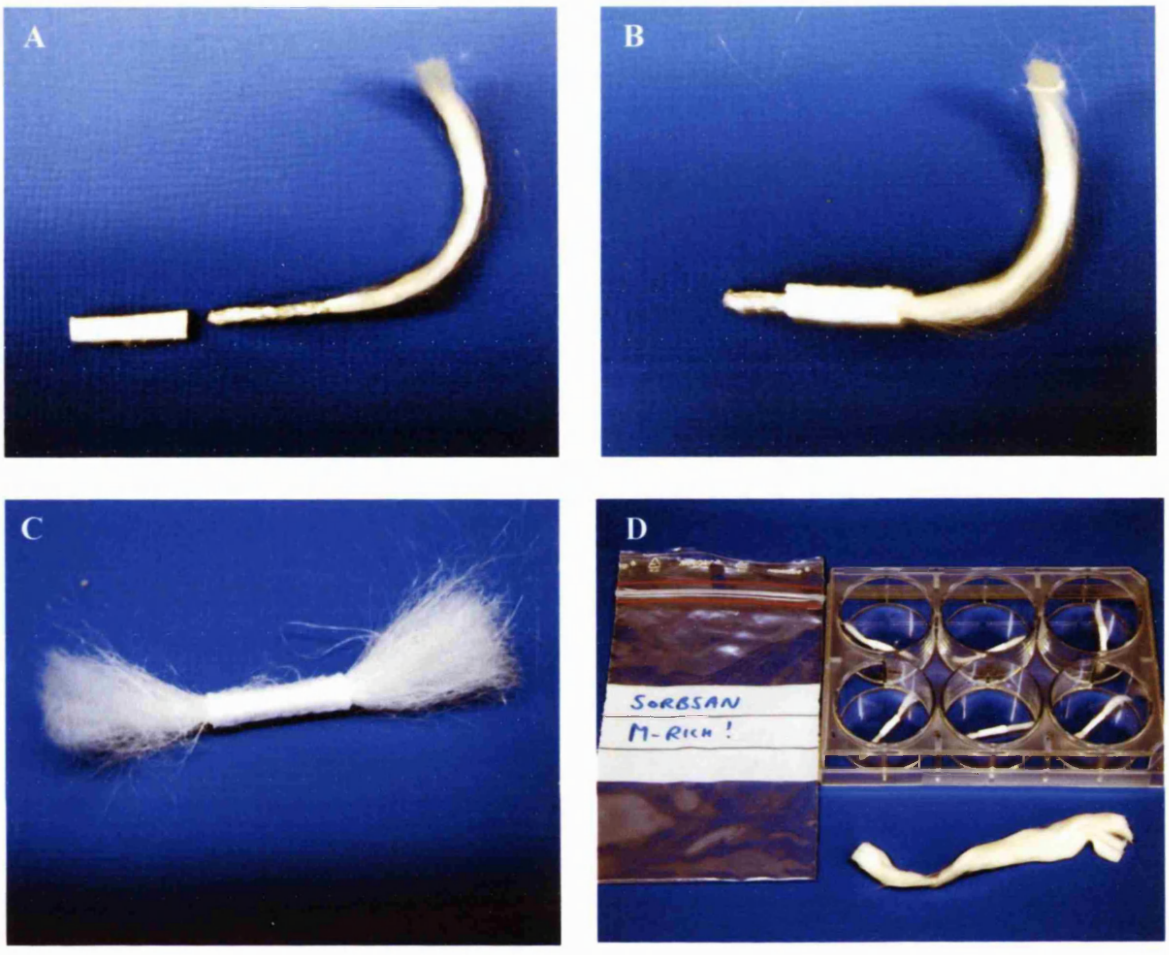

Figure 2.4 Loading of PHB conduits with alginate fibres. (A) Aluminium foil is tightly wrapped around the alginate fibres forming a sharp tip. (B) Using the tip the fibres are guided through the conduit. (C) For *in vitro* studies 1cm long tails are left at either end of the tube. (D) The alginate fibre loaded conduits are placed in a six well culture tray.

2.5 POLYHYDROXYALKATONE (PHA)

Porous PHA4400 (Poly-4-hydroxybutyric acid) sheets (Tepha, Cambridge, MA, USA) prepared by four different methods (A-D) were used. The different types of PHA available were as follows:

A - TIPS (Thermally Induced Phase Separation) foam with water leaching

B - TIPS (Thermally Induced Phase Separation) foam with Tween80 leaching

C - Ethanol precipitate with water leaching

D - Ethanol precipitate with Tween80 leaching

The samples were prepared by Tepha as follows. A stock solution of PHA4400 was prepared in dioxane containing 5% w/v of PHA4400. Sodium chloride particles of between 100-250μm were sieved and combined with the polymer solution, at a weight ratio of 1:2 and mixed well. 10-12g of the salt/polymer mixture were sandwiched between two Mylar sheets (Carver Inc., Wabash, USA) and pressed together in a Carver press using 500 or 300μm steel spacers and frozen uniformly at –26°C between aluminium plates. The top Mylar sheet was removed whilst frozen. For samples A and B, the polymer foam and bottom Mylar sheet were transferred to the lyophilizer to be freeze-dried overnight. For samples C and D the polymer foam and bottom Mylar sheet were placed in an ethanol bath to allow the dioxane to soak out and the polymer to crystallize. The lyophilised or crystallized foam was removed from the Mylar backing and the salt leached out. For samples A and C only water was used, whereas for samples

B and D 0.025% Tween80 was added to the water as a polymer wetting agent. Therefore configurations AB and CD are identical except for the salt leaching step. The samples were finally dried and sterilized using cold ethylene oxide gas giving a final pore size range of between 25-150μm.

2.5.1 PHA conduit preparation

Like the PHB material the PHA sheets are soft and flexible, thus the conduits were prepared in a similar way (cf 2.4.1). Under sterile conditions the PHA was cut into rectangular sheets measuring 8mm x 14mm. These were dipped in normal saline and rolled around a 16G intravenous cannula thus standardising the internal diameter of the conduits at 1.6mm as before. Orientation was thought unnecessary as the PHA has no fibres and its pores appeared to have no macroscopic orientation. The rolled sheets were heat sealed longitudinally with a fine tipped soldering iron. The conduits, still rolled around the cannula were pre-soaked in normal saline to allow expansion of the PHA without a reduction in the internal diameter of the conduit.

2.6 NEW ZEALAND WHITE RABBIT MODEL

Female New Zealand White rabbits weighing between 2.5 and 3.0kg, were used to assess axonal regeneration across gaps in the common peroneal nerve using PHB conduits (cf 2.6.2) The common peroneal nerve was chosen to avoid the problems of autotomy and trophic ulceration that often occur with the use of the full sciatic nerve.

The animals were sacrificed at 21, 42, 63 and 120 days after implantation. All procedures were carried out in accordance with UK Home Office regulations.

2.6.1 Anaesthesia, pre-operative preparation, recovery and euthanasia

The rabbits were anaesthetised by an intramuscular injection of diazepam (1mg; Phoenix Pharmaceuticals Ltd., UK) and Hypnorm™ (0.5mls/kg, fentanyl citrate 0.315mg/ml and fluanisone 10mg/ml; Jansen Animal Health, UK). Antibiotic prophylaxis was given at induction Baytril® (0.5ml s.c., 50mg enrofloxacin/ml; Bayer plc, UK). After induction of anaesthesia, the left lateral thigh of the rabbit was shaved and the skin prepared with povidone iodine solution. The rabbit was transferred to the operating table, placed in the right lateral position on a warming mat and administered oxygen via a face mask at 2 l/min throughout the procedure. Periodically the rabbit was checked for depth of anaesthesia, heart rate and breathing.

At the end of the procedure an intramuscular injection of Temgesic® (0.2ml, buprenorphine 0.3mg/ml; Reckitt and Colman Products Ltd., UK) was given for post-operative analgesia and to reverse the anaesthetic agent. The rabbits were transferred to the recovery room where they were kept overnight. The following day they were returned to their cages in the holding room for the remainder of the time period. Post-operatively the rabbits received the following analgesic and antibiotic regime for a total of three days: Temgesic® (0.2ml o.d., s.c., buprenorphine 0.3mg/ml; Reckitt and

Colman Products Ltd., UK) and Baytril® (0.5ml o.d., s.c., 50mg enrofloxacin/ml; Bayer plc, UK).

At the time of harvesting, the rabbits were sacrificed with an intravenous overdose of Sagatal™ (10mls pentobarbitone sodium, 60mg/ml; Rhöne Mérieux Ltd., UK).

2.6.2 Operative Procedure

Under sterile operating conditions a longitudinal lateral skin incision was made along the left thigh and the left sciatic nerve was exposed by a muscle splitting incision between the *m. abductor cruris cranialis* and *m. bicepsfemoris* (Figure 2.5). Under the operating microscope the loose connective tissue between tibial and common peroneal portions of the sciatic nerve were divided from the lower border of *m. quadratusfemoris* in the proximal part of the intermuscular cleft, to the level of the knee distally. This amount of dissection was performed in every case to standardise the blood supply to the regenerating nerve. The proximal resection margin was kept constant at a distance 10mm distal to the lower border of *m. quadratusfemoris*. The common peroneal nerve was divided with sharp microsurgery scissors. The repair was carried out using either an empty PHB conduit, a PHB conduit loaded with 2% w/v alginate, a PHB conduit loaded with 2% w/v alginate and GGF or a PHB conduit loaded with alginate fibres (cf 2.4.2 and 2.4.3). The proximal nerve stump was inset into the PHB conduit by a distance of 2mm and secured by two 9/0 epineural sutures (Ethilon®, UK) (Figure 2.6 and 2.7). The PHB tube was then placed adjacent to the tibial nerve in its anatomical position, and a

length of common peroneal nerve was resected to create a nerve gap of either 2 or 4cm allowing the distal stump to be similarly inset into the PHB tube without tension at the anastomoses (Figure 2.8). In all groups the muscle fascia was closed with continuous 4/0 Vicryl® (Johnson and Johnson Intl., Belgium), the skin was closed with interrupted 3/0 Vicryl® (Johnson and Johnson Intl., Belgium) sutures and the wound dressed with OpSite™ spray (Smith and Nephew, UK).

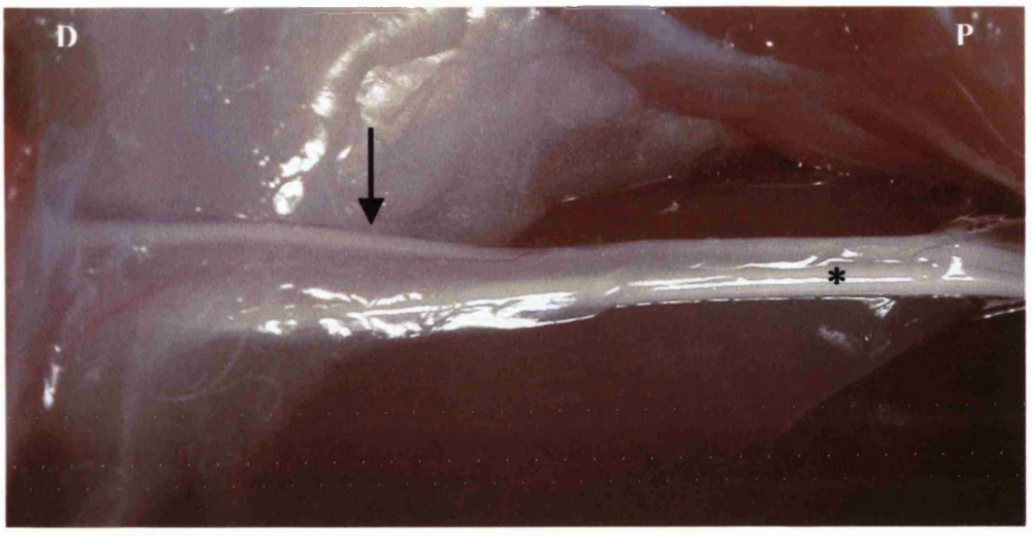

Figure 2.5 The sciatic nerve of the rabbit in the thigh prior to separation of the common peroneal (arrow) and tibial nerves (*) which are joined by loose connective tissue (P = proximal, D = distal).

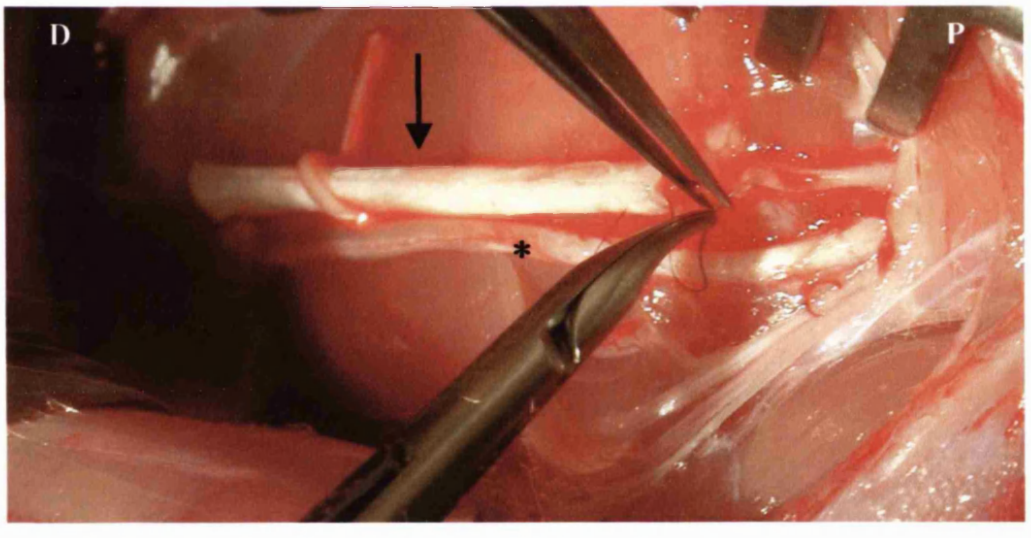

Figure 2.6 Entubulation of the proximal stump of the common peroneal nerve into a 4.4cm PHB conduit (arrow). The cut common peroneal nerve lies across the PHB conduit (arrow). The tibial nerve (*) lies adjacent to the conduit (P = proximal, D = distal).

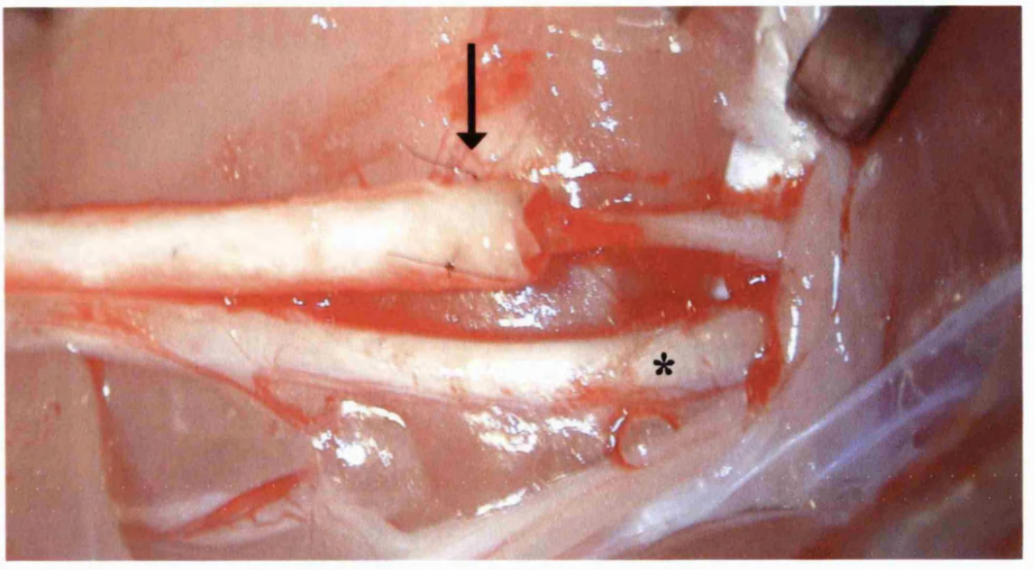

Figure 2.7 Detail of the proximal anastomosis showing the 9/0 sutures used to secure the PHB conduit to the proximal nerve stump (arrow). (* = Tibial nerve).

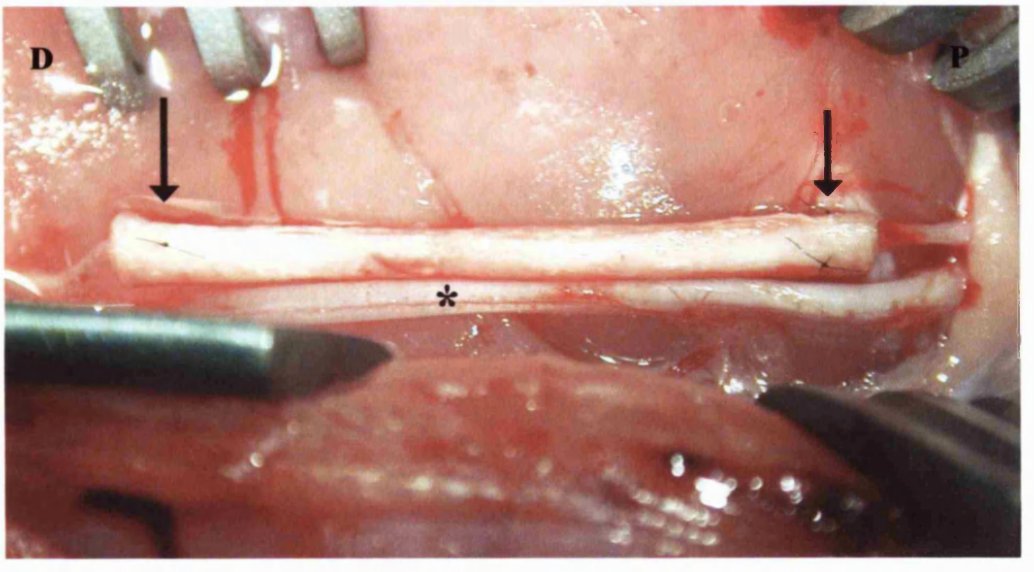

Figure 2.8 A 4.4cm PHB conduit at implantation. The epineural sutures (arrows) are seen at the proximal (P) and distal (D) ends with the PHB conduit lying next to the tibial nerve (*).

2.7 SPRAGUE-DAWLEY RAT MODEL

Eight week-old male inbred Sprague-Dawley rats weighing between 180-200g, were used to assess axonal regeneration across a 10mm gap in the sciatic nerve through empty PHA conduits (cf 2.5.1) and nerve autografts. The animals were sacrificed 10 and 20 days after implantation. All procedures were carried out in accordance with UK Home Office regulations.

2.7.1 Anaesthesia, pre-operative preparation, recovery and euthanasia

The rats were anaesthetised by an intramuscular injection of Hypnorm^{TM} (0.3ml/kg, fentanyl citrate 0.315mg/ml and fluanisone 10mg/ml; Jansen Animal Health, UK) and an intraperitoneal injection of diazepam (2.5mg/kg: Phoenix Pharmaceuticals Ltd., UK). After induction of anaesthesia, the left gluteal and thigh region were shaved with an electric hair clipper and the skin prepared with povidone iodine solution. The rat was transferred to the operating table, placed in the right lateral position on a warming mat and periodically checked for depth of anaesthesia (tail pinch), heart rate and breathing. After the operative procedure (cf 2.7.2) the animal was transferred to the recovery room, placed on soft bedding on it's right flank and kept there overnight before re-colonisation.

At the time of harvesting, the rats were sacrificed by placing them in a chamber with increasing concentration of CO_2. When the animal was unresponsive, cervical dislocation was performed.

2.7.2 Operative procedure

A 4cm lateral skin incision was made along the left thigh, starting 1cm below the sciatic notch. The underlying gluteal muscles were split along the fibres to expose the sciatic nerve. Adequate operative exposure was provided using skin hooks to keep the muscles and skin apart. Under the operating microscope the sciatic nerve was dissected free from the loose connective tissue surrounding it, to allow manipulation along its length. The sciatic nerve was divided 5mm distal to the sciatic notch using sharp microsurgery scissors and repaired using either a nerve autograft or one of the four types of PHA conduit (cf 2.5.1).

In the PHA group the proximal nerve stump was inset into the 1.4cm conduit by a distance of 2mm and secured by two 9/0 epineural sutures (Ethilon®, UK). The PHA conduit was placed in the anatomical position of the sciatic nerve, and a length of nerve was resected to create a 1cm gap, allowing the distal stump to be similarly inset into the PHA tube without tension at the anastomoses. For nerve autografting the distal nerve was placed on a calibrated template and divided to produce a nerve autograft of 10mm. The resected length of nerve was reversed and re-anastomosed using 9/0 epineural sutures (Ethilon®, UK). In all groups the retracted muscles were re-opposed but not

sutured. The skin was closed with interrupted 3/0 Vicryl® sutures (Johnson and Johnson Intl., Belgium) and the wound dressed with OpSite™ spray (Smith and Nephew, UK).

2.8 TISSUE COLLECTION

2.8.1 New Zealand White rabbit model

2.8.1.1 Short-term group

At 21, 42 and 63 days, the rabbits were sacrificed and the lateral thigh skin was shaved and the old incision opened and extended proximally and distally. The insertion of the *m.bicepsfemoris* was then divided and the muscle reflected to open up the intramuscular cleft and reveal the nerve repair site. The PHB conduit was dissected free from the tibial nerve and the pseudocapsule which had formed around it. The suture material was removed to prevent tissue damage during cryostat cutting (cf 2.9). The proximal and distal nerve stumps were cut and the specimen comprised of the graft and a segment of proximal and distal nerve was pinned out to length on a plastic splint in order to prevent shrinkage and to maintain its shape during fixation (Figure 2.9). The tissue was immersed in Zamboni's solution for 6 hours at room temperature. Following fixation, the samples were washed in 0.01M PBS containing 15% (w/v) sucrose and 0.1% (w/v) sodium azide three times at 24-hour intervals, or until the solution was no longer discoloured by excess Zamboni's fixative. They were then stored at 4°C prior to blocking for cryostat sections.

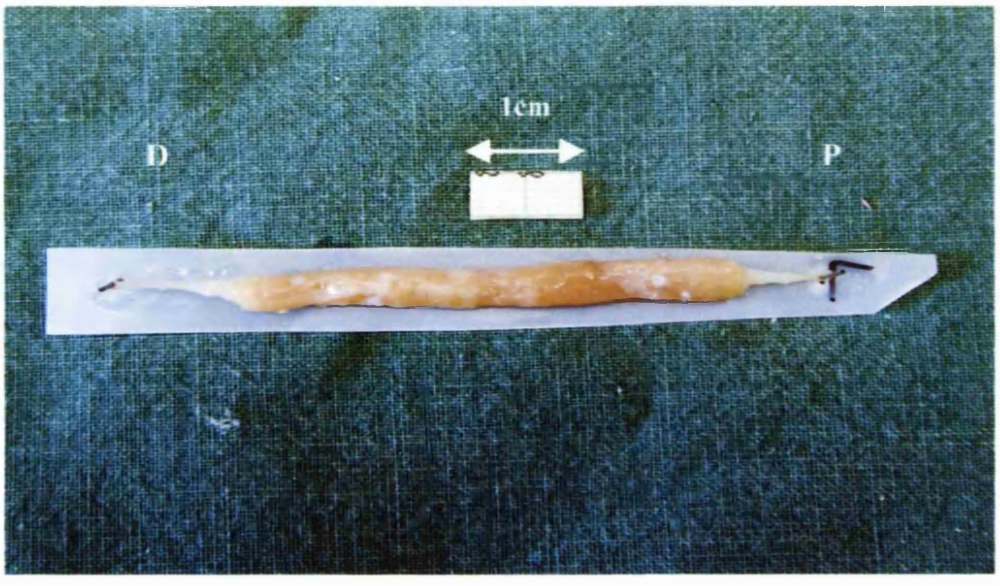

Figure 2.9 4.4cm conduit harvested at 42 days. To avoid shrinkage of the conduit during fixation the proximal (P) and distal (D) nerve stumps are pinned out to length on a plastic splint.

2.8.1.2 Long-term group

The animals were sacrificed at 120 days and the lateral aspects of both legs and thighs and the dorsum of both feet were shaved. The skin was incised on the lateral aspect of the lower leg from the level of the knee joint to the ankle. The neurovascular bundle to the muscle containing the common peroneal nerve was tied off, and the left and right tibialis anterior (TA) and extensor digitorum longus (EDL) muscle complexes were excised with the knee and ankle joint in 90° of flexion, and weighed immediately. The combined weight of the two muscles were recorded for each side and the percentage muscle atrophy and percentage loss of muscle mass were calculated for each group as follows;

$$\% \text{ muscle atrophy} = \frac{\text{left side (operated) percentage muscle weight}}{\text{right side (unoperated) percentage muscle weight}} \times 100$$

% loss of muscle mass = 100 - % muscle atrophy

Following muscle harvest the lateral thigh skin was incised and the thigh compartment was opened. The conduit was dissected free from the surrounding tissue between the sciatic notch and the level of the knee. A marker suture was placed 10mm distal to the distal end of the conduit (Figure 2.10). The distal nerve was then divided 5mm proximal to this suture and immediately distal to it (Figure 2.10). This length of distal stump was

fixed in 1% paraformaldehyde and 1.5% glutaraldehyde in phosphate buffer, and prepared for semithin sections (cf 2.10.2). The conduit graft was excised with a length of proximal stump and distal stump and divided into three pieces (Figure 2.10). The first piece was a 2mm transverse slice taken 12mm distal from the proximal end of the conduit, which was fixed in 1% paraformaldehyde and 1.5% glutaraldehyde in phosphate buffer overnight and prepared for semithin and ultrathin sections. The length of grafted conduit and nerve end distal to the transverse slice were pinned out to length on a plastic splint and fixed by immersion for 6 hours in Zamboni's solution. The third piece was the proximal part of the PHB graft and the accompanying proximal nerve end which, were also pinned out to length on a plastic splint and fixed in Zamboni's solution as above. Following fixation, the samples were washed in 0.01M PBS-15% (w/v) sucrose-0.1% (w/v) sodium azide solution three times at 24-hour intervals, or until the solution was no longer discoloured by excess Zamboni's fixative. The samples were then stored at 4°C prior to blocking for cryostat sections.

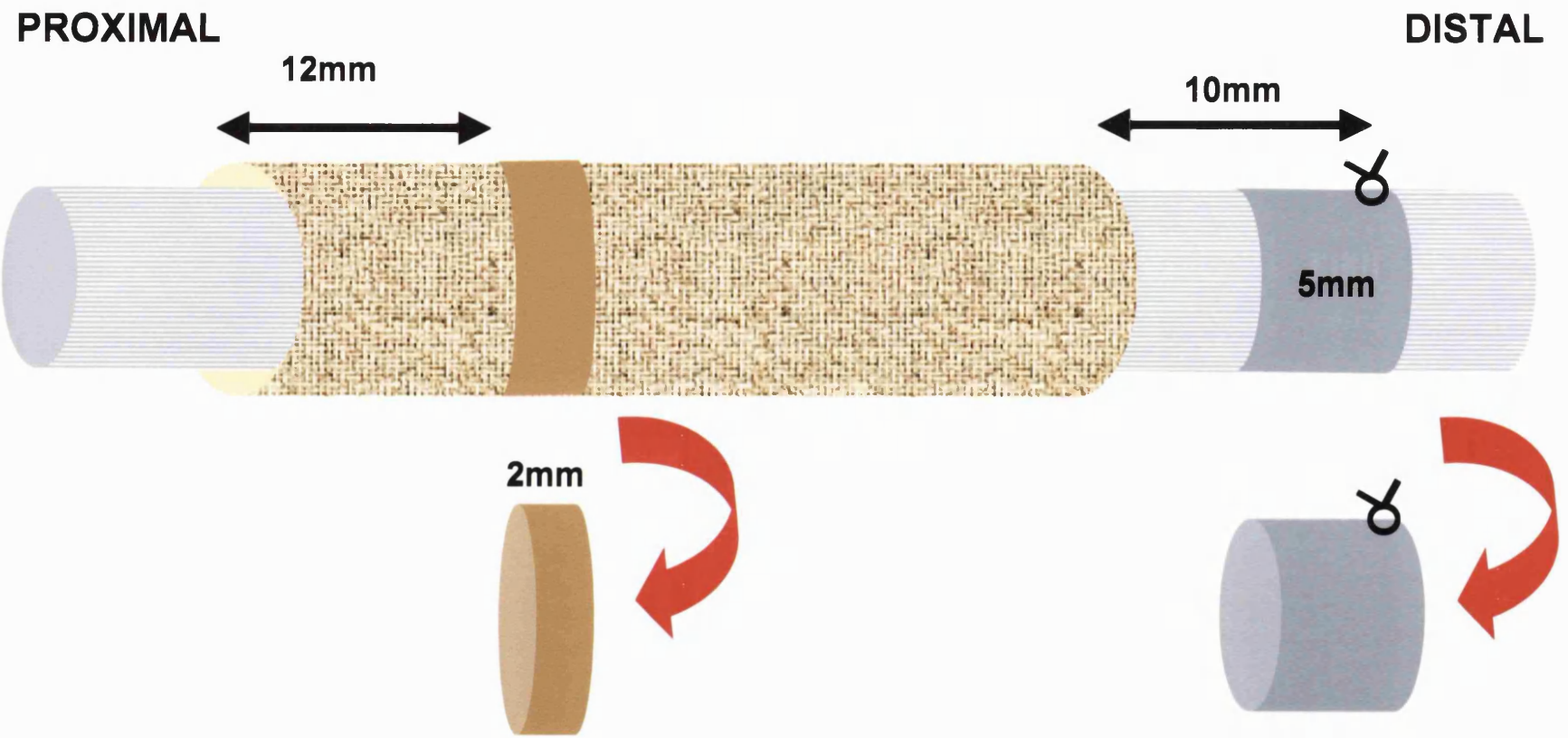

Figure 2.10 Long-term group nerve repair tissue collection. A marker suture is placed 10mm distal to the distal end of the conduit. The distal nerve is divided 5mm proximal to this suture and immediately distal to it and this specimen is collected. The conduit graft is excised with a length of proximal and distal stump and divided into three pieces. The first piece is a 2mm transverse slice taken 12mm distal from the proximal end of the conduit. The second and third pieces are the segment of conduit and nerve stump proximal and distal to the transverse slice.

2.8.2 Sprague-Dawley rat model

At 10 and 20 days, the rats were sacrificed and the lateral thigh skin was shaved and the old incision opened and extended proximally and distally. The insertion of the *m.bicepsfemoris* was then divided and the muscle reflected to open up the intramuscular cleft and reveal the nerve repair site. The PHA conduit and nerve autograft repairs were dissected free along with a length of proximal and distal nerve. The suture material was removed to prevent tissue damage during cryostat cutting. The proximal and distal nerve stumps were cut and the specimen pinned out to length on a plastic splint in order to prevent shrinkage and to maintain its shape during fixation. The tissue was immersed in Zamboni's solution for 6 hours at room temperature. Following this period of fixation the samples were washed in 0.01M PBS containing 15% (w/v) sucrose and 0.1% (w/v) sodium azide three times at 24-hour intervals, or until the solution was no longer discoloured by excess Zamboni's fixative. They were then stored at 4°C prior to blocking for cryostat sections.

2.9 TISSUE BLOCKING AND SECTIONING

The grafted nerve samples were blocked in OCT compound (Tissue-Tek™, Sakura, Japan) and quick-frozen in liquid nitrogen. The samples were sectioned longitudinally to include the grafted conduit and the two nerve ends. Hence, the orientation of all grafts was marked by placing a piece of liver at the proximal nerve end. The PHA conduits, nerve autografts, 2cm short-term PHB conduits and 2cm PHB alginate fibre conduits

were blocked whole for cryostat sectioning. The short-term 4cm PHB conduits were divided in two and blocked as a proximal and a distal specimen maintaining the orientation. The long-term 2 and 4cm PHB tubes were also blocked as a proximal and distal specimen. The frozen blocks were stored at –40°C ready for cryostat sectioning. The specimens were cut longitudinally and trimmed until the nerve stump and the central regeneration zone were identified by quick haematoxylin staining (cf Appendix). 15μm thick frozen sections were then sequentially collected onto coded Vectabond™ coated slides (Vector Lab., USA) and the numbers noted for subsequent analysis. The slides were allowed to dry at 36°C overnight prior to immunostaining.

The distal nerve segment and the 2mm transverse slice of the conduit collected at 120 days were prepared for transverse semithin sectioning. In addition, the 2mm transverse slice of the conduit was prepared for ultrathin sectioning. These methodologies and the viewing of ultrathin sections (cf 2.10.3) were carried out in collaboration with the Electron Microscopy Unit, Royal Free and University College Medical School, Royal Free Campus, London. The specimens were processed using a Lynx tissue processor. This involved fixation in osmium tetroxide followed by serial alcohol dehydration and finally resin infiltration (cf Appendix). 1μm semithin sections were cut using glass knives, collected onto glass microscope slides and dried for one hour on a hotplate ready for staining (cf 2.10.2). Ultrathin sections were cut using a diamond knife and collected onto 200HS, 3.05mm copper grids (Gilder) prior to processing for transmission electron microscopy (cf 2.10.3)

2.10 MORPHOLOGICAL ASSESSMENT

2.10.1 Immunohistochemistry

Immunohistochemical staining of grafted nerve sections was carried out using the avidin-biotin complex (ABC) nickel enhancement technique (cf Appendix) (Hsu *et al.* 1981; Shu *et al.* 1988). Immunoreactive structures stain dark blue/black against non-stained white background.

In the rabbit tissue, axonal regeneration was assessed by staining with monoclonal antibodies to pan neurofilaments (PanNF) (Affiniti, UK, dilution of 1/5000), a general pan-neuronal marker. Staining with monoclonal antibodies to S100 (Affiniti, UK, dilution of 1/2000) was used to assess SC regeneration.

In the rat tissue, polyclonal antibody to protein gene product 9.5 (PGP) (Affiniti, UK, dilution of 1/5000) was used to assess axonal regeneration and polyclonal antibodies to S100 (Affiniti, UK, dilution of 1/8000) demonstrated SC regeneration.

2.10.2 Histological staining

Normal histology of the PHA and PHB conduits and the nerve autografts was assessed using haematoxylin and eosin staining. This was performed on at least the first and last sections that were collected from each sample (cf Appendix).

Transverse semithin sections and distal stumps of the long-term PHB conduits were stained with Humphrey's stain (cf Appendix) and were viewed by light microscopy (Olympus BX60) in order to assess nerve regeneration through the PHB conduit.

2.10.3 Electron Microscopy

Transverse ultrathin sections 12mm from the proximal end of the conduit were prepared (cf Appendix). These sections were stained with saturated uranyl acetate in 50% ethanol (TAAB Laboratories Equipment Ltd., UK) followed by Reynold's lead citrate (cf Appendix). The ultrathin sections were viewed and photographed on a Phillips 201 transmission electron microscope. The sections were assessed for the presence of myelinated and unmyelinated nerve fibres, Bands of Büngner and other cellular and structural elements present.

2.11 QUANTIFICATION

PanNF and PGP 9.5 immunostaining were used to quantify the distance and area of axonal regeneration at all time points whilst S100 immunostaining was used to quantify the distance and area of SC regeneration at all time points. Two random non-consecutive sections were analysed per antibody per animal using an Olympus microscope (BH60, Japan) connected to a computerised image analyser via a digital camera (Spot™, Diagnostic Instruments Inc., USA).

2.11.1 Regeneration distance

The axonal and SC regeneration distance was assessed using a calibrated ocular graticule at x4 magnification. The absolute distance of penetration was measured in mm from the proximal end of the conduit, or the proximal anastomosis of the nerve autograft, to the most distal PanNF or PGP 9.5 positive fibres (axonal regeneration distance) or S100 positive cells (SC regeneration distance) (Figure 2.11). The measurement was expressed to the nearest 0.25mm as this corresponded to one division of the graticule. For the PHA conduits and nerve autografts in the rats, it was not possible to measure accurately the regeneration distance as the grafts had already been bridged at the chosen time points.

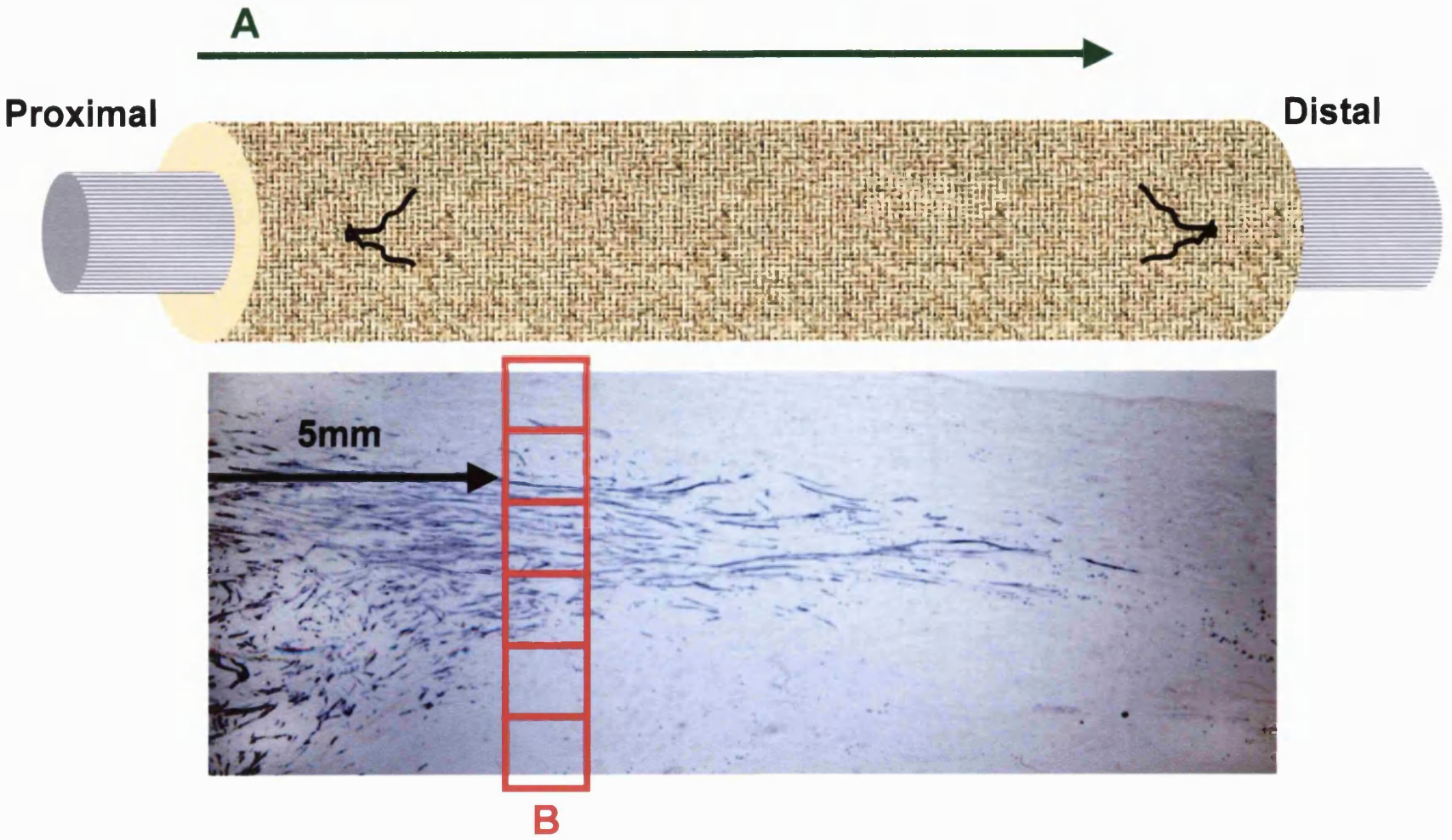

Figure 2.11 Measurement of axonal and SC (A) regeneration distance (mm) and (B) regeneration area (μm^2) within the conduit.

2.11.2 Regeneration area

The area of PanNF, PGP and S100 immunostaining were used as an indication of the quantity of axonal and SC regeneration. The area of immunostaining was assessed using a computerised image analysis program (Image-Pro® Plus 4, Media Cybernetics®, U.S.A.). The measurements were carried out at a fixed point in all the experimental groups, to allow direct comparison between the experimental treatments at different time points. For PHB conduits, adjacent frames were captured across the whole width of the longitudinal section at a distance of 5mm from the proximal end of the graft, using a digital camera at x10 objective (Figure 2.11). For the PHA conduits and nerve autografts adjacent frames were captured across the width of the distal stump. The overlap between adjacent images was identified and edited out to avoid counting the same structure twice. The images were converted to 8 bit grey scale (Figure 2.12) before thresholding to select the stained regenerating nerve fibres (Figure 2.12). Objects below the threshold value were set to zero and not counted whilst objects equal to or greater than the threshold value were selected and counted. Thresholding was performed automatically and kept within a narrow range for all specimens in order to select regenerating nerve fibres without operator bias. Any background objects above the threshold limit were manually edited prior to counting by comparing the original and thresholded images. The total area of staining and the total area of all the frames captured were quantified and expressed in μm^2. The staining per-area was calculated by dividing these two values and expressed as a percentage. The per-area figure allows a comparison to be made

between PHB conduits, which may have undergone a varying degree of swelling with time, in terms of regeneration per unit of area available for regeneration.

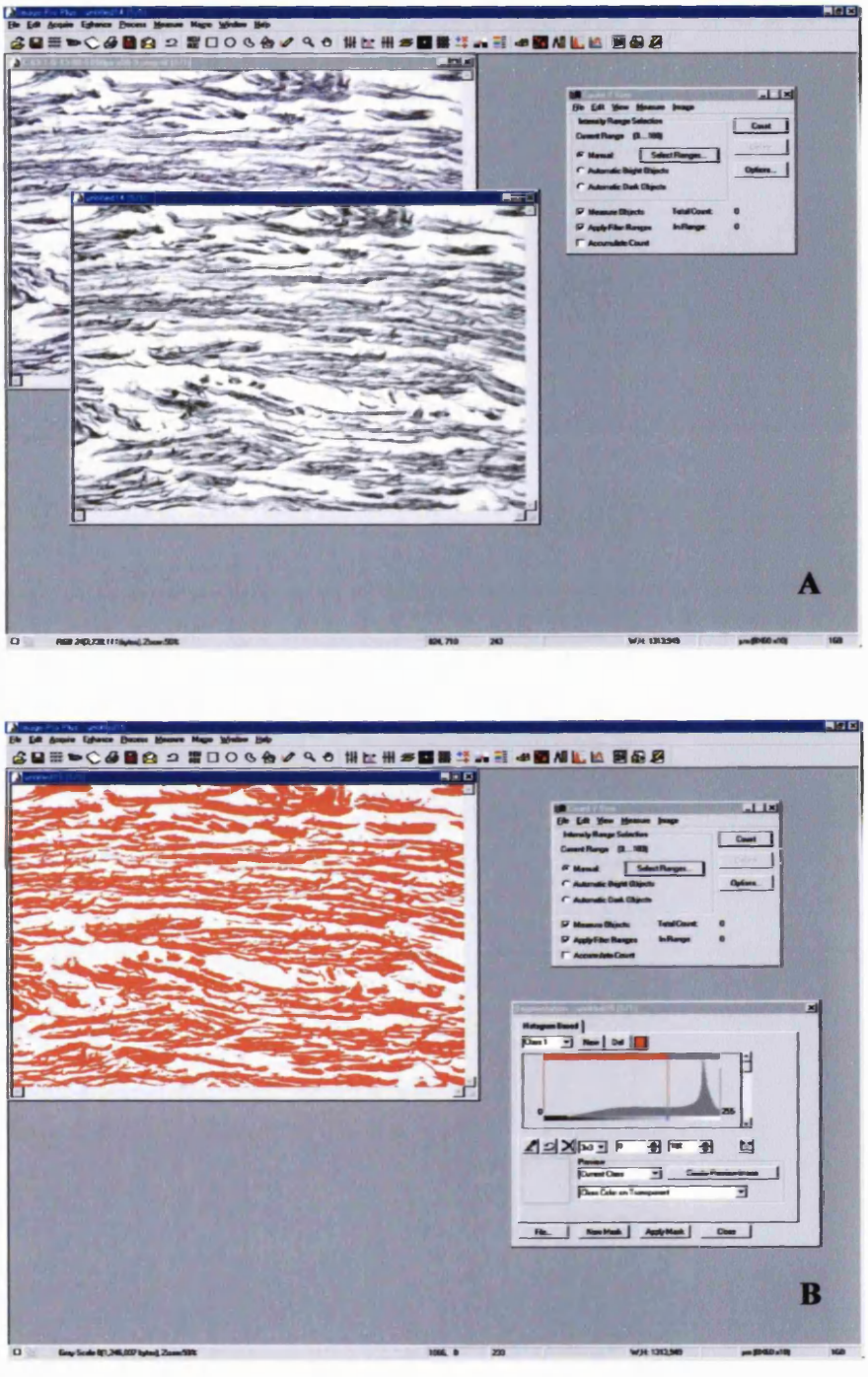

Figure 2.12 Computerised image analysis. The images are converted to an 8 bit grey scale (A), prior to thresholding to select the stained regenerating fibres (B).

2.12 STATISTICAL ANALYSIS

Statistical analysis was carried out in collaboration with Mrs. Deborah Ridout, Senior Research Fellow and Statistician, Institute of Child Health, Great Ormond Street Hospital, London.

2.12.1 Regeneration distance

Many of the regeneration distances were censored observations as the regeneration had entered the distal nerve by some of the harvest dates. Therefore a non-parametric method of analysis was used. To investigate whether there was an effect of gap length a Mann-Whitney U one way ANOVA was used to compare gap lengths for each combination of graft type and day. To investigate the difference in regeneration distance between the graft types a Kruskal-Wallis test was performed for each combination of gap length and day. To look at the effect of time on the regeneration distance within each graft type a Kruskal-Wallis test was performed for each gap length. All the results were corrected for multiple comparisons using the Bonferroni Correction. The statistical analysis was performed using Stata^{TM} Release 7 statistical software (Stata Corporation, USA).

2.12.2 Regeneration area

2.12.2.1 Short-term group

Statistical analysis was performed for the S100 and PanNF staining areas and the percentage staining areas (cf 2.11.3). Firstly a three way analysis of variance (ANOVA) was performed with factors day (21, 42 and 63), graft type (E-PHB, PHB-ALG and PHB-GGF), gap length (2 or 4cm) and their interactions. For example, the interaction term assessed whether the difference between the graft types varied with gap length. The normality assumption of the ANOVA was checked using the Shapiro-Francia W dash test and Bartletts test was used to check the equal variances assumptions. The assumptions from the analysis on the untransformed data were not valid, therefore a square root transformation of the data was carried out. As no significant difference was identified for S100 and PanNF staining area and percentage staining area, by increasing the gap length from 2 to 4cm the data was presented using the group total values rather than the values for each individual gap length. A two way ANOVA was performed on the transformed total area of staining and total percentage area of staining with factors graft type (E-PHB, PHB-ALG and PHB-GGF) and day (21, 42 and 63) and their interaction. Following analysis of the initial results a Student's t-test was used and the P values of the post-hoc tests were corrected for multiple comparisons using the Bonferroni Correction. The statistical analysis was performed using Stata™ Release 7 statistical software (Stata Corporation, USA).

2.12.2.2 Long-term group

Statistical analysis was performed for the S100 and PanNF staining areas and the percentage staining areas (cf 2.11.3). Firstly a two way analysis of variance (ANOVA) was performed with factors graft type (E-PHB, PHB-ALG and PHB-GGF) and gap length (2 or 4cm) and their interactions. For example, the interaction term assessed whether the difference between the graft types varied with gap length. The normality assumption of the ANOVA was checked using the Shapiro-Francia W dash test and Bartletts test was used to check the equal variances assumptions. The assumptions from the analysis on the untransformed data were not valid, therefore a square root transformation of the data was carried out. For both the PanNF and S100 transformed staining areas and percentage staining areas there was a significant difference between graft types but not gap length. There was no graft-gap interaction for S100 percentage staining area but for S100 and PanNF staining areas and PanNF percentage staining area the interaction was significant Therefore for the S100 percentage staining area the data was presented using the group total values rather than the values for each individual gap length. A one way ANOVA was performed on the transformed S100 total percentage staining area with factor graft type (E-PHB, PHB-ALG and PHB-GGF). For the transformed S100 and PanNF staining areas and PanNF percentage staining area a one way ANOVA was performed for each graft type looking at the factor gap length (2 and 4cm) as well as for each gap length looking at the factor graft type (E-PHB, PHB-ALG and PHB-GGF). Following analysis of the initial results a Student's t-test was used and the P values of the post-hoc tests were corrected for multiple comparisons using the

Bonferroni Correction. The statistical analysis was performed using Stata™ Release 7 statistical software (Stata Corporation, USA).

In addition, for the transformed PanNF and S100 staining areas and percentage staining areas a three way analysis of variance (ANOVA) was performed with factors day (63 and 120), graft type (E-PHB, PHB-ALG and PHB-GGF), gap length (2 or 4cm) and their interactions. To further study the effect of time on the PanNF and S100 staining areas and percentage staining areas, a one way ANOVA was performed for each graft type looking at the factor time (63 and 120). The data was presented using the group total values rather than the values for each individual gap length. Following analysis of the initial results a Student's t-test was used and the P values of the post-hoc tests were corrected for multiple comparisons using the Bonferroni Correction. The statistical analysis was performed using Stata™ Release 7 statistical software (Stata Corporation, USA).

2.12.3 Motor reinnervation

The percentage atrophy and percentage loss of muscle mass was calculated for each left sided (operated) TA/EDL muscle complex (cf 2.8.1.2). To investigate the difference between the percentage loss of muscle mass between the three groups a one way ANOVA was performed for each gap length looking at the factor graft type (PHB-GGF, PHB-ALG and EPHB). Following analysis of the initial results Bonferroni's multiple comparison test was performed. In addition, unpaired t-tests were carried out for each

graft type looking at the factor gap length (2 and 4cm) and its effect on the percentage loss of muscle mass. The statistical analysis was performed using Prism® Version 2.01 statistical software (Graph Pad Software, USA).

CHAPTER 3

SHORT-TERM ASSESSMENT OF SHORT AND LONG NERVE GAP REPAIR WITH A PHB/GGF CONDUIT

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3.5 DISCUSSION

3.1 INTRODUCTION

Following nerve injury a gap may arise between the nerve ends. The type of nerve repair performed is dependent on the size of the gap between the proximal and distal stumps. Short gaps of up to 2cm can be repaired directly by mobilisation of the proximal and distal stumps and end-to-end coaptation. Long nerve gaps of greater than 2cm require additional material to bridge the defect (Terzis & Smith 1990b). The current gold standard is the use of nerve autografts which, provides the regenerating axons with a natural guidance channel populated with functioning SC surrounded by their basal lamina. Nerve autografting, however, is far from an optimal treatment.

An alternative to nerve autografting is the use of conduits. Previous work in the rabbit model has shown regeneration up to 4cm in both biological and synthetic nerve conduits (Fawcett & Keynes 1986; Hems & Glasby 1992; Hems & Glasby 1993; Brown *et al.* 1996; Strauch *et al.* 1996). Regeneration across longer gaps in this model has not been successful without the addition of SC (Whitworth *et al.* 1995b).

Previous work from our research group has shown PHB to be a promising material. However, regeneration across 4cm gaps did not result in functional target organ reinnervation. Therefore there is a need to increase the rate and quantity of regeneration through the PHB conduits with the addition of trophic factors, thereby improving the level of reinnervation.

SC are not only important for neuronal survival and axonal specialisation during development (Jessen & Mirsky 1999) but are essential for axonal regrowth following injury (Hall 1986b). GGF is a trophic factor specific for SC, which may promote neuronal survival and proliferation indirectly, by promoting glial cell-neuron interaction (Reynolds & Woolf 1993).

Thus, it might be possible to enhance long gap peripheral nerve regeneration using a bioengineered composite conduit. This construct would act through the contact guidance provided by the PHB material and through the GGF-enhanced axonal regeneration promoted indirectly by its action on SC.

3.2 AIMS

The aim of this part of the study was to assess axonal and SC regeneration in a composite PHB-GGF conduit bridging 2 and 4cm gaps in the rabbit common peroneal nerve. Immunohistochemical assessment of axonal and SC regeneration distance and area was carried out in PHB-GGF conduits, in comparison to empty PHB conduits and PHB conduits filled with alginate alone.

3.3 MATERIALS AND METHODS

Ninety female New Zealand White rabbits were used, divided into 3 groups: PHB conduit filled with GGF in alginate (PHB-GGF), empty PHB conduit (E-PHB) and PHB

conduit filled with alginate alone (PHB-ALG) (cf 2.1, 2.3 and 2.4). In each group, nerve gaps of 2 and 4cm (cf 2.6) were studied and the animals were sacrificed at 21, 42 and 63 days post-operatively. Therefore 5 animals underwent a conduit repair for each gap length, type of conduit and time point.

At the designated time points the animals were sacrificed and the conduit repair sites harvested (cf 2.8.1.1). After fixation the tissue was blocked, sectioned (cf 2.9) and then stained with monoclonal antibodies to PanNF and S100 (cf 2.10.1). The axonal and SC regeneration distance and area were then quantified (cf 2.11).

3.4 RESULTS

At the time of harvest all wounds were healthy. There was no evidence of superficial or deep wound infections and no macroscopic evidence of inflammation. There were no anastomotic failures or evidence of ulceration in the distribution of the common peroneal nerve. From 21 days onwards, it was macroscopically evident that the PHB tubes had become well vascularised in all 3 groups. Numerous blood vessels encircled the outer surface of the conduits and appeared to be arising form the adjacent tibial nerve (Figure 3.1). The number and size of blood vessels increased during the study period. At all harvest points the PHB tubes were noted to be covered in a very thin pseudocapsule, which could be easily removed and the PHB tube explanted (Figure 3.2). The conduits had not become adherent to underlying muscles. On microscopic examination a trifurcation of regenerating fibres was seen at the proximal stump with

axons and SC regenerating parallel to the long axis of the PHB conduit, both along the walls and through the centre of the tube. No regenerating fibres were seen to grow out through the wall of the PHB conduits, although they were freely permeable.

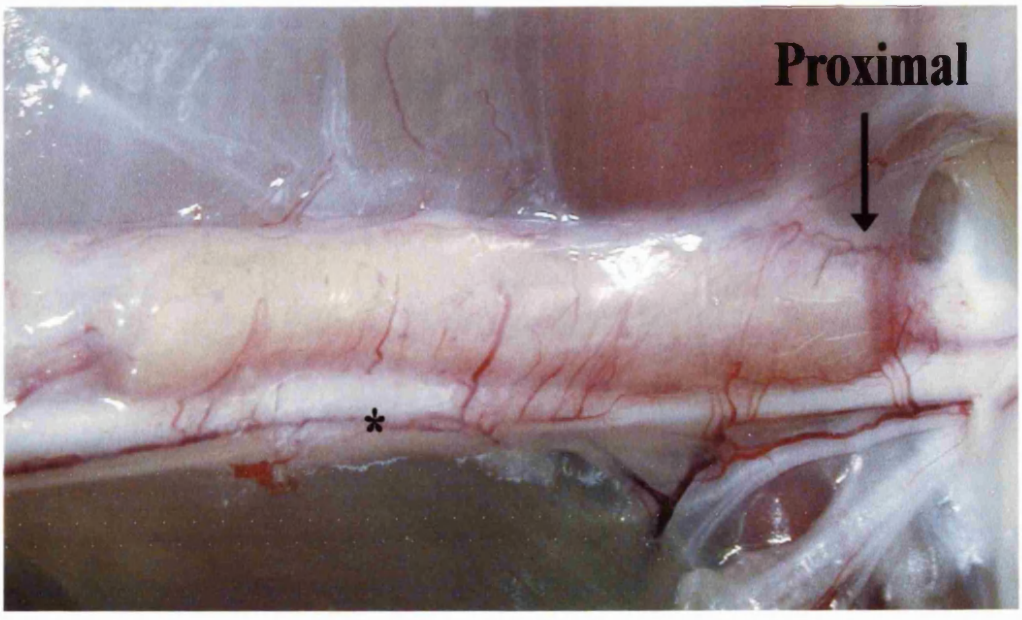

Figure 3.1 PHB conduit 42 days after implantation. Numerous blood vessels encircle the outer surface of the conduit and appear to arise from the adjacent tibial nerve (*).

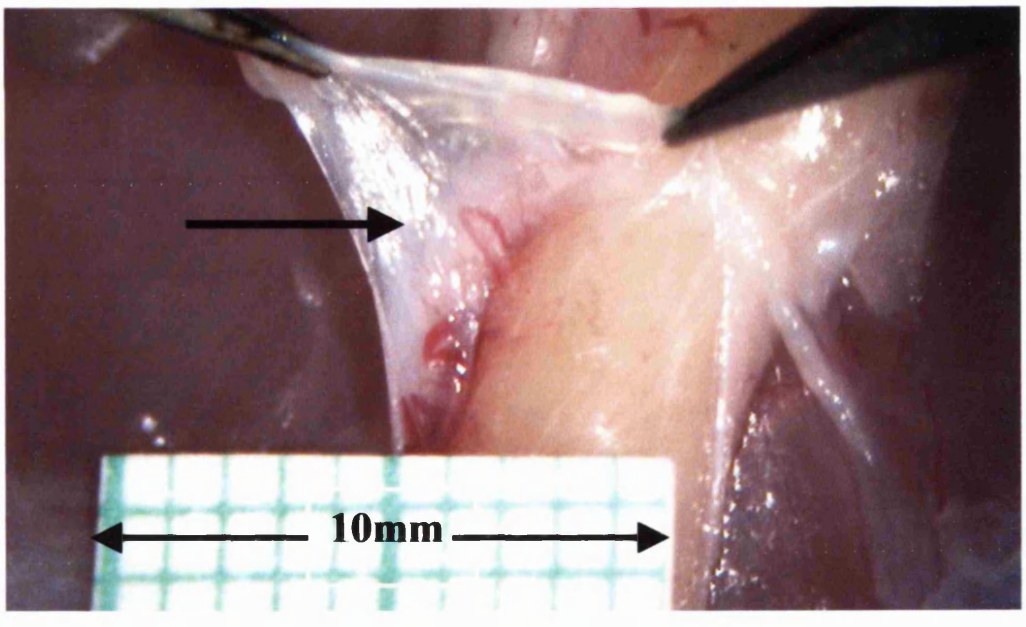

Figure 3.2 At 21 days after implantation a pseudocapsule (arrow) has formed around the PHB conduit.

3.4.1 Regeneration distance

The distance reached into the conduit by the furthermost PanNF and S100 positive fibres were measured at 21, 42 and 63 days for each group (Table 3.1 and 3.2). In the E-PHB conduits both the 2 and 4cm gaps were crossed by SC and axons by 42 days. However, in the PHB-GGF conduits the SC had bridged the 2cm gap by 42 days and the axons had entered the distal stump by 63 days. Neither the 2 or 4cm gaps had been crossed in the PHB-ALG tubes by 63 days. As a result, many of the regeneration distances were censored observations as the regeneration had entered the distal nerve by some of the harvest dates. Therefore a non-parametric method of analysis was used and all the results were corrected for multiple comparisons using the Bonferroni Correction.

AXONAL REGENERATION DISTANCE					
Group	Day	Gap / cm			
		2		4	
		Median	Range	Median	Range
E-PHB	**21**	5.5	5.5-6.0	5.25	5.5-7.5
PHB-ALG	**21**	5.0	2.75-5.5	5.0	4.25-5.5
PHB-GGF	**21**	7.0	6.25-8.5	7.5	6.75-8.25
E-PHB	**42**	>20	All>20	>40	All>40
PHB-ALG	**42**	8.75	8.0-11.0	9.75	8.5-11.00
PHB-GGF	**42**	14.0	12.0->20	12.5	11.75-14.75
E-PHB	**63**	>20	All>20	>40	All>40
PHB-ALG	**63**	15	10.0-18.0	13	10.0-15.0
PHB-GGF	**63**	>20	All>20	15.25	14.5-19.0

Table 3.1 The axonal regeneration distances (mm) in the E-PHB, PHB-ALG and PHB-GGF conduits for the 2 and 4cm nerve repairs at 21, 42 and 63 days. The censored values indicate the minimum regeneration distances reached by the regenerating axons.

SCHWANN CELL REGENERATION DISTANCE					
Group	Day	Gap / cm			
		2		4	
		Median	Range	Median	Range
E-PHB	21	5.5	4.0-6.75	4.5	4.25-7.00
PHB-ALG	21	5.25	3.75-5.5	5.5	4.75-6.75
PHB-GGF	21	8.5	6.25-8.75	8.0	7.5-8.25
E-PHB	42	>20	All>20	>40	All>40
PHB-ALG	42	10.0	8.5-10.5	10.0	6.25-12
PHB-GGF	42	>20	All>20	15.0	12.5-18.0
E-PHB	63	>20	All>20	>40	All>40
PHB-ALG	63	16.5	12.0->20	14.0	11.0-15.0
PHB-GGF	63	>20	All>20	17.0	16.5-22.0

Table 3.2 The SC regeneration distances (mm) in the E-PHB, PHB-ALG and PHB-GGF conduits for the 2 and 4cm nerve repairs at 21, 42 and 63 days. The censored values indicate the minimum regeneration distances reached by the regenerating SC fibres.

3.4.1.1 Comparison between gap lengths (Figures 3.3-3.6)

To investigate whether there was an effect of gap length a Mann-Whitney U one way ANOVA was performed to compare gap lengths for each combination of graft type and day. For S100 staining there was a significant difference between the different gap lengths in the E-PHB grafts at 42 and 63 days ($p=0.03$, 2 vs 4cm) and in the PHB-GGF grafts at 42 days only ($p=0.05$, 2 vs 4cm). For PanNF staining there was a significant difference between the different gap lengths in the E-PHB grafts again at 42 and 63 days ($p=0.03$, 2 vs 4cm) and in the PHB-GGF grafts at 63 days only ($p=0.05$, 2 vs 4cm).

3.4.1.2 Comparison between groups

To investigate the difference in regeneration distance between the graft types a Kruskal-Wallis one way ANOVA test was performed for each combination of gap length and day, as explained in detail below.

3.4.1.1.1 S100 & PanNF regeneration distance for the 2cm gap (Figures 3.3 & 3.4)

At 21 and 42 days there was a significantly greater distance of SC regeneration in the PHB-GGF conduits compared to the PHB-ALG grafts ($p\leq0.03$, PHB-GGF vs PHB-ALG). In addition the SC regeneration distance in the PHB-GGF grafts at 21 days was significantly greater than the E-PHB tubes ($p<0.05$, PHB-GGF vs E-PHB). The SC

regeneration distance in the E-PHB tubes at 42 days was significantly greater than the PHB-ALG grafts (p=0.02, E-PHB vs PHB-ALG).

At 21, 42 and 63 days the PHB-GGF conduits resulted in a significantly greater distance of axonal regeneration compared to the PHB-ALG grafts (p≤0.03, PHB-GGF vs PHB-ALG). The axonal regeneration distance in the PHB-GGF conduits was significantly greater than the E-PHB grafts at 21 days (p=0.02, PHB-GGF vs E-PHB). The E-PHB conduits resulted in a significantly greater distance of axonal regeneration compared to the PHB-ALG tubes at 42 and 63 days (p=0.02, E-PHB vs PHB-ALG).

3.4.1.1.2 S100 and PanNF regeneration distance for the 4cm gap (Figures 3.5 & 3.6)

At 21 days there was a significantly greater distance of SC regeneration in the PHB-GGF conduits compared to the PHB-ALG and E-PHB tubes (p=0.03, PHB-GGF vs PHB-ALG and E-PHB) At 42 and 63 days the SC regeneration distance in the PHB-GGF grafts was greater than the PHB-ALG tubes (p≤0.04, PHB-GGF vs PHB-ALG). The SC regeneration distance in the E-PHB tubes at 42 and 63 days was significantly greater than the PHB-GGF and PHB-ALG grafts (p=0.02, E-PHB vs PHB-GGF and PHB-ALG).

The axonal regeneration distance in the PHB-GGF tubes at 21 days was significantly greater than the PHB-ALG and E-PHB grafts (p=0.03, PHB-GGF vs PHB-ALG and E-PHB). At 42 days the PHB-GGF conduits resulted in a significantly greater distance of

axonal regeneration than the PHB-ALG tubes ($p \leq 0.05$, PHB-GGF vs PHB-ALG). The E-PHB conduits resulted in a significantly greater distance of axonal regeneration compared to the PHB-GGF and PHB-ALG tubes at 42 and 63 days ($p=0.02$, E-PHB vs PHB-GGF and PHB-ALG).

3.4.1.2 Comparison between time points

To look at the effect of time on the regeneration distance within each graft type a Kruskal-Wallis test was performed for each gap length as detailed below. It is important to note that once the gap length had been bridged no further progression in regeneration distance could be observed with time.

3.4.1.3.1 S100 and PanNF regeneration distance for the 2cm gap (Figures 3.3 & 3.4)

For the PHB-GGF and E-PHB conduits the SC regeneration distance at 42 and 63 days was significantly greater than at 21 days ($p \leq 0.02$, 21 vs 42 and 21 vs 63 days). In the PHB-ALG grafts the SC regeneration distance significantly increased with time ($p=0.03$, 21 vs 42 vs 63 days).

Similarly the axonal regeneration distance in the PHB-GGF and E-PHB conduits was significantly greater at 42 and 63 days than at 21 days ($p \leq 0.03$, 21 vs 42 and 21 vs 63 days). For the PHB-ALG grafts the axonal regeneration distance significantly increased with time ($p \leq 0.05$, 21 vs 42 vs 63 days).

3.4.1.3.2 S100 and PanNF regeneration distance for the 4cm gap (Figures 3.5 & 3.6)

For all three graft types the SC regeneration distance at 42 and 63 days was significantly greater than at 21 days ($p \leq 0.05$, 21 vs 42 and 21 vs 63 days).

In the PHB-ALG and E-PHB grafts the axonal regeneration distance at 42 and 63 days was significantly greater than at 21 days ($p \leq 0.03$, 21 vs 42 and 21 vs 63 days). The axonal regeneration distance in the PHB-GGF conduits significantly increased with time ($p \leq 0.05$, 21 vs 42 vs 63 days).

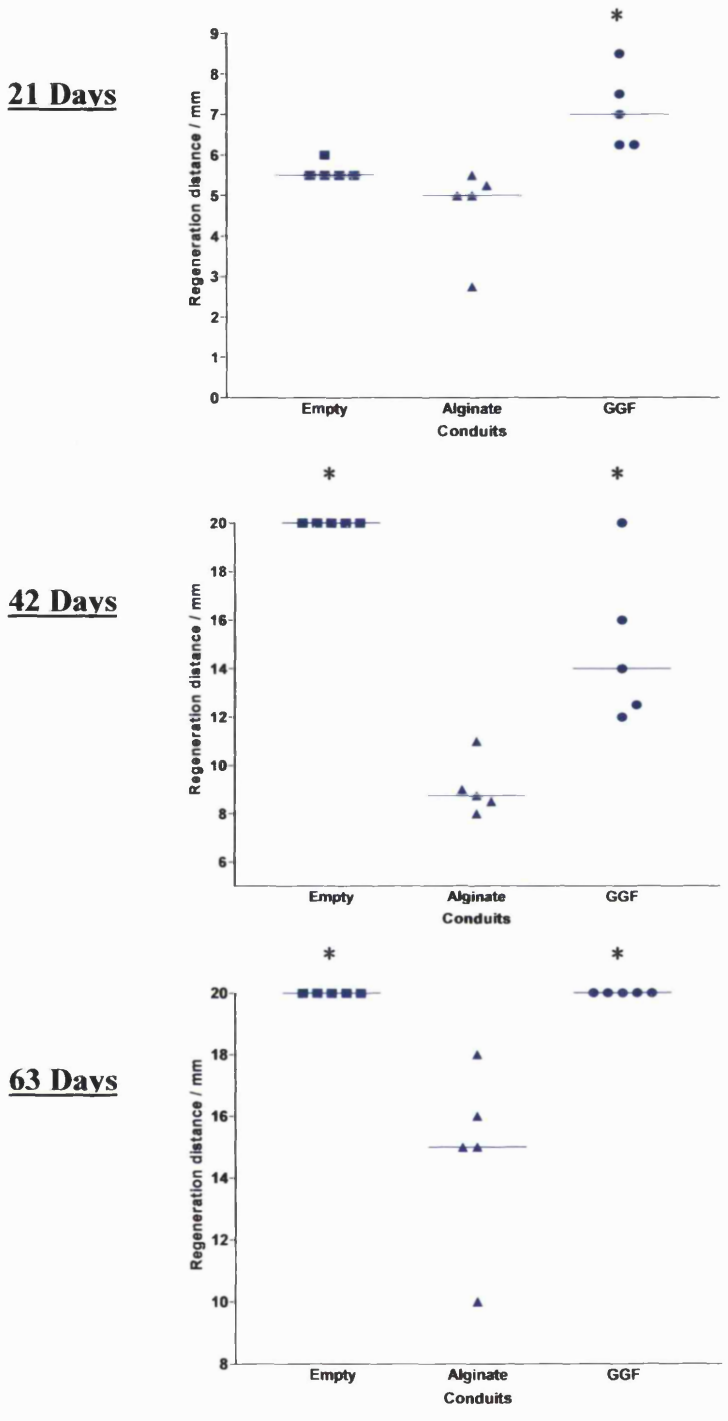

Figure 3.3 Axonal regeneration distance at 21, 42 and 63 days in the 2cm Empty, Alginate and GGF conduit groups. Raw data expressed with median values highlighted. *p≤0.03 GGF vs Empty and Alginate at 21 days (Kruskal-Wallis test). *p≤0.03 GGF vs Alginate and Empty vs Alginate at 42 days (Kruskal-Wallis test). *p=0.02 GGF vs Alginate and Empty vs Alginate at 63 days (Kruskal-Wallis test). N=5 for each group.

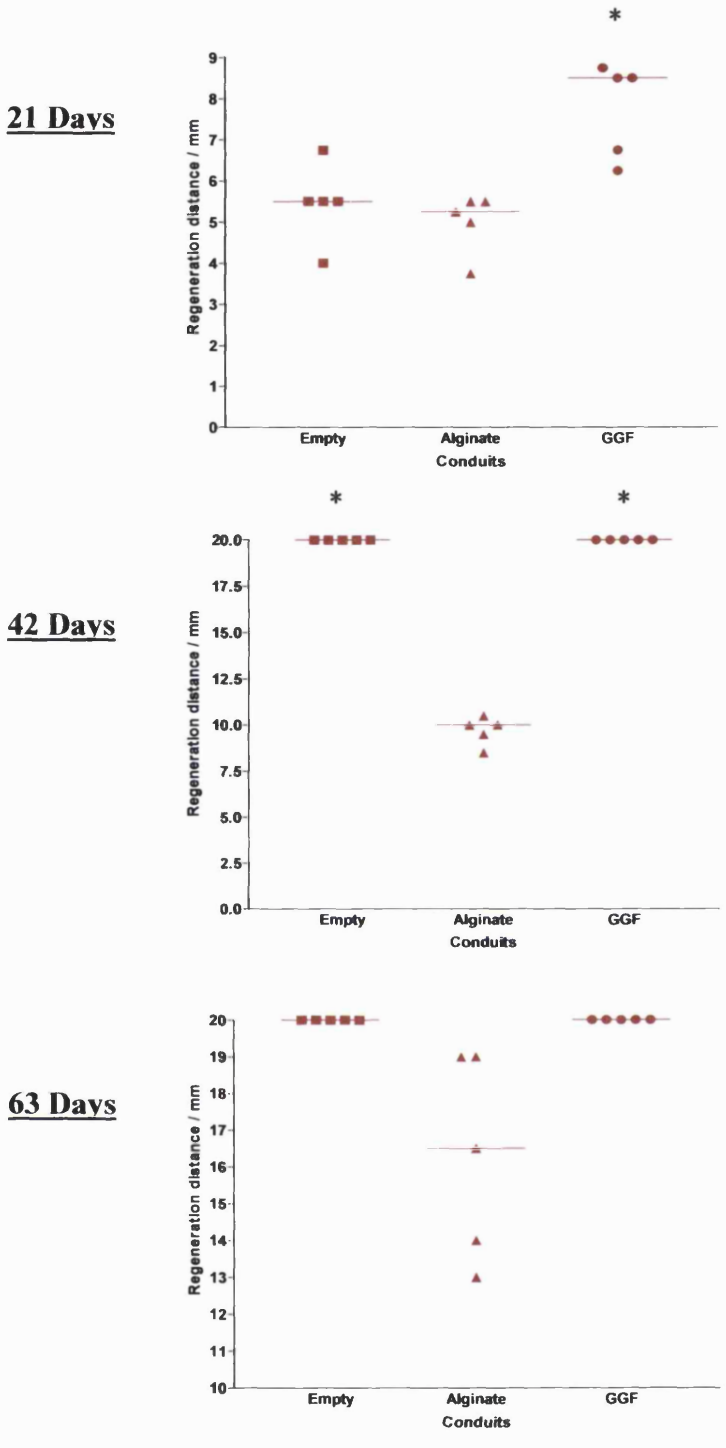

Figure 3.4 SC regeneration distance at 21, 42 and 63 days in the 2cm Empty, Alginate and GGF conduit groups. Raw data expressed with median values highlighted. *p≤0.05 GGF vs Empty and Alginate at 21 days (Kruskal-Wallis test). *p=0.02 GGF vs Alginate and Empty vs Alginate at 42 days (Kruskal-Wallis test). N=5 for each group.

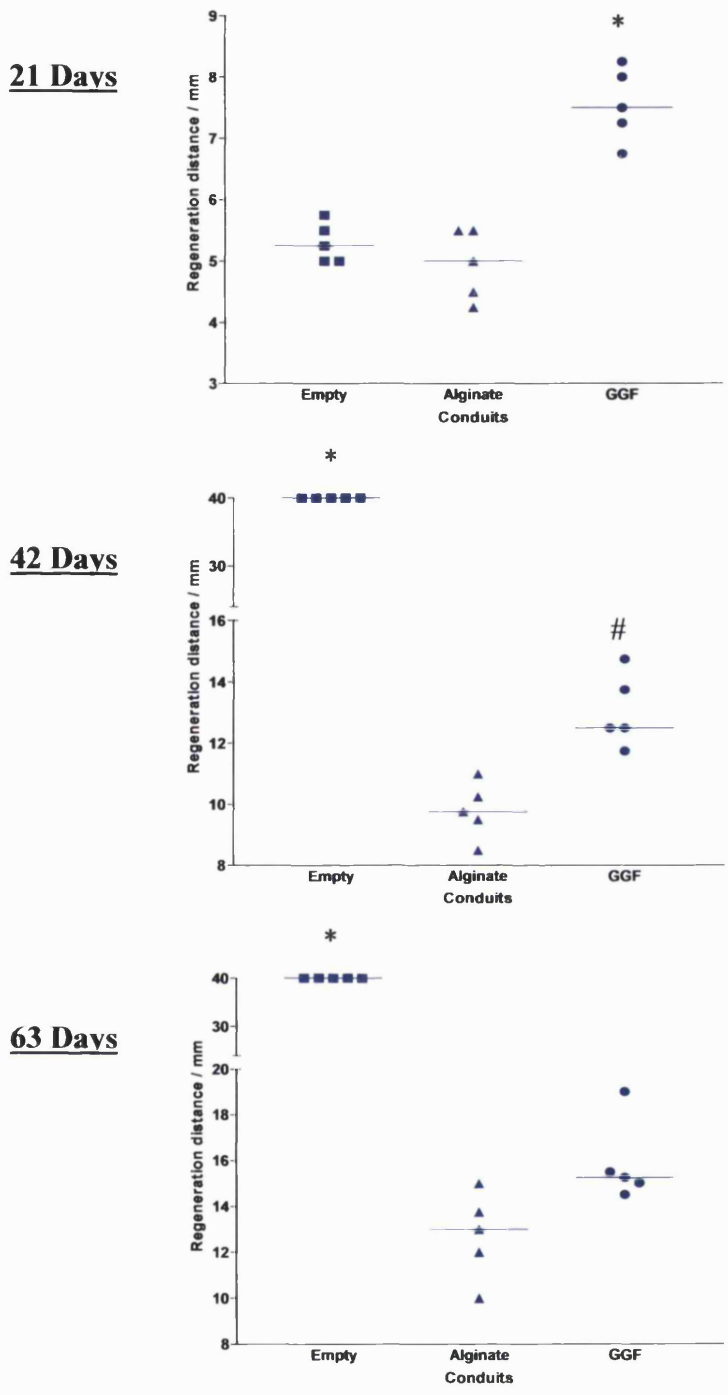

Figure 3.5 Axonal regeneration distance at 21, 42 and 63 days in the 4cm Empty, Alginate and GGF conduit groups. Raw data expressed with median value highlighted. *p=0.03 GGF vs Empty and Alginate at 21 days (Kruskal-Wallis test). #p<0.05 GGF vs Alginate and *p=0.02 Empty vs GGF and Alginate at 42 days (Kruskal-Wallis test). *p=0.02 Empty vs GGF and Alginate at 63 days. (Kruskal-Wallis test). N=5 for each group.

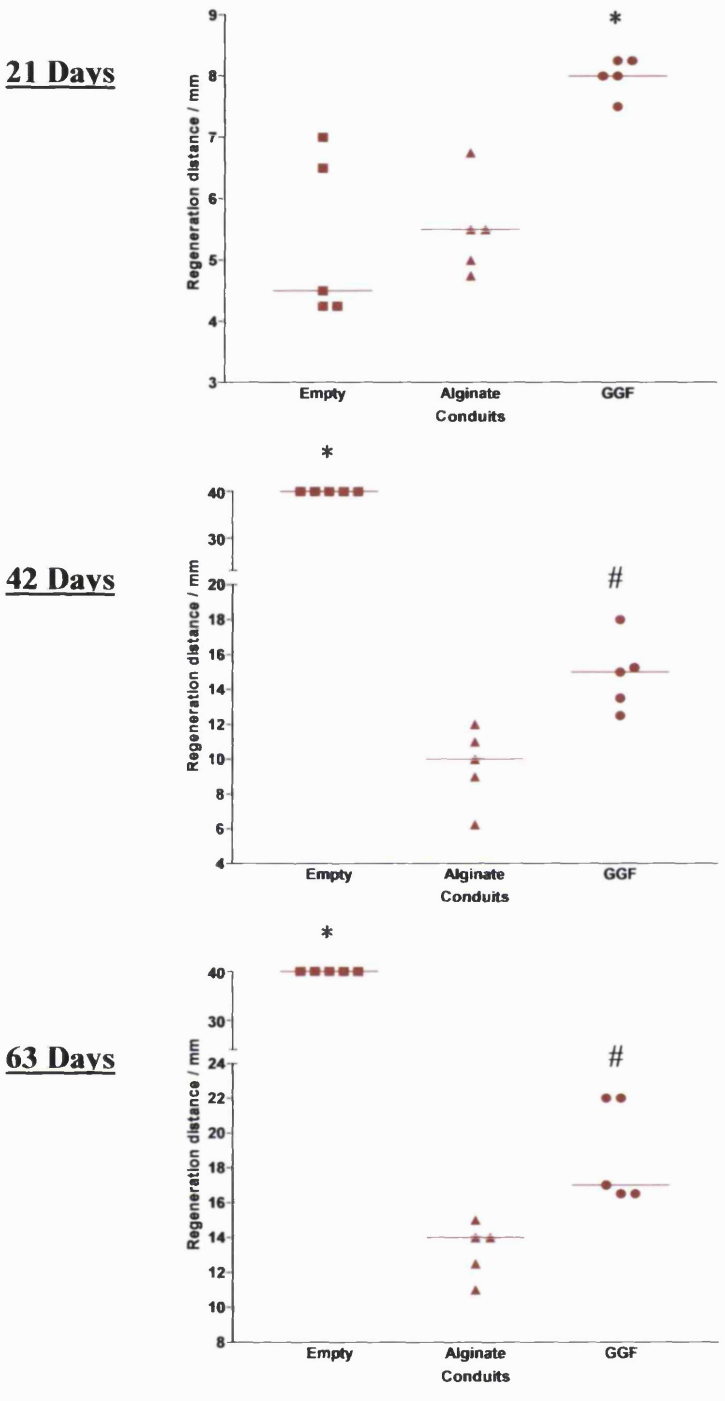

Figure 3.6 SC regeneration distance at 21, 42 and 63 days in the 4cm Empty, Alginate and GGF conduit groups. Raw data expressed with median values highlighted. *p=0.03 GGF vs Empty and Alginate at 21 days (Kruskal-Wallis test). #p=0.04 GGF vs Alginate and *p=0.02 Empty vs GGF and Alginate at 42 days (Kruskal-Wallis test). #p=0.03 GGF vs Alginate and *p=0.02 Empty vs GGF and Alginate at 63 days. (Kruskal-Wallis test). N=5 for each group.

3.4.2 Regeneration area

The PanNF and S100 staining areas and the percentage staining areas were quantified at a point 5mm distal to the proximal end of the conduit, at 21, 42 and 63 days for the three groups. A three way analysis of variance (ANOVA) was performed with factors day (21, 42 and 63), graft type (E-PHB, PHB-ALG and PHB-GGF), gap length (2 or 4cm) and their interactions. The normality assumption of the ANOVA was checked using the Shapiro-Francia W dash test and Bartletts test was used to check the equal variances assumptions. The assumptions from the analysis on the untransformed data were not valid, therefore a square root transformation of the data was carried out. For both PanNF and S100 transformed staining areas and percentage staining areas there was a significant difference between graft types ($p<0.001$, E-PHB vs PHB-ALG vs PHB-GGF, three way ANOVA) and between days ($p<0.001$, 21 vs 42 vs 63, three way ANOVA). There were no significant effects of gap length. For S100 staining areas there were no graft-day, graft-gap or day-gap interactions. For PanNF staining areas there was a graft-day interaction but no graft-gap or day-gap interactions. These results demonstrate that there was a significant difference between the graft types and between time points, but there was no significant difference across the different gap lengths in each group. As no significant difference was identified for staining area and percentage staining area by increasing the gap length from 2 to 4cm, the following data is presented using the group total values rather than the values for each individual gap length. A two way ANOVA was performed on the transformed total area of staining and total percentage area of staining with factors graft type (E-PHB, PHB-ALG and PHB-GGF)

and day (21, 42 and 63) and their interaction. Following analysis of the initial results a Student's t-test was used and the P values of the post-hoc tests were corrected for multiple comparisons using the Bonferroni Correction.

3.4.2.1 Axonal regeneration area

3.4.2.1.1 Area of PanNF staining

A two way ANOVA was performed to compare the transformed data of total area of staining in the E-PHB, PHB-ALG and PHB-GGF grafts at 21, 42 and 63 days (Figure 3.7). At 21 days there was a significantly greater area of staining in the PHB-GGF grafts than the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB). Also the E-PHB grafts had a significantly greater area of staining than the PHB-ALG grafts ($p=0.02$, E-PHB vs PHB-ALG). By 42 days the staining area in the PHB-GGF and E-PHB grafts was significantly greater than the PHB-ALG grafts ($p<0.001$, PHB-GGF vs PHB-ALG, $p=0.015$, E-PHB vs PHB-ALG). At 63 days there was a significantly greater area of staining in the PHB-GGF grafts than the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB). Also the E-PHB grafts had a significantly greater area of staining than the PHB-ALG grafts ($p<0.001$, E-PHB vs PHB-ALG).

Looking at the effect of time on the total area of staining, there was a significant increase from day 21 to day 63 for each of the three graft types: E-PHB and PHB-GGF

grafts ($p<0.001$, 21 vs 42 vs 63 days) and PHB-ALG grafts ($p<0.001$, 21 vs 42 days and 21 vs 63 days; $p=0.02$ 42 vs 63 days).

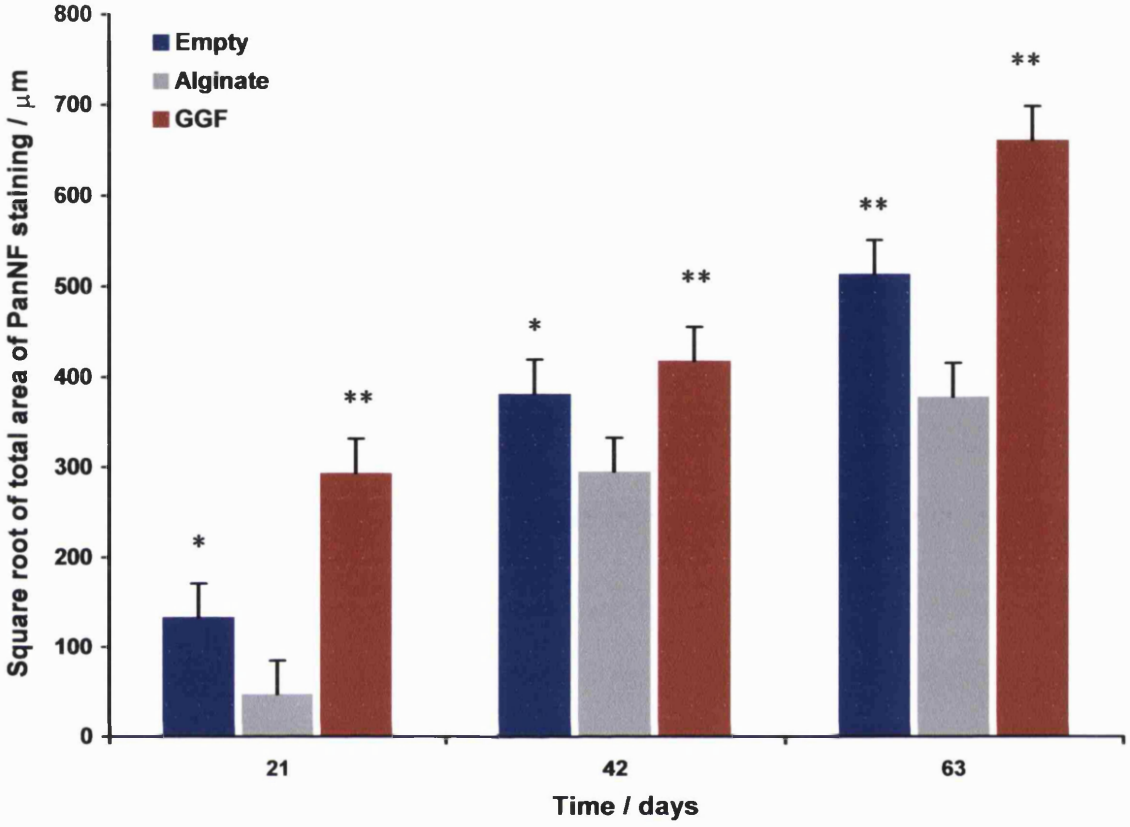

Figure 3.7 Total area of PanNF staining at 21, 42 and 63 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty at 21 and 63 days, GGF vs Alginate at 21, 42 and 63 days and Empty vs Alginate at 63days (post-hoc t-test). *$p\leq0.02$ Empty vs Alginate at 21 and 42 days (post-hoc t-test). N=10 for each group.

3.4.2.1.2 Percentage area of PanNF staining

A two way ANOVA was performed to compare the transformed data of total percentage area occupied by the regenerating nerve in the E-PHB, PHB-ALG and PHB-GGF grafts at 21, 42 and 63 days (Figure 3.8). At 21 days there was a significantly greater percentage area of staining in the PHB-GGF grafts than the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB). Also the E-PHB grafts had a significantly greater area of staining than the PHB-ALG grafts ($p=0.004$, E-PHB vs PHB-ALG). By 42 days there was a significantly greater percentage area of staining in the PHB-GGF and E-PHB grafts than the PHB-ALG grafts ($p<0.001$, PHB-GGF vs PHB-ALG; $p=0.017$, E-PHB vs PHB-ALG). At 63 days there was a significantly greater percentage area of staining in the PHB-GGF grafts than the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB). Also the E-PHB grafts had a significantly greater percentage area of staining than the PHB-ALG grafts ($p<0.001$, E-PHB vs PHB-ALG).

Looking at the effect of time on the total percentage area of staining, there was a significant increase from day 21 to day 63 for each of the three graft types: E-PHB and PHB-GGF grafts ($p<0.001$, 21 vs 42 vs 63 days) and PHB-ALG grafts ($p<0.001$, 21 vs 42 days and 21 vs 63 days; $p<0.01$ 42 vs 63 days).

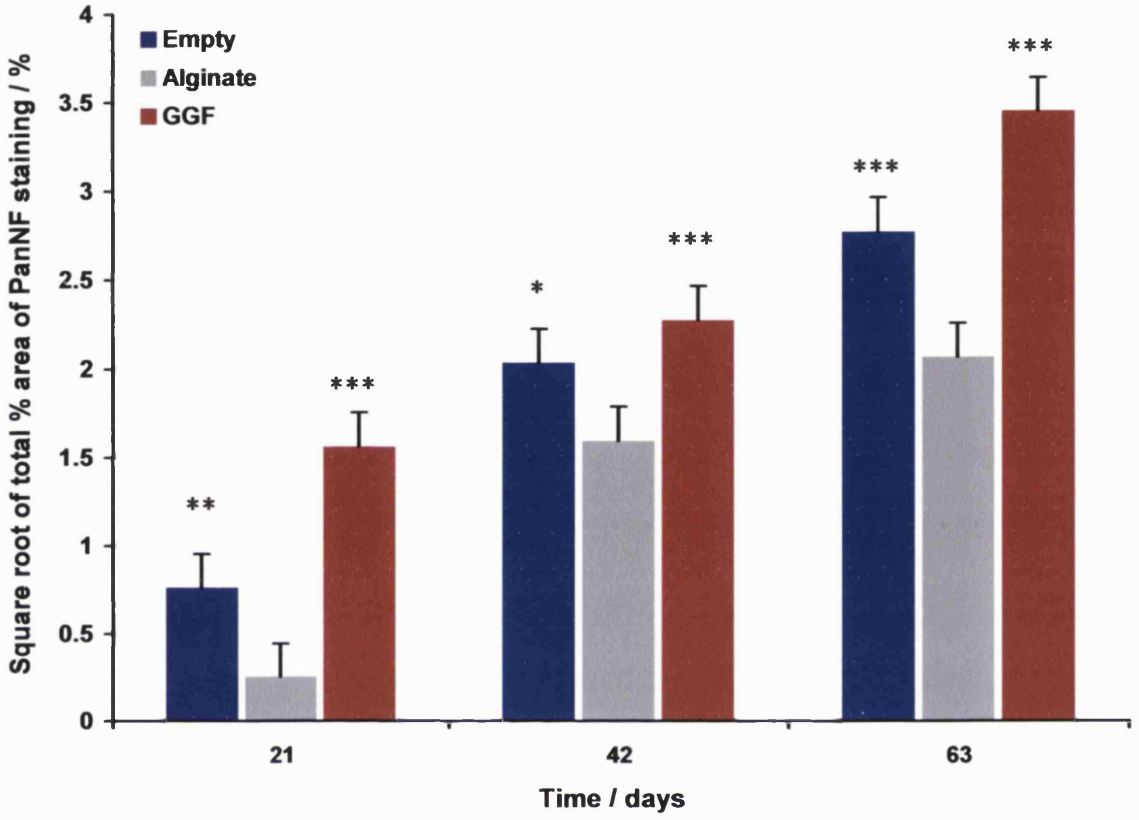

Figure 3.8 Total percentage area of PanNF staining at 21, 42 and 63 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. ***$p<0.001$ GGF vs Empty at 21 and 63 days, GGF vs Alginate at 21, 42 and 63 days and Empty vs Alginate at 63 days (post-hoc t-test). **$p=0.004$ Empty vs Alginate at 21 days and *$p=0.017$ Empty vs Alginate at 42 days (post-hoc t-test). N=10 for each group.

3.4.2.2 SC regeneration area

3.4.2.2.1 Area of S100 staining

A two way ANOVA was performed to compare the transformed data of total area of staining in the E-PHB, PHB-ALG and PHB-GGF grafts at 21, 42 and 63 days (Figure 3.9). At 21, 42 and 63 days there was a significantly greater area of staining in the PHB-GGF grafts than the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB).

Looking at the effect of time on the total area of staining, there was a significant increase from day 21 to day 63 for each of the three graft types ($p<0.001$, 21 vs 42 vs 63 days).

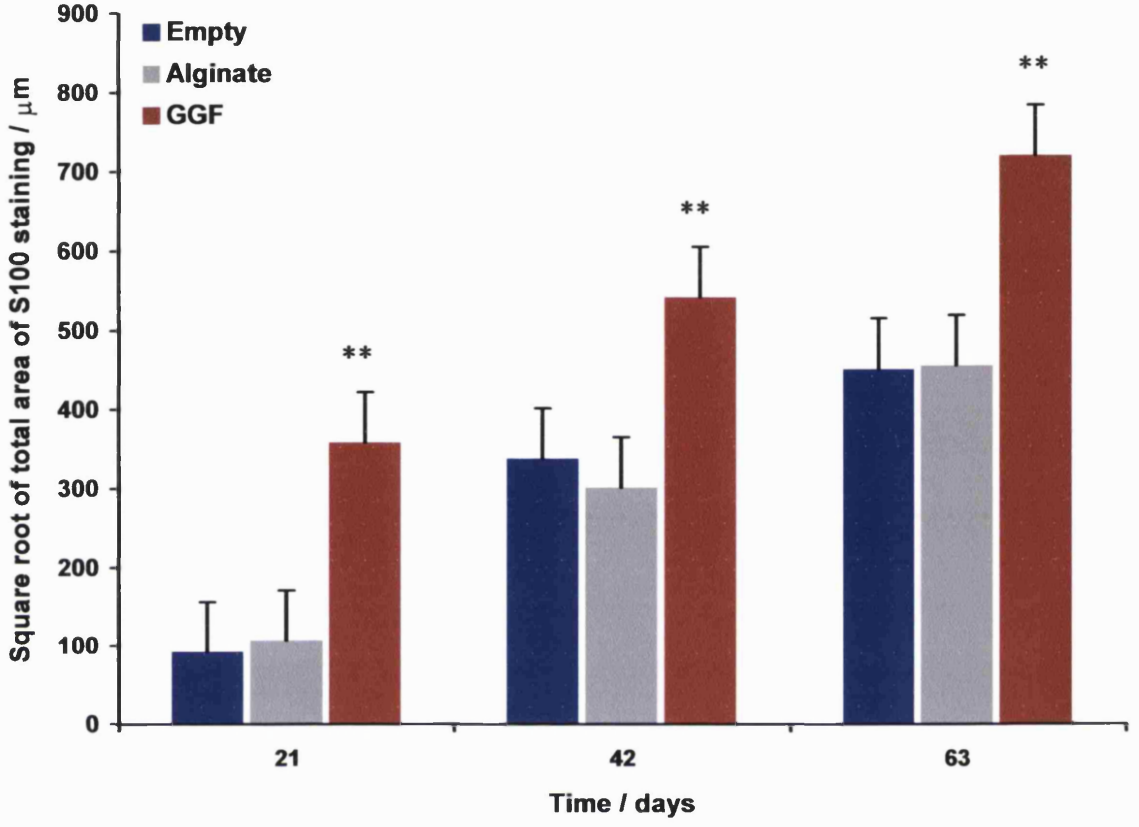

Figure 3.9 Total area of S100 staining at 21, 42 and 63 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and Alginate at 21, 42 and 63 days (post-hoc t-test). N=10 for each group.

3.4.2.2.2 Percentage area of S100 staining

A two way ANOVA was performed to compare the transformed data of total percentage area occupied by the regenerating SC in the E-PHB, PHB-ALG and PHB-GGF grafts at 21, 42 and 63 days (Figure 3.10). Over time there was a significant increase of percentage staining area in the PHB-GGF grafts compared to controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB).

Looking at the effect of time on the total percentage area of staining, there was a significant increase from day 21 to day 63 for each of the three graft types ($p<0.001$, 21 vs 42 vs 63 days).

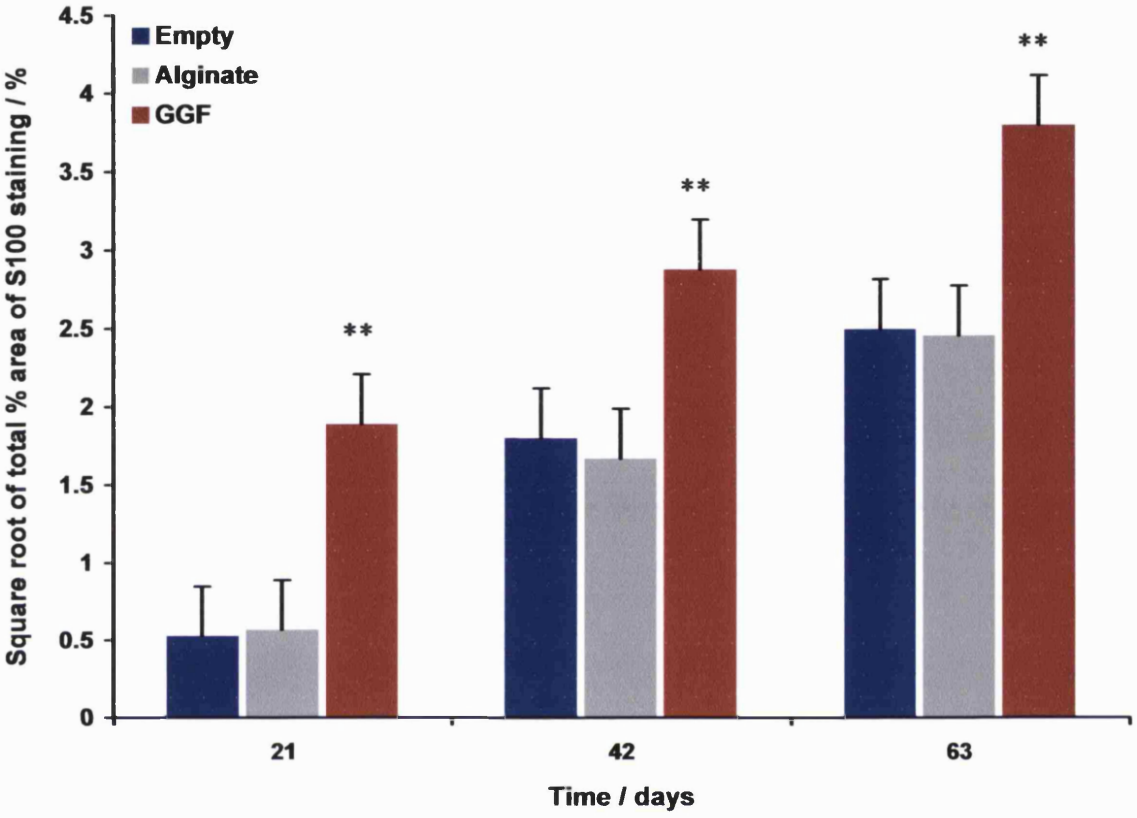

Figure 3.10 Total percentage area of S100 staining at 21, 42 and 63 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and Alginate at 21, 42 and 63 days (post-hoc t-test). N=10 for each group.

3.5 DISCUSSION

The physical characteristics of PHB suggest that it is suitable for use as a peripheral nerve conduit. Indeed, it has been used experimentally as an alternative to direct epineural repair and to bridge short gaps in the rat sciatic nerve model (Hazari *et al.* 1999a; Hazari *et al.* 1999b). Also PHB has been demonstrated to support axonal regeneration across long gap repairs, although it did not result in functional target organ reinnervation (Young *et al.* 2002). The possible reasons for this are that the regenerating axons reached the distal stump too late to enter healthy endoneurial tubes by which time the SC have started to die, or that the number of axons reaching the distal stump were too few to result in correct and successful target organ reinnervation. These results are in keeping with the work of other researchers who have demonstrated that the upper limit for spontaneous regeneration through an empty biological or synthetic conduit is 4cm in the rabbit model (Hems & Glasby 1992; Hems & Glasby 1993; Strauch *et al.* 1996) and 5cm in the primate model (Mackinnon *et al.* 1985; Dellon & Mackinnon 1988; Mackinnon & Dellon 1990a). However, since axons will grow many centimetres within a distal stump, this suggests that the microenvironment of the axons within the conduit may contribute to this failure (Mackinnon & Dellon 1988b).

Manipulation of the local environment has met with some success in improving regeneration over short gaps but there are very few reports in the literature concerning neurotrophic factors and long gap nerve repair. The majority of work on short gap repair has focused on the use of a single exogenous neurotrophic factor acting on specific

neurons (Whitworth *et al.* 1996; Sterne *et al.* 1997a; Tham *et al.* 1997; Bryan *et al.* 2000). However, the use of GGF as an exogenous trophic agent acting directly on SC could trigger a spectrum of mechanisms, which may indirectly enhance peripheral nerve regeneration. GGF promotes SC proliferation (Dong *et al.* 1995) and induces the release of various neurotrophic factors such as NGF, BDNF, CNTF, LIF, PDGF and IGF from the increased number of SC (Heumann *et al.* 1987; Friedman *et al.* 1992; Eccleston *et al.* 1993; Acheson *et al.* 1995; Cheng *et al.* 1996; Mahanthappa *et al.* 1996; Matsuoka *et al.* 1997). These neurotrophic factors are retrogradely transported to the neuronal cell body where they promote survival, increase protein synthesis and proliferation (Terenghi 1999).

Following axotomy, axonal and SC regeneration from the proximal stump normally progresses at a similar rate, or with SC slightly ahead of the neurites (Jessen & Mirsky 1998). This was demonstrated by the comparable distance of axonal and SC regeneration into the E-PHB and PHB-ALG conduits at all three time points (Table 3.1 and 3.2). However, in the PHB-GGF grafts at 42 days the SC have bridged the 2cm gap whilst the axons have regenerated three-quarters of the way into the conduit (Table 3.1 and 3.2). After axotomy the expression of erbB2 and erbB3 receptors on SC in the proximal and distal stumps is rapidly upregulated (Li *et al.* 1997; Bryan *et al.* 2000). GGF released from the alginate within the lumen of the conduit acts on these receptors resulting in the stimulation of SC migration and proliferation (Mahanthappa *et al.* 1996). This results in the acceleration of SC regeneration ahead of axonal regeneration in the PHB-GGF conduits. As a result of the continuous SC scaffold forming in the PHB-GGF

conduits and a spectrum of endogenous neurotrophic factors being released from these SC, a guidance path is provided along which the axons can regenerate more rapidly and in increased amounts to the distal stump. Cell adhesion molecules such as NCAM, L1 and laminin, which are expressed on the inner surface of the SC are upregulated after axotomy (Martini *et al.* 1994) and mediate SC interaction with the counterpart molecules present on the growth cone (Jessen & Richardson 1996). It is conceivable that this contact guidance, and the GGF enhanced neurotrophic and neurotropic support provided by the SC, promoted axonal regeneration such that the axons had bridged the 2cm gap in the PHB-GGF tubes in one animal at 42 days and in all five animals by 63 days, whilst axons had not yet crossed the 2cm PHB-ALG grafts even at 63 days. Although the presence of PanNF positive axons in the distal stump of the 2cm PHB-GGF conduits was observed at 63 days, it is likely that they had reached the distal stump earlier. Indeed between 21 and 42 days their regeneration rate was 0.37mm/day, and if this rate were maintained then the axons would have bridged the gap by 56 days. The effects of GGF on the rate of SC regeneration, and thereby axonal regeneration, were comparable for both the 2 and 4cm gaps. Although the response was most marked in the first 21days, it was sustained until 63 days as was demonstrated by the increase in regeneration distance with time.

It is interesting to note that the SC and axons in the 2 and 4cm E-PHB conduits bridge the gaps by 42 days even if at 21 days the regeneration rate was significantly slower than that observed in the PHB-GGF conduits. In the E-PHB grafts the topographical and microgeometric cues are provided by the individual polymer fibres, which are aligned

longitudinally along the wall of the conduit, and gradually separate from each other over the first 63 days to occupy a proportion of the original lumen (Hazari *et al.* 1999a; Young *et al.* 2002) thus increasing the surface area for cellular alignment and guidance. However, in the PHB-GGF and PHB-ALG conduits the PHB fibres can not readily migrate into the lumen because of the presence of the alginate gel, thus resulting in an initial decrease in contact guidance cues provided by the PHB fibres. However, this initial disadvantage is compensated by the action of GGF, which results in the enhancement of the quantity of SC and axonal regeneration.

It is also possible that the high mannuronic content alginate gel is behaving as a physical barrier owing to its small pore size. In this alginate there is a high proportion of M-blocks (cf 1.6.1) which themselves are short and linked together by long segments which are elastic and result in greater collapse of the M-block structures, ultimately resulting in a smaller pore size. Hence the regenerating SC and axons may find it more difficult to find a pathway of low resistance, thereby slowing down the rate of regeneration. Indeed on microscopic examination the regenerating SC and axons appear to regenerate along the outer surface of the hydrogel and do not penetrate it. In addition, the presence of the alginate gel may impede the neurotropic effect of the growth factors released from the distal stump particularly over longer gap lengths where there is a larger volume of alginate within the conduit. Similar observations were made by other investigators when collagen and laminin gels were used as an intraluminal matrix (Valentini *et al.* 1987; Labrador *et al.* 1998). They presumed that the gel remnants were blocking neurotropic factor diffusion and therefore axonal regeneration and suggested

that reducing the concentration of the protein gel may overcome this problem (Valentini *et al.* 1987; Labrador *et al.* 1998). Consistently at 63 days there was evidence of the presence of alginate within the conduits, which is expected as mammalian cells lack enzymes to digest alginate and the slow renal excretion is dependant on the loss of gel structure occurring as the calcium ions dissolve out.

At 21 days the SC and axonal regeneration distance in the PHB-GGF conduits were significantly greater than in the E-PHB grafts. This may be a reflection of the GGF release profile from the alginate gel, with the maximal release occurring in the first 21 days. Secondly in the E-PHB grafts the PHB fibre separation and migration into the conduit lumen may not be sufficient to enhance regeneration by contact guidance during the initial 21 days. Lastly, when the proximal and distal stumps are inset by 2mm into the PHB-GGF and PHB-ALG conduits a volume of alginate is displaced out of the conduit to accommodate for this and therefore there maybe no initial physical resistance provided by the alginate in the close proximity of the proximal and distal stumps.

The quantity of SC regeneration was significantly greater in the PHB-GGF grafts at all three time points when compared to the controls. For axonal regeneration the addition of GGF significantly increased the quantity of PanNF staining in comparison to the PHB-ALG grafts at all three time points, and at 21 and 63 days when compared to the E-PHB grafts. Although the addition of GGF produced the most dramatic increase in the quantity of SC and axonal regeneration at 21 days, this effect was sustained up to 63 days, as was demonstrated by a continuous increase in the quantity of regeneration.

Interestingly the quantity of axonal regeneration in the PHB-ALG grafts was significantly less than in the E-PHB grafts at all time points, possibly indicating that the alginate behaves as a barrier and prevents full axonal regeneration.

Interestingly no significant statistical difference was observed in the quantity of SC and axonal regeneration within each group at any time point on increasing the gap length form 2 to 4cm. With respect to regeneration distance there was no significant effect of gap length prior to the SC and axons bridging the 2 and 4cm gaps. But when the nerve gaps had been bridged the effect of gap length on the regeneration distance appeared to be significant. However, one needs to interpret this with caution as it is likely that the axons in the 2 and 4cm conduits have actually regenerated a similar distance, as would be expected from their comparable rate of regeneration at the earlier time points, but the regeneration distance can not be accurately assessed as it is beyond the conduit and into the distal nerve. This would suggest that the nerve gap does not influence the neurotropic and neurotrophic effects produced by the distal stump on the proximal stump. However, there is some debate regarding the distance over which the distal stump can influence the proximal regenerating stump and this may be as little as 5mm in the rat (Politis *et al.* 1982; Longo *et al.* 1983; Brunelli *et al.* 1994; Hefti 1994; Frey *et al.* 1996; Kiyotani *et al.* 1996; Strauch *et al.* 1996). Therefore the ability of the proximal stump to regenerate may be influenced by the other two main factors already mentioned; contact guidance provided by the PHB fibres, the GGF induced increase quantity of the SC scaffold within the conduit, as well as the inherent neurotrophism which is further

enhanced by the GGF triggered release of neurotrophic factors from SC (Mahanthappa *et al.* 1996).

In conclusion this study has demonstrated that the addition of GGF significantly increases the quantity of SC and axonal regeneration over short and long gaps when compared to E-PHB and PHB-ALG conduits. Although by 63 days the SC and axons have not bridged the longer gap, they are regenerating at a rate comparable to those, which have bridged the 2cm gap. Functional recovery following nerve repair is not only dependent on the speed of regeneration but also on the quantity of regeneration, correct target organ reinnervation and maturation of the regenerated nerve fibres. In the PHB-GGF conduits the rate of regeneration is inferior to the empty tubes at the later time points, but the quantity is significantly greater which in theory should increase the chances of appropriate target organ reinnervation, endogenous neurotrophic support and therefore survival and maturation of the axons. The results of the PHB-ALG tubes were inferior to the E-PHB conduits for both gap lengths and time points, suggesting that the presence of alginate maybe damping down the full effects of GGF.

CHAPTER 4

LONG-TERM ASSESSMENT OF SHORT AND LONG NERVE GAP REPAIR WITH A PHB/GGF CONDUIT

4.1 INTRODUCTION

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4.4.4 PHB conduit transverse ultrathin sections

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4.4.6 Muscle reinnervation

4.5 DISCUSSION

4.1 INTRODUCTION

In Chapter 3 the distance and area of SC and axonal regeneration through PHB-GGF conduits bridging nerve gaps of 2 and 4cm was assessed up to 63 days post operatively and compared to E-PHB and PHB-ALG tubes. This investigation demonstrated that the area of regeneration in the PHB-GGF conduits was greater than the E-PHB and PHB-ALG conduits at nearly all time points. Although the 4cm gap in the PHB-GGF tubes had as yet not been bridged, the rate of regeneration was comparable to that of the 2cm tubes implying that the SC and axons would reach the distal nerve stump by a later time point.

Following transection of a peripheral nerve the motor and sensory organs undergo denervation trophy. In muscle this manifests itself as atrophy with a discernible loss of muscle mass (Pellegrino & Franzini 1963). Reinnervation of the muscle results in a return of muscle mass (Bertelli & Mira 1995), which correlates well with the force of contraction (Gillespie *et al.* 1987). In this study the muscle mass was measured to assess the level of motor reinnervation following short and long nerve gap repair using PHB-GGF, PHB-ALG and E-PHB conduits.

Young *et al.* demonstrated that in empty PHB conduits there was abundant axonal regeneration across short and long gaps in the short-term studies, but at long-term time points there were fewer myelinated axons in the distal stump of the long gaps, associated with poorer target organ reinnervation (Young *et al.* 2002). As the addition of

GGF in the PHB conduits, increased the quantity of axonal regeneration (cf 3.4.1), it was expected that once the axons reached the distal stump there would be a greater probability of appropriate target organ reinnervation, neurotrophic support and axonal survival.

4.2 AIMS

The aim of this study was to assess whether the regeneration observed within the 2cm and 4cm PHB-GGF conduits at early time points was sustained at 120 days and whether the regeneration within the conduit and the distal stump was proportionate to the reinnervation of muscle.

4.3 MATERIALS AND METHODS

Thirty female New Zealand White rabbits were used for this study. The animals were divided into 3 groups according to whether the nerve gap was repaired with either PHB-GGF, PHB-ALG or E-PHB conduits (cf 2.4). Furthermore, in each group nerve gaps of 2 and 4cm were repaired (cf 2.6) therefore 5 animals underwent a specific conduit nerve repair at each gap length. At 120 days post-operatively the animals were sacrificed and the combined tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were excised under standard conditions and weighed (cf 2.8.1.2). The combined weight of the two muscles was recorded for each side and the percentage muscle atrophy was calculated for each animal and subtracted from 100 to obtain the percentage loss of

muscle mass (cf 2.8.1.2). After harvesting the muscle the conduit was dissected free from the surrounding tissue between the sciatic notch and the level of the knee and a section of distal nerve stump was harvested from a point 5mm distal to the distal anastomosis of the gap repair. This segment of distal nerve was marked for orientation and fixed in 1% paraformaldehyde and 1.5% glutaraldehyde in phosphate buffer overnight (cf 2.8.1.2). The nerve conduits were explanted and a 2mm transverse slice was removed from a point 12mm distal to the proximal end of the tube. This slice was also fixed in 1% paraformaldehyde and 1.5% glutaraldehyde in phosphate buffer overnight. Semithin and ultrathin sections of the conduit transverse slice and semithin sections of the distal nerve stump were prepared. Humphrey's stain was used to demonstrate regenerating fascicles and myelinated fibres in the semithin sections (cf 2.10.2) and the ultrathin sections were prepared for transmission electron microscopy to assess morphology (cf 2.10.3). These sections were qualitatively assessed. The conduit and associated segment of nerve, proximal and distal to the transverse slice were placed on a splint and fixed in Zamboni's solution (cf 2.8.1.2). Using the cryostat longitudinal sections were made and stained using the avidin-biotin complex (ABC) nickel enhancement technique with antiserum to PanNF and S100 (cf 2.10.1) and then assessed for axonal and SC regeneration distance (cf 2.11.1) and area (cf 2.11.2).

4.4 RESULTS

4.4.1 General observations

All animals survived until the planned harvest time. There was no evidence of trophic ulceration and none of the animals appeared to be disabled when moving around their cages. The original scar and muscle splitting incision were both well healed with no evidence of superficial or deep infection. In the intermuscular cleft the nerve repair site was easily identifiable. The conduits were covered in a fibrous capsule, which had thickened compared to the pseudocapsule seen previously at 63 days, but were still well vascularised (Figure 4.1a). The capsule now also involved the tibial nerve (Figure 4.1a). There was no macroscopic evidence of tissue inflammation and once the capsule had been incised the PHB nerve conduit could be easily removed (Figure 4.1b). There were no anastomotic failures with the proximal and distal stumps identified entering the PHB tubes. The 2.4cm PHB conduits were still cylindrical in shape along their entire length, however the 4.4cm conduits had a subtle hour glass appearance with some narrowing in their mid portion but there was no obvious kink (Figure 4.2). All the conduits had maintained their original length. The distal nerves of the three groups were approximately of the same diameter but appeared slightly smaller and paler in comparison to the proximal nerves. The PHB tubes themselves were flexible, firm in consistency and non-friable. On microscopic examination regeneration had occurred across the 2 and 4cm gaps in all 30 animals. The pattern of regeneration was as seen at the earlier time points (cf 3.4), parallel to the long axis of the PHB conduits with fibres

growing along the internal aspect of the conduit itself as well as through the centre of the tube. No regenerating fibres were seen to grow out through the wall of the PHB conduits, although they were freely permeable.

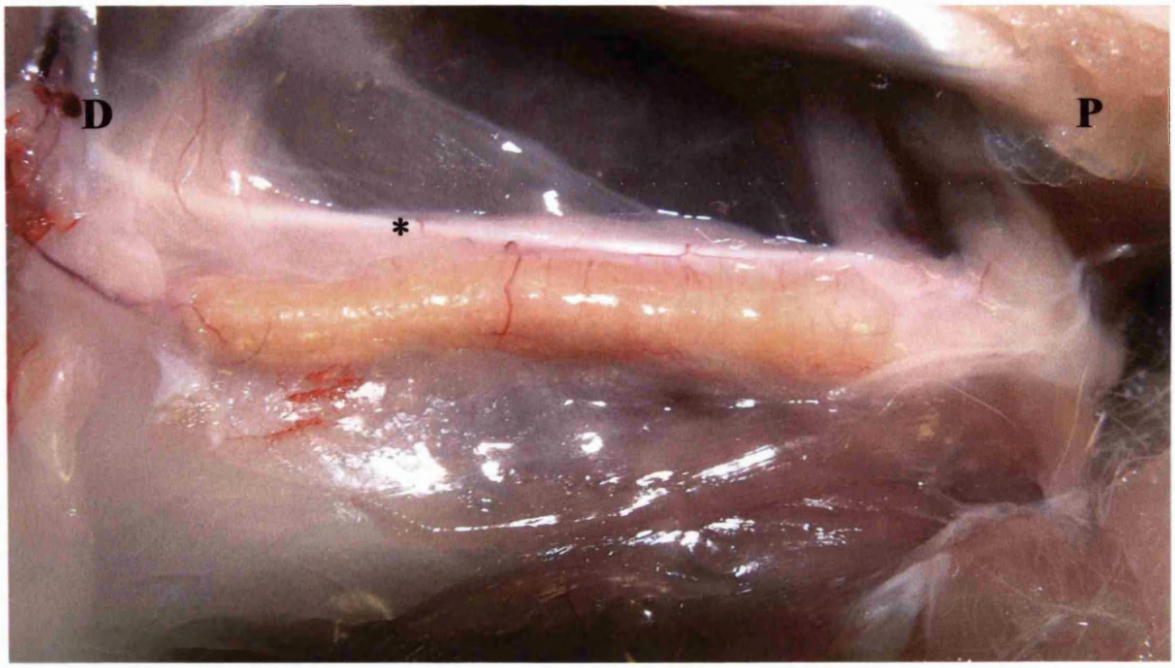

Figure 4.1a The pseudocapsule surrounding the PHB conduit is thicker at 120 days and also involves the adjacent tibial nerve (*). (P = proximal, D = distal).

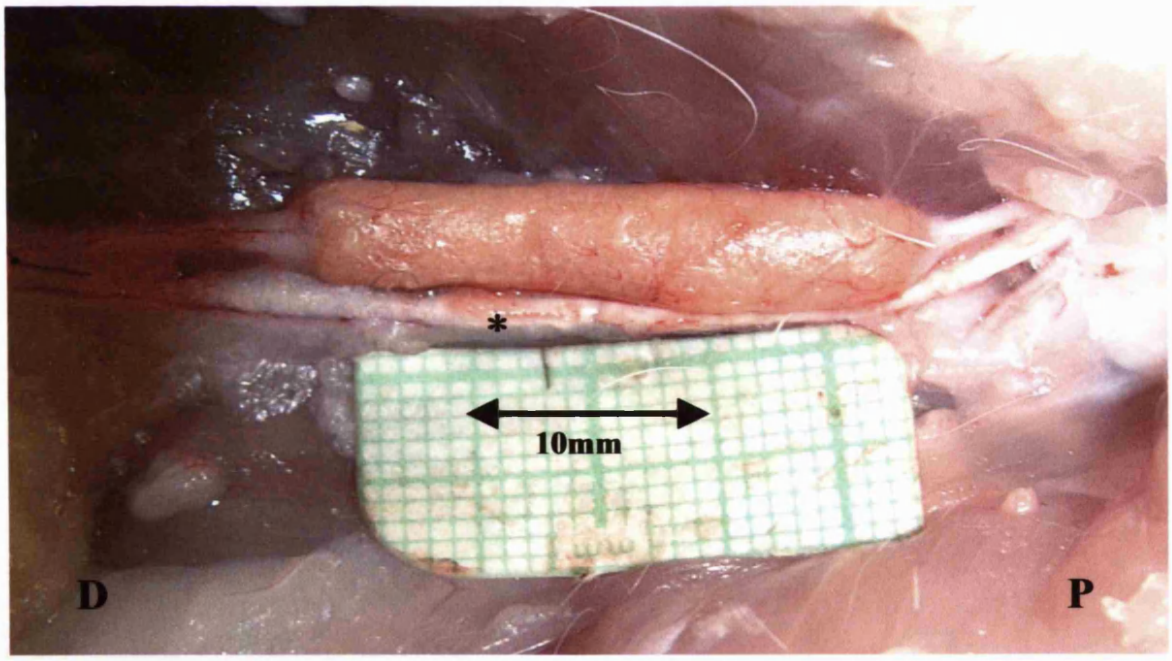

Figure 4.1b PHB conduit after explantation from the pseudocapsule. (* = Tibial nerve, P = proximal, D = distal).

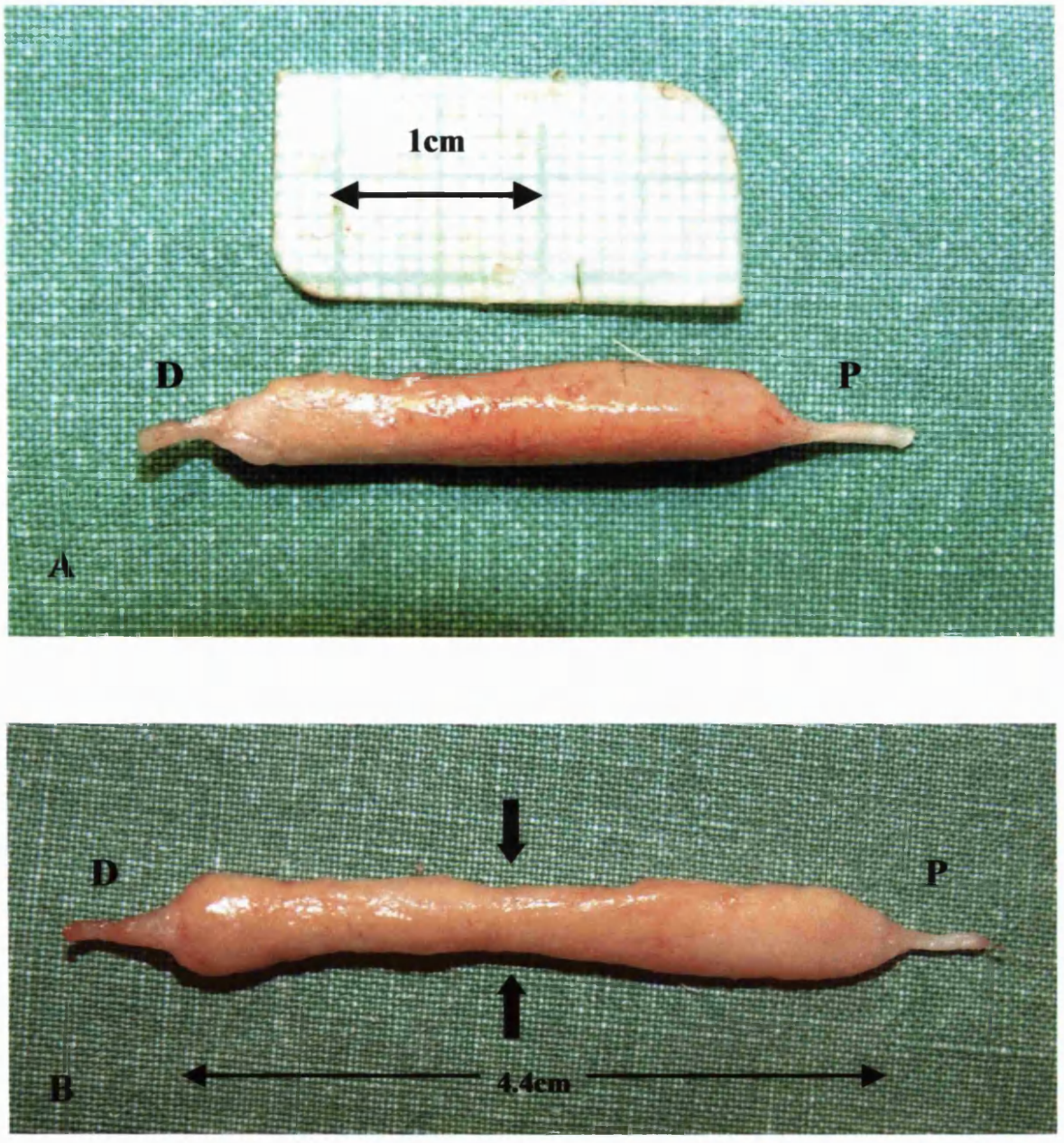

Figure 4.2 (A) A 2.4cm PHB conduit 120 days after implantation is still cylindrical in shape along its entire length. (B) A 4.4cm PHB conduit harvested at the same time point has a slight hour glass appearance with some narrowing in its mid portion (arrows).

4.4.2 Regeneration area

The PanNF and S100 staining areas and the percentage staining areas were quantified at a point 5mm distal to the proximal end of the conduit, at 120 days for the three groups (cf 2.11.3). Firstly, a two way analysis of variance (ANOVA) was performed with factors graft type (E-PHB, PHB-ALG and PHB-GGF) and gap length (2 or 4cm) and their interactions. For example, the interaction analysis assessed whether the difference between the graft types varied with gap length. The normality assumption of the ANOVA was checked using the Shapiro-Francia W dash test and Bartletts test was used to check the equal variances assumptions. The assumptions from the analysis on the untransformed data were not valid, therefore a square root transformation of the data was carried out. For both the PanNF and S100 transformed staining areas and percentage staining areas there was a significant difference between graft types ($p<0.001$, E-PHB vs PHB-ALG vs PHB-GGF) but not gap length. There was no graft-gap interaction for S100 percentage staining area but for S100 staining area ($p=0.04$), PanNF staining area ($p=0.03$) and PanNF percentage staining area ($p<0.001$) the interaction was significant. Therefore for the S100 percentage staining area only, the data was presented using the group total values rather than the values for each individual gap length. Following analysis of the initial results a Student's t-test was used and the P values of the post-hoc tests were corrected for multiple comparisons using the Bonferroni Correction.

4.4.2.1 Axonal regeneration area

4.4.2.1.1 Area of PanNF staining

A one way ANOVA was performed on the transformed data for PanNF staining area for each graft type looking at the factor gap length (2 and 4cm) (Figure 4.3). For the E-PHB and PHB-ALG conduits there was no significant difference in the percentage area of staining between the 2 and 4cm nerve gaps. However, with the PHB-GGF grafts there was a significantly greater percentage area of staining in the 4cm compared to the 2cm nerve gaps ($p=0.008$, 2 vs 4cm).

To study the effect of graft type on PanNF staining, a one way ANOVA was performed for each of the gap lengths looking at the factor graft type (E-PHB, PHB-ALG and PHB-GGF) (Figures 4.4 and 4.5). For both the 2 and 4cm nerve gaps there was a significantly greater area of staining in the PHB-GGF conduits compared to the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB, 2 and 4cm gaps). For the 4cm nerve gaps only, there was a significantly greater area of staining in the E-PHB tubes compared to the PHB-ALG grafts ($p=0.008$, E-PHB vs PHB-ALG).

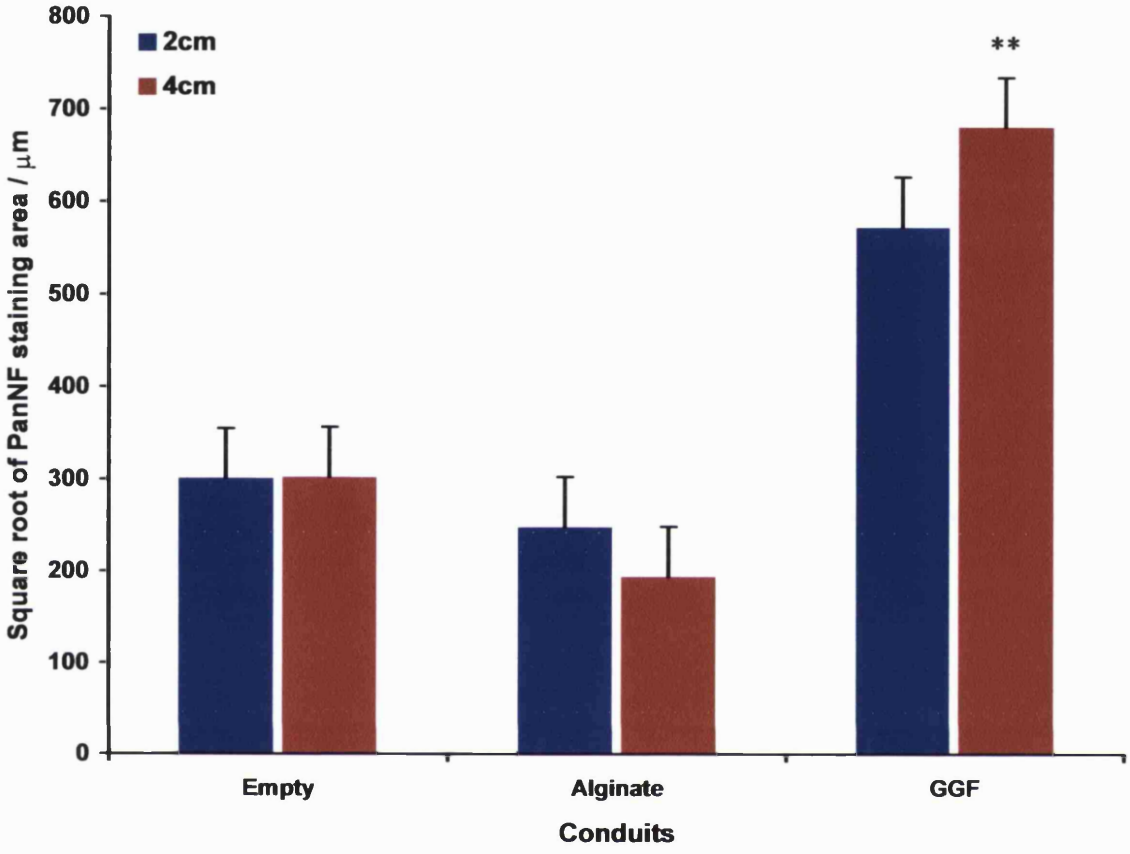

Figure 4.3 Area of PanNF staining for the 2 and 4cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **p=0.008 2cm vs 4cm GGF conduits (post-hoc t-test). N=5 for each group.

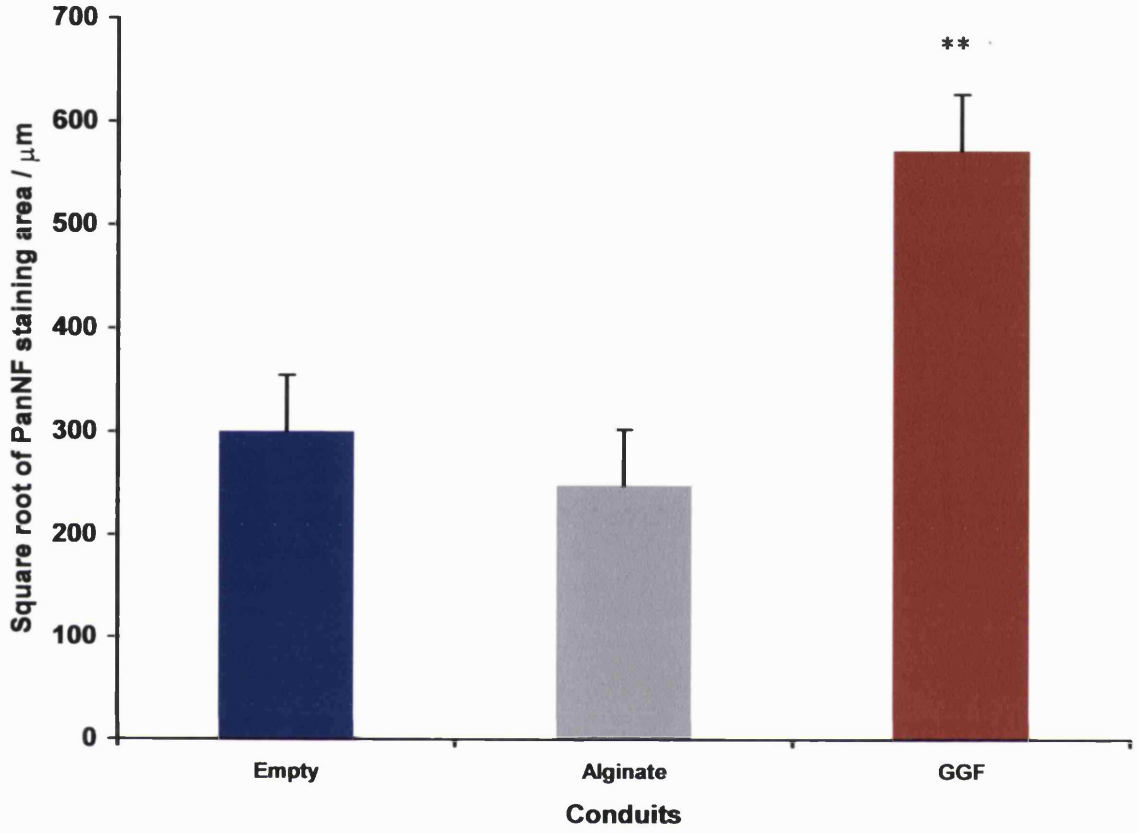

Figure 4.4 Area of PanNF staining for the 2cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and Alginate (post-hoc t-test). N=5 for each group.

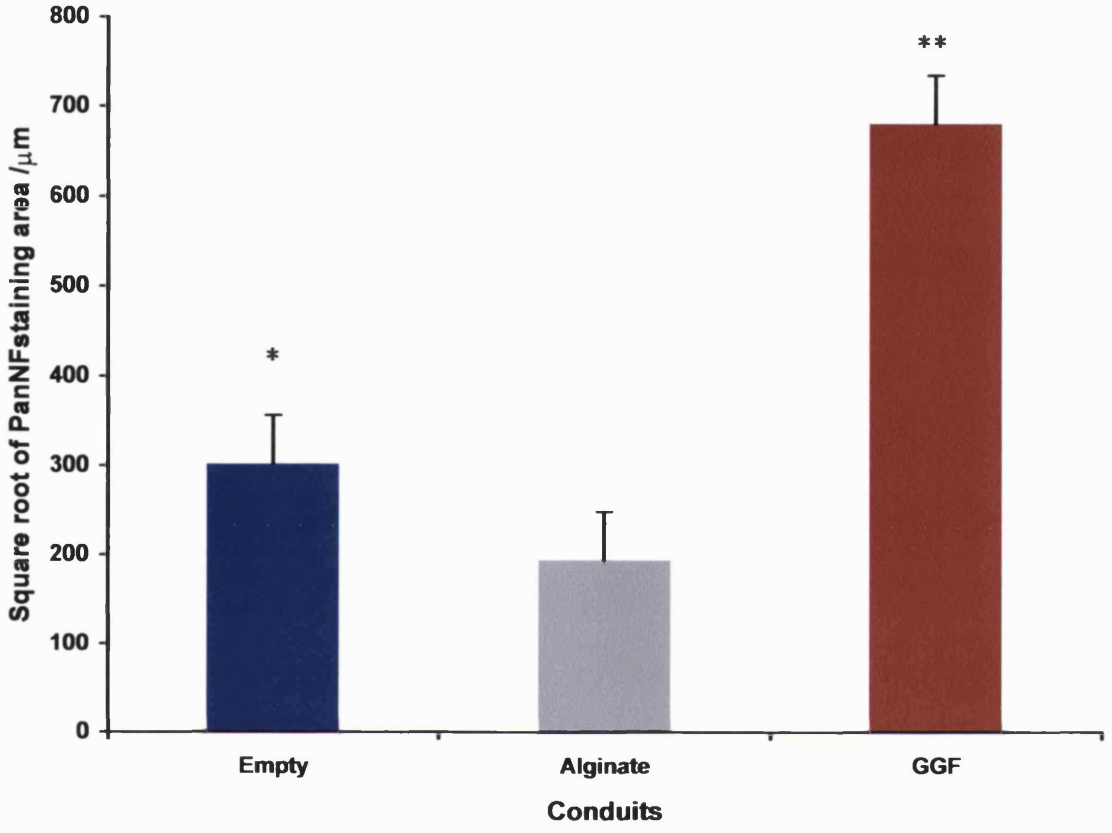

Figure 4.5 Area of PanNF staining for the 4cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and Alginate (post-hoc t-test). *$p=0.008$ Empty vs Alginate (post-hoc t-test). N=5 for each group.

4.4.2.1.2 Percentage area of PanNF staining

A one way ANOVA was performed on the transformed data for percentage area of PanNF staining for each graft type looking at the factor gap length (2 and 4cm) (Figure 4.6). For the E-PHB and PHB-ALG conduits there was no significant difference in the percentage area of staining between the 2 and 4cm nerve gaps. However, with the PHB-GGF grafts there was a significantly greater percentage area of staining in the 4cm compared to the 2cm nerve gaps ($p=0.003$, 2 vs 4cm).

To study the effect of graft type on the transformed data for percentage area of staining, a one way ANOVA was performed for each of the gap lengths looking at the factor graft type (E-PHB, PHB-ALG and PHB-GGF) (Figures 4.7 and 4.8). For both the 2 and 4cm nerve gaps there was a significantly greater percentage area of staining in the PHB-GGF conduits compared to the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB, 2 and 4cm gaps). For both gap lengths the E-PHB tubes had a significantly greater percentage area of staining compared to the PHB-ALG grafts ($p\leq0.003$, E-PHB vs PHB-ALG, 2 and 4cm gaps).

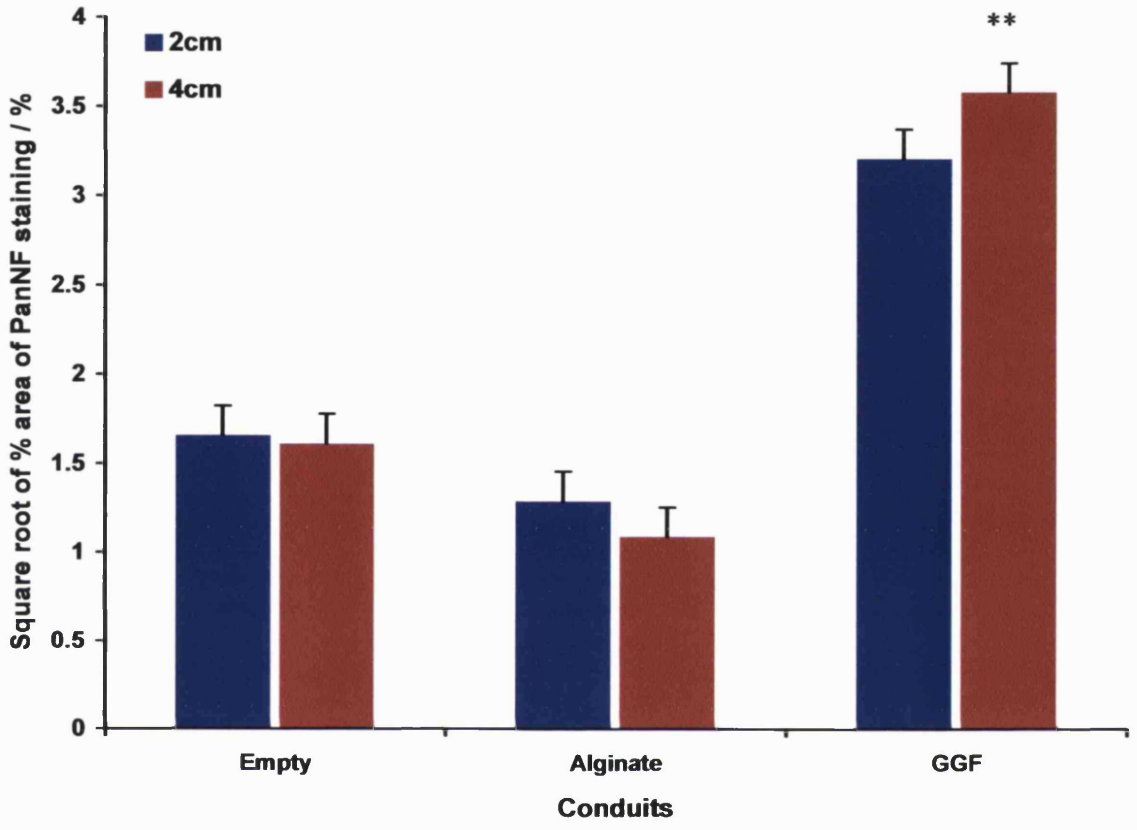

Figure 4.6 Percentage area of PanNF staining for the 2 and 4cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **p=0.003 2cm vs 4cm GGF conduits (post-hoc t-test). N=5 for each group.

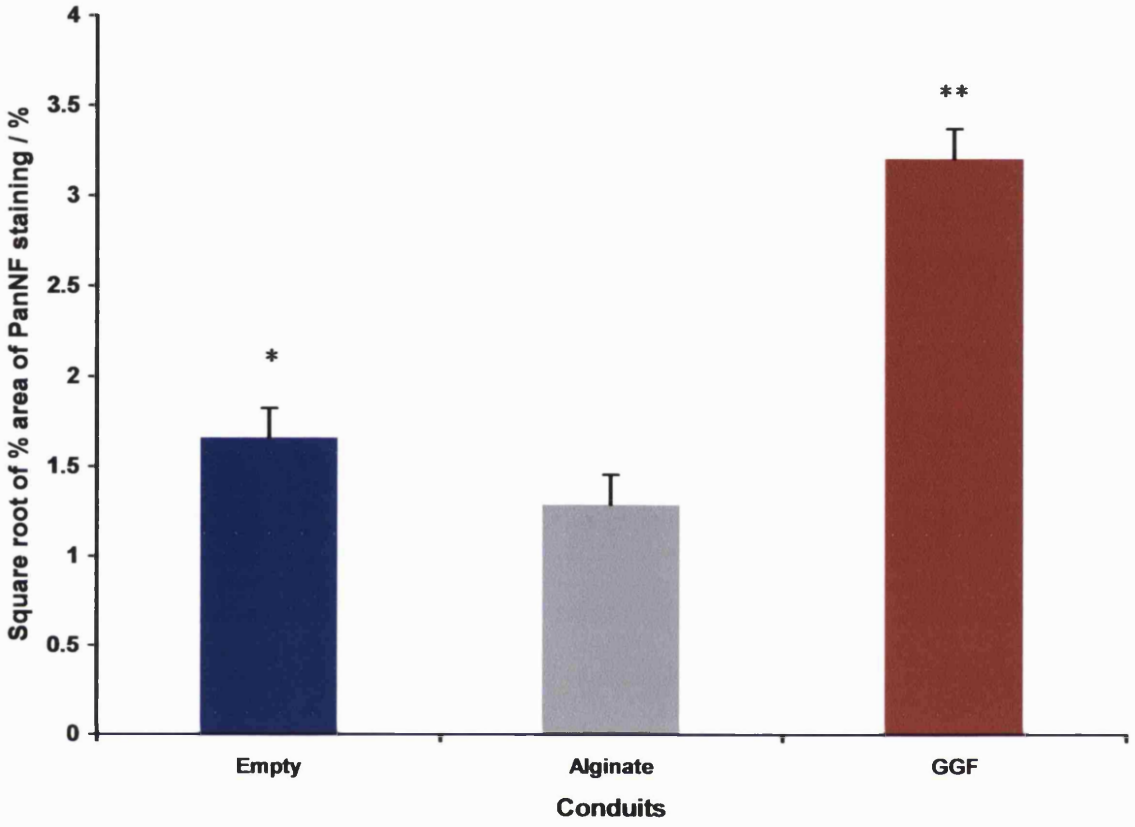

Figure 4.7 Percentage area of PanNF staining for the 2cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and Alginate (post-hoc t-test). *$p=0.003$ Empty vs Alginate (post-hoc t-test). N=5 for each group.

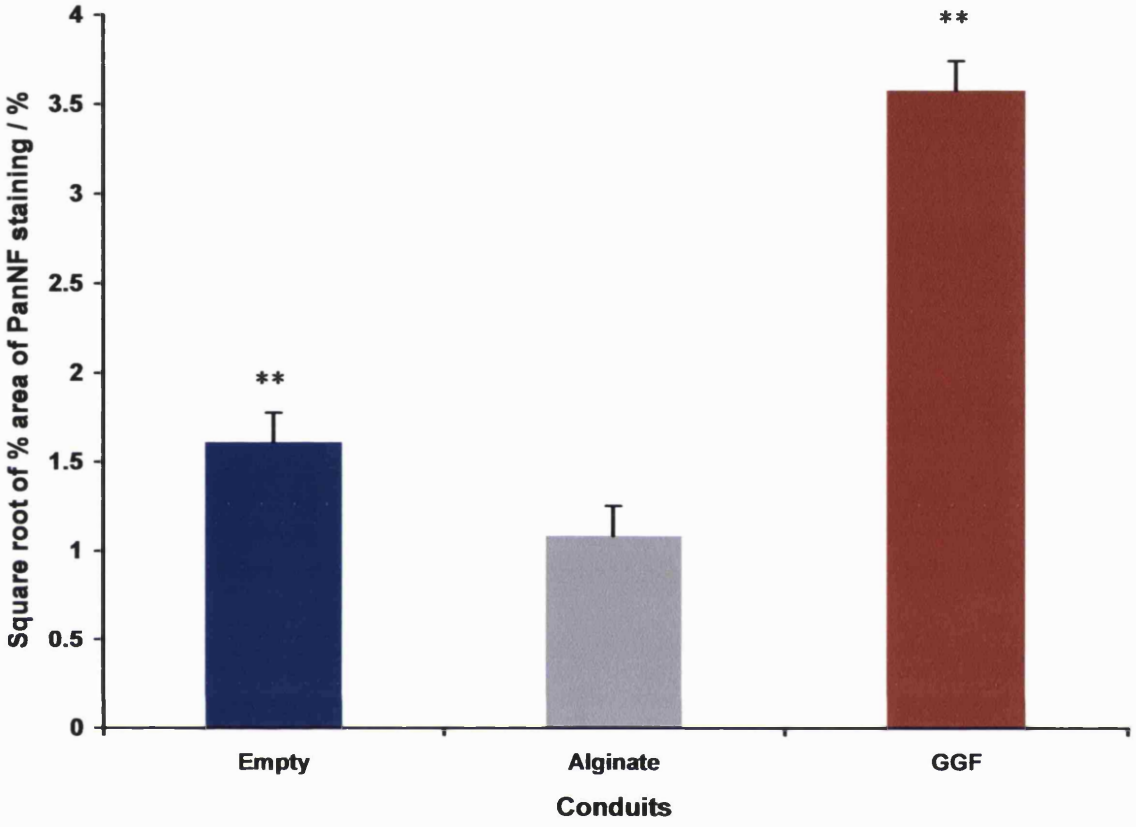

Figure 4.8 Percentage area of PanNF staining for the 4cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and Alginate, and Empty vs Alginate (post-hoc t-test). N=5 for each group.

4.4.2.2 SC regeneration area

4.4.2.2.1 Area of S100 staining

A one way ANOVA was performed on the transformed data for S100 staining area for each graft type looking at the factor gap length (2 and 4cm) (Figure 4.9). There was no significant difference in the percentage area of staining between the 2 and 4cm nerve gaps for each type of conduit.

To study the effect of graft type on S100 staining, a one way ANOVA was performed for each of the gap lengths looking at the factor graft type (E-PHB, PHB-ALG and PHB-GGF) (Figures 4.10 and 4.11). For the 2cm nerve gaps there was a significantly greater percentage area of staining in the PHB-GGF conduits compared to the PHB-ALG tubes ($p<0.001$, PHB-GGF vs PHB-ALG). However, for the 4cm gaps there was a significantly greater percentage area of staining in the PHB-GGF conduits compared to both controls ($p<0.001$, PHB-GGF vs E-PHB and PHB-ALG).

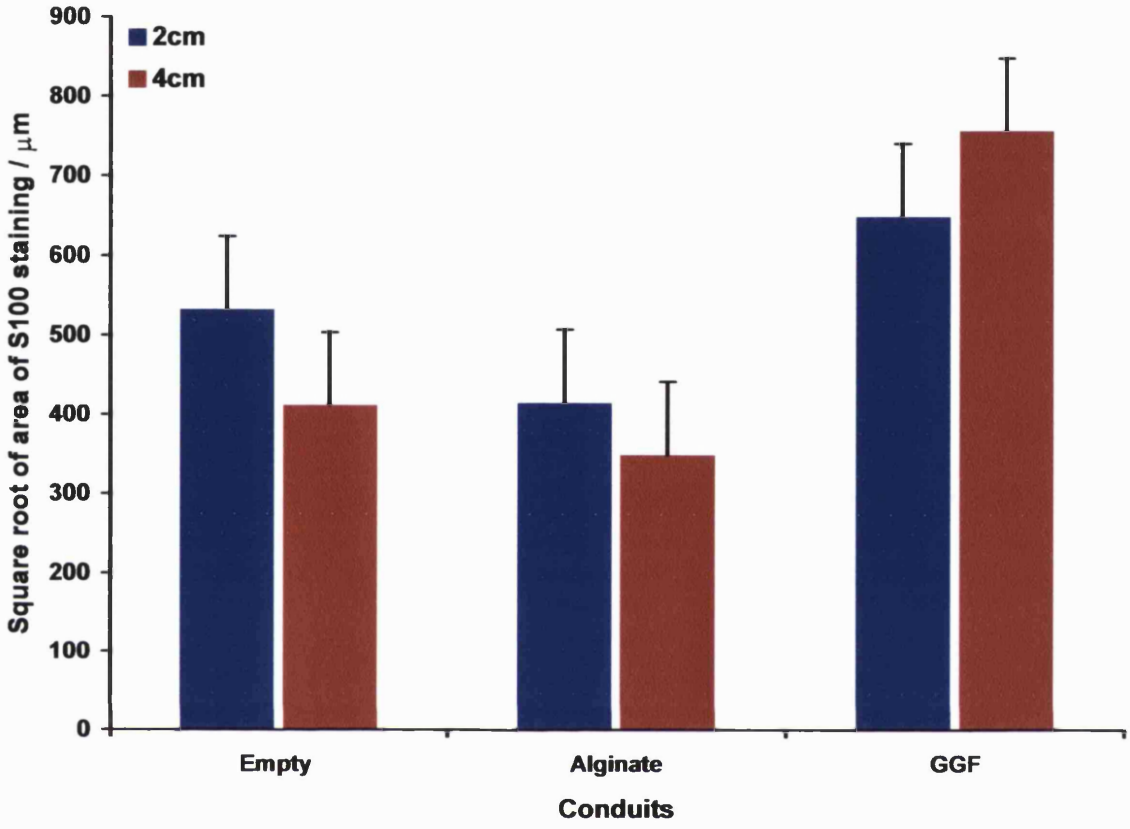

Figure 4.9 Area of S100 staining for the 2 and 4cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. No significant interaction was observed. N=5 for each group.

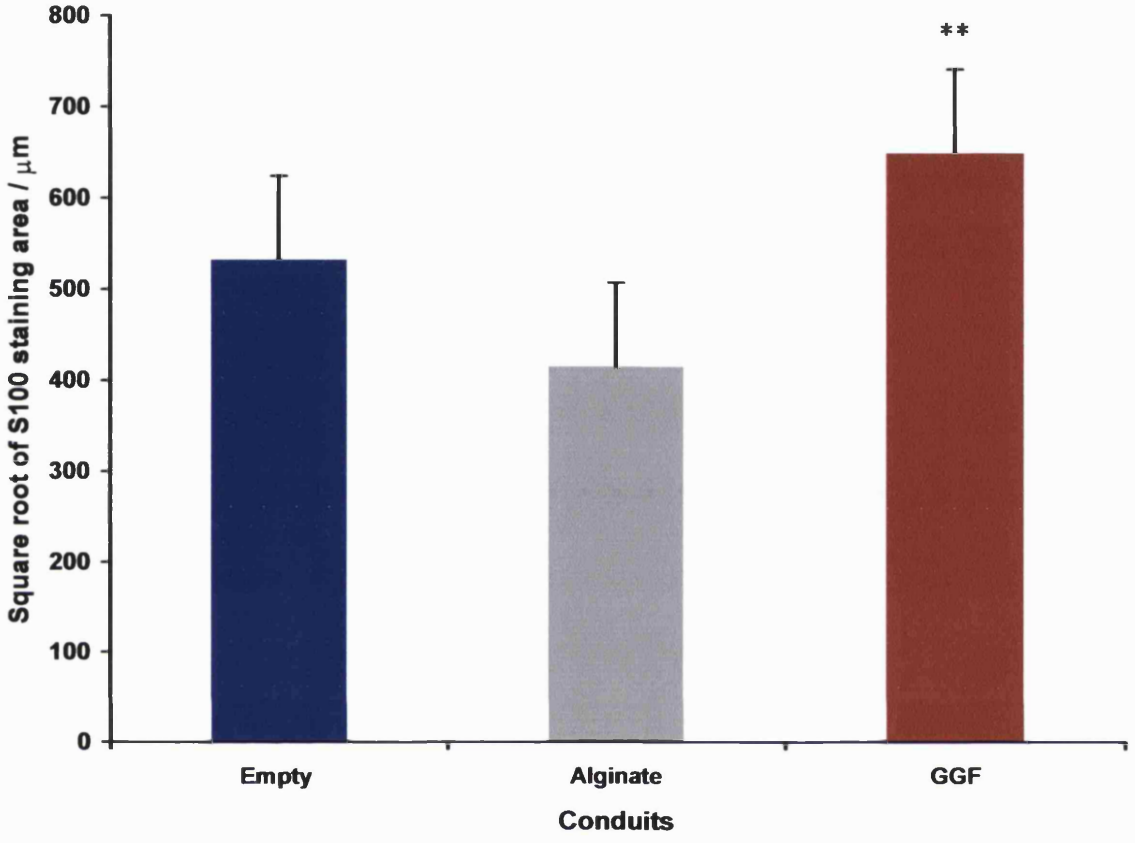

Figure 4.10 Area of S100 staining for the 2cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Alginate (post-hoc t-test). N=5 for each group.

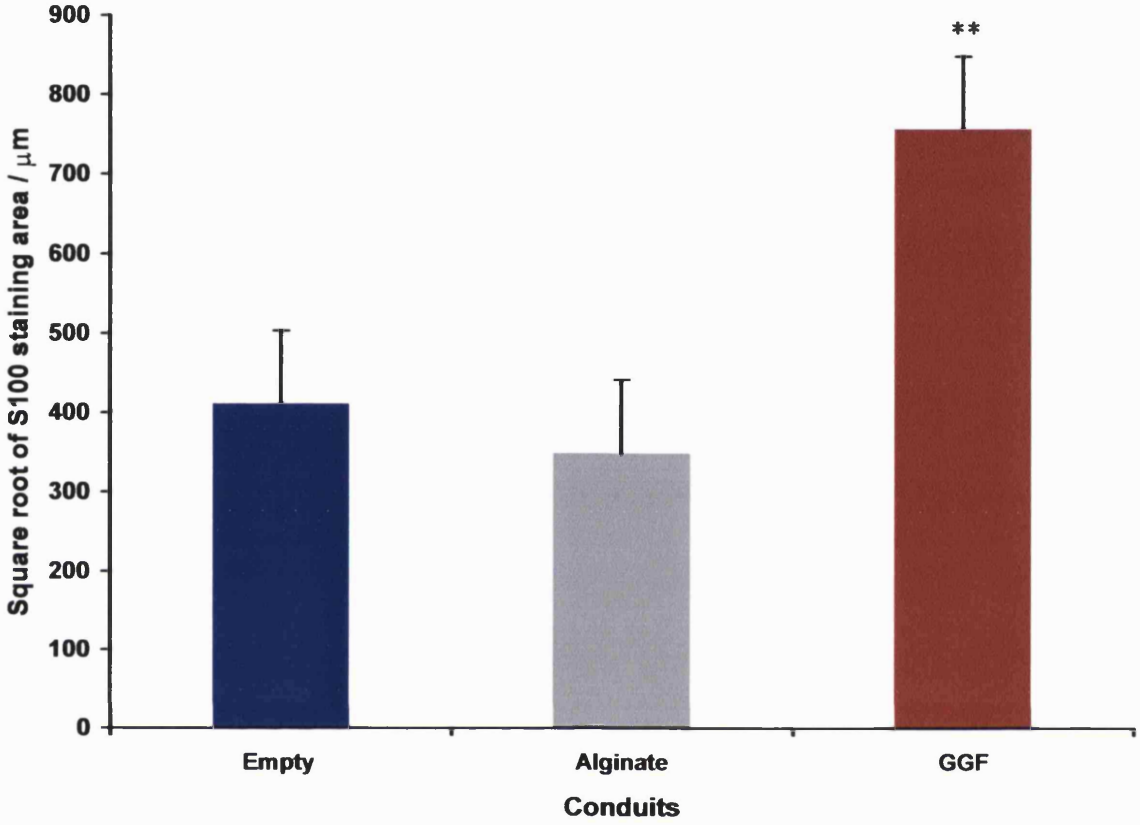

Figure 4.11 Area of S100 staining for the 4cm nerve gaps at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and Alginate (post-hoc t-test). N=5 for each group.

4.4.2.2.2 Percentage area of S100 staining

A one way ANOVA was performed to compare the transformed data for total percentage area of S100 staining (2 and 4cm grafts combined) in the E-PHB, PHB-ALG and PHB-GGF groups (Figures 4.12). There was a significantly greater percentage area of staining in the PHB-GGF conduits compared to the controls ($p<0.001$, PHB-GGF vs PHB-ALG and E-PHB). Also the E-PHB grafts had a significantly greater area of staining than the PHB-ALG tubes ($p=0.04$, E-PHB vs PHB-ALG).

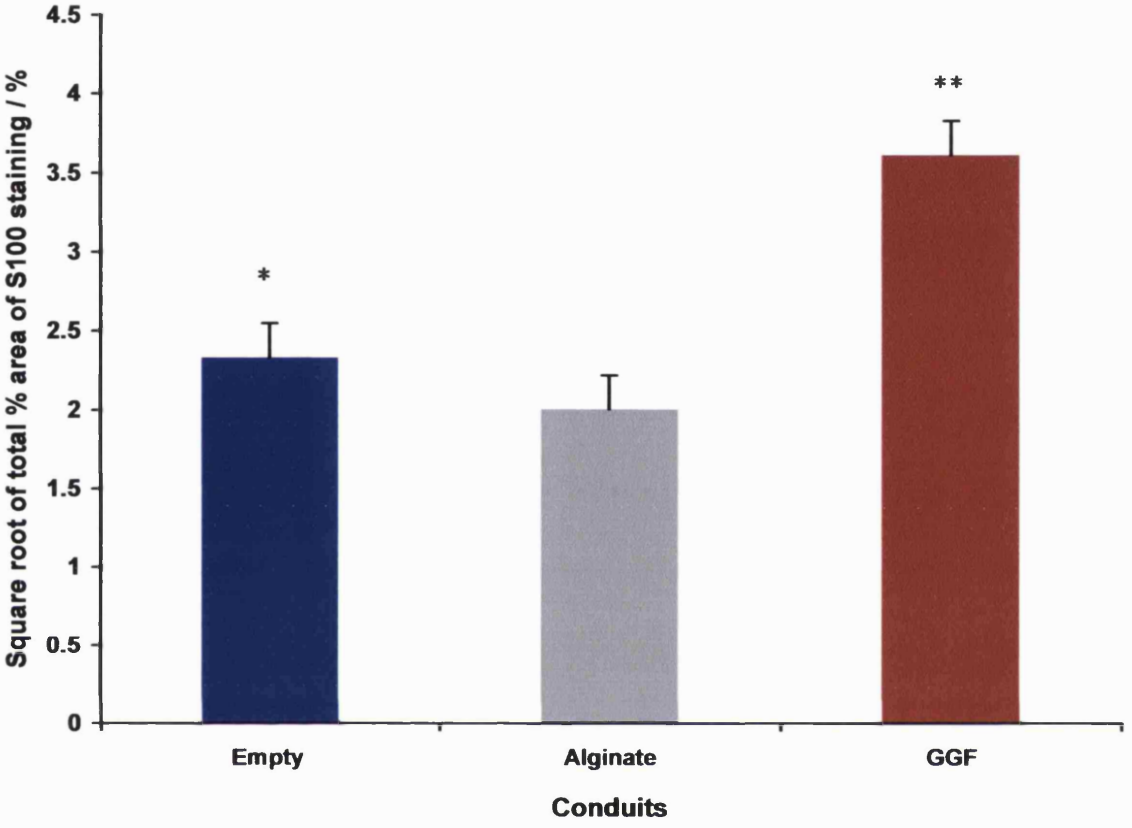

Figure 4.12 Total percentage area of S100 staining at 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ GGF vs Empty and GGF vs Alginate (post-hoc t-test). *$p=0.04$ Empty vs Alginate (post-hoc t-test). N=10 for each group.

4.4.2.3 Axonal and SC regeneration area at 63 days vs 120 days

In addition, a three way analysis of variance (ANOVA) was performed on the transformed data for PanNF and S100 staining areas and percentage staining areas with factors day (63 and 120), graft type (E-PHB, PHB-ALG and PHB-GGF), gap length (2 or 4cm) and their interactions. For both PanNF and S100 staining areas and percentage staining areas there was a significant difference between graft types ($p<0.001$, E-PHB vs PHB-ALG vs PHB-GGF). For PanNF staining area and percentage staining area there was a significant difference between days ($p<0.001$, 63 vs 120 for PanNF) but for S100 this was only significant for the percentage staining area ($p=0.02$, 63 vs 120 for S100). There was a significant effect of gap length on the PanNF staining area ($p=0.03$, 2 vs 4cm for PanNF). For S100 there were no significant graft-day, graft-gap or gap-day interactions. For PanNF the graft-day ($p<0.001$) and graft-gap ($p\leq0.02$) interactions were significant. Further analysis was carried out on the graft-day interaction and the data is presented using the group total values rather than the values for each individual gap length.

To study the effect of time on the PanNF and S100 staining areas and percentage staining areas, a one way ANOVA was performed for each graft type looking at the factor time (63 and 120). For both the E-PHB and PHB-ALG conduits there was a significant decrease in the PanNF staining area and percentage staining area with time ($p<0.001$, 63 vs 120) (Figures 4.13 and 4.14). However for the S100 staining area and

percentage staining area there was no significant difference with time for any of the three graft types (Figures 4.15 and 4.16)

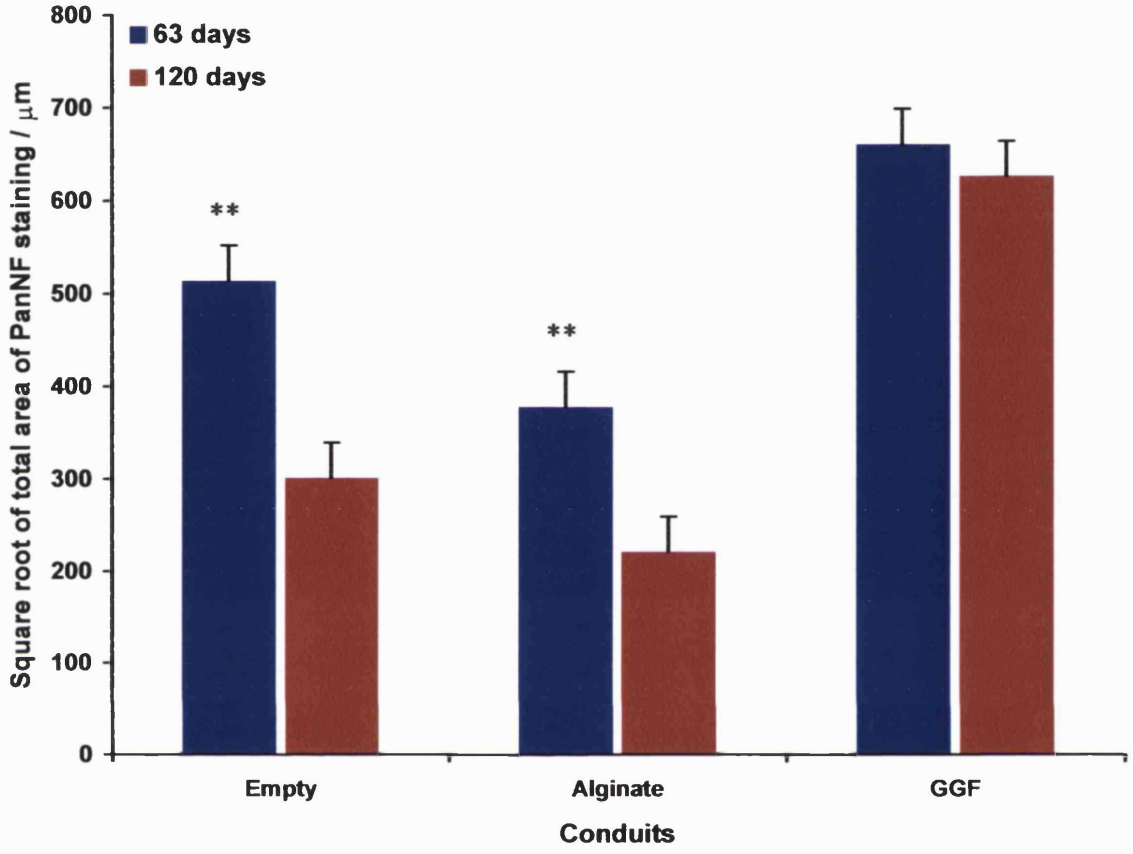

Figure 4.13 Total area of PanNF staining at 63 and 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ 63 days vs 120 days for Empty and Alginate conduits (post-hoc t-test). N=10 for each group.

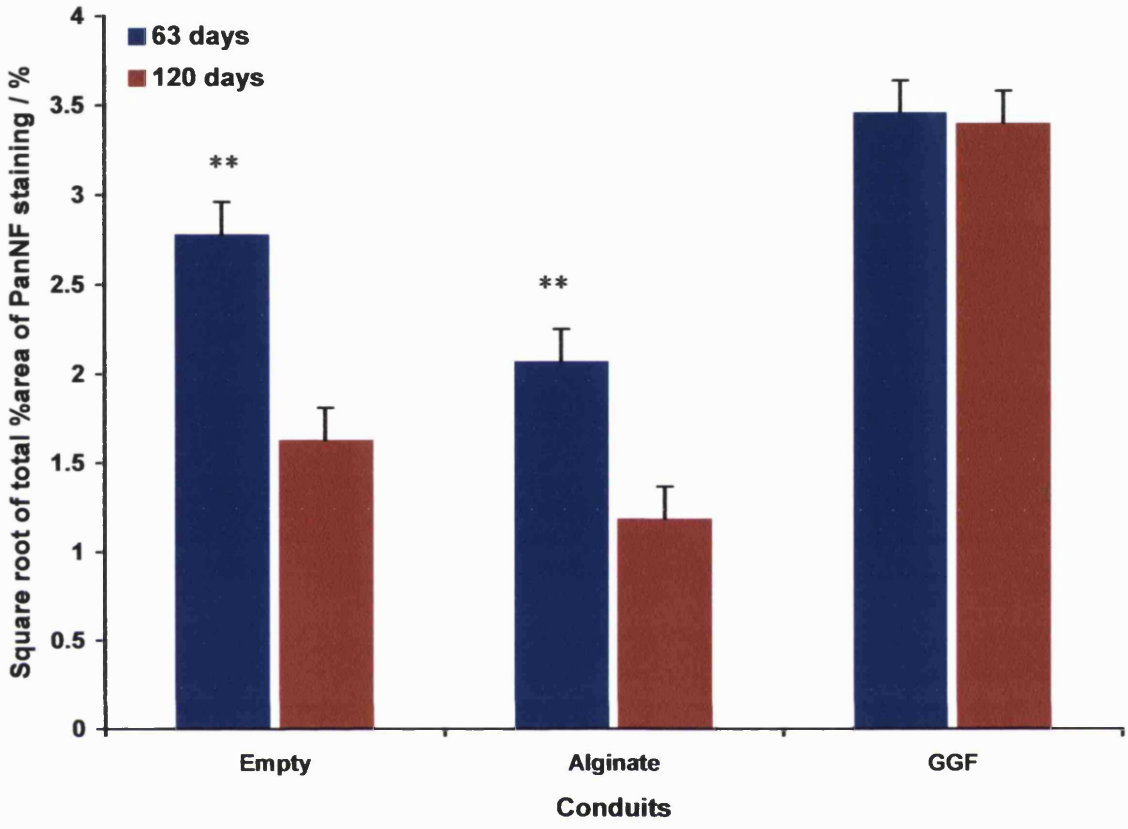

Figure 4.14 Total percentage area of PanNF staining at 63 and 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. **$p<0.001$ 63 days vs 120 days for Empty and Alginate conduits (post-hoc t-test). N=10 for each group.

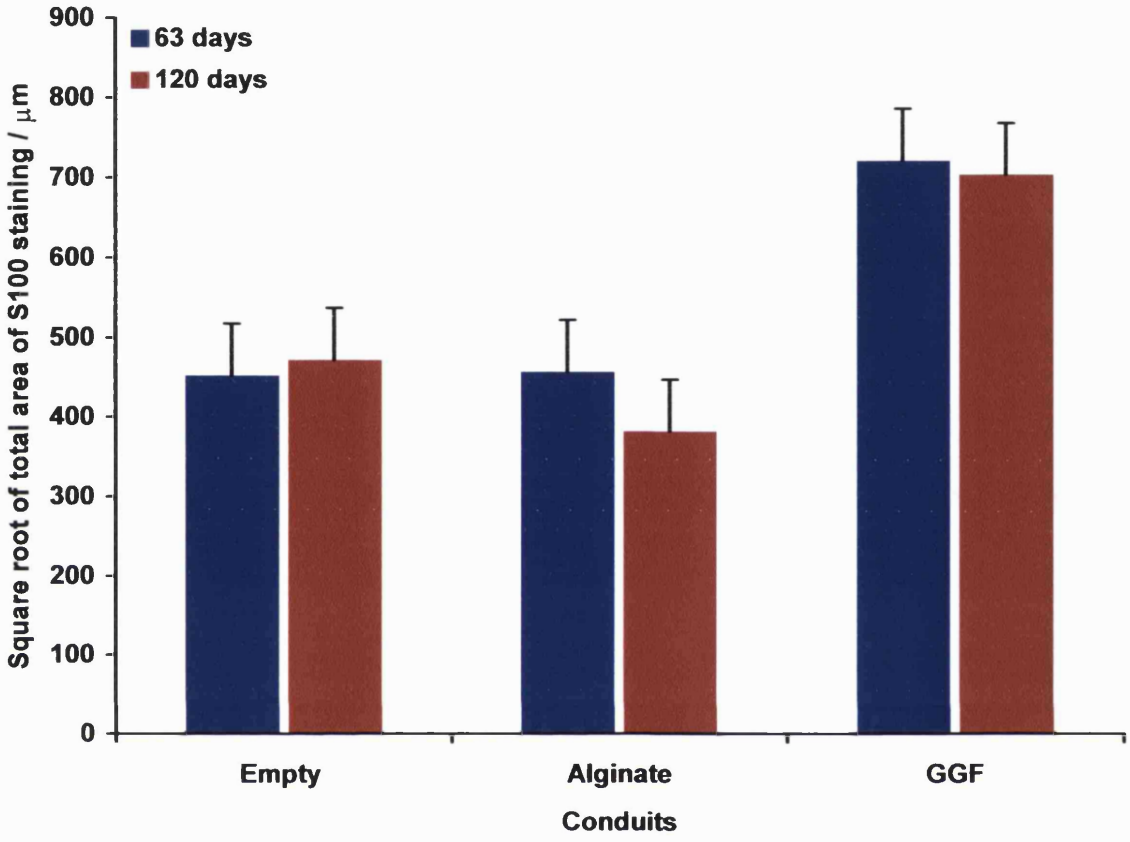

Figure 4.15 Total area of S100 staining at 63 and 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. No significant difference was observed over time. N=10 for each group.

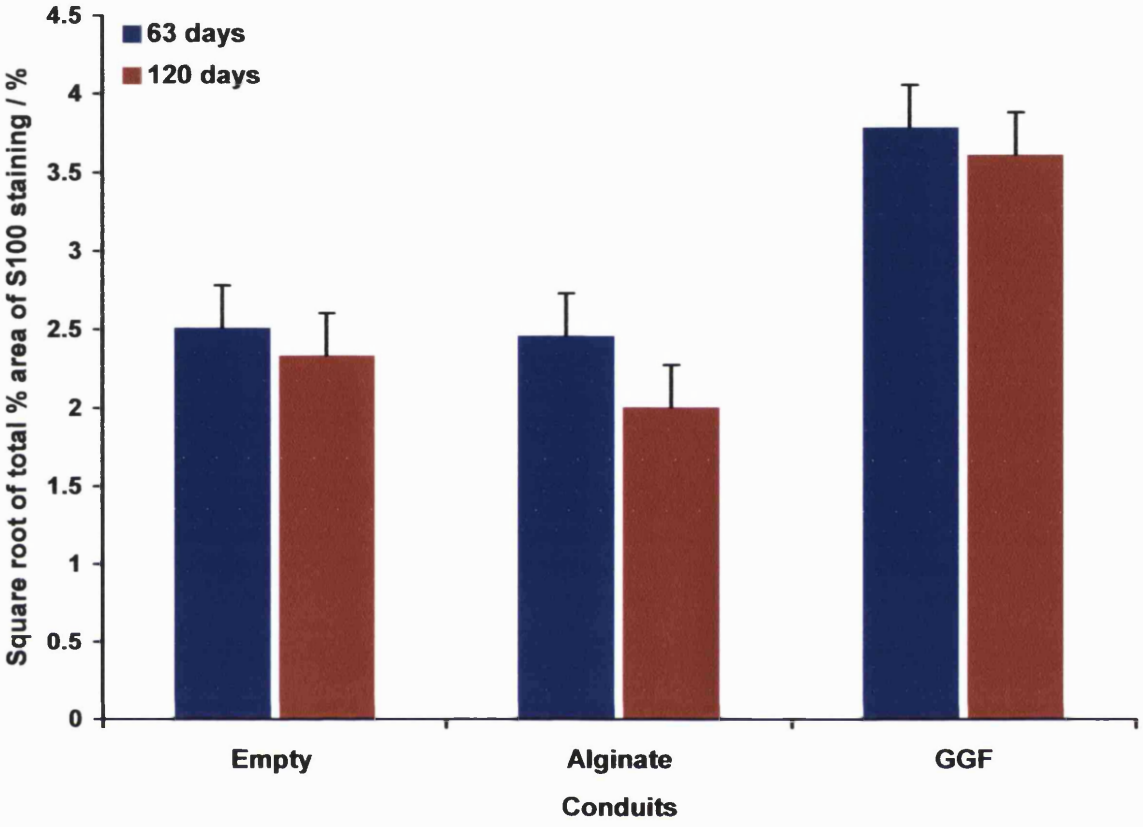

Figure 4.16 Total percentage area of S100 staining at 63 and 120 days after implantation in the Empty, Alginate and GGF conduit groups. Square root transformed data expressed as group mean values with 95% confidence intervals. No significant difference was observed over time. N=10 for each group.

4.4.3 PHB conduit transverse semithin sections

The transverse semithin sections of the conduits were qualitatively assessed for the presence of myelinated fibres, their arrangement and any other cells present within the conduit at a distance of 12mm from the proximal end. For all three graft types the observations in the 2 and 4cm conduits were very similar and the minifascicles of fibres were generally seen in a restricted area of the conduit cross-section. In the PHB-GGF conduits there were numerous groups of mini-fascicles containing more than 10 myelinated fibres in each (Figures 4.17a and b). In the PHB-ALG (Figures 4.18a and b) and E-PHB (Figures 4.19a and b) grafts there were fewer minifascicles, which generally contained less myelinated fibres. As the number of fibres were small compared to the size of the graft section no quantification was carried out. In addition there were numerous macrophages distributed throughout the cross-section, often surrounding PHB fibre fragments (Figure 4.18b).

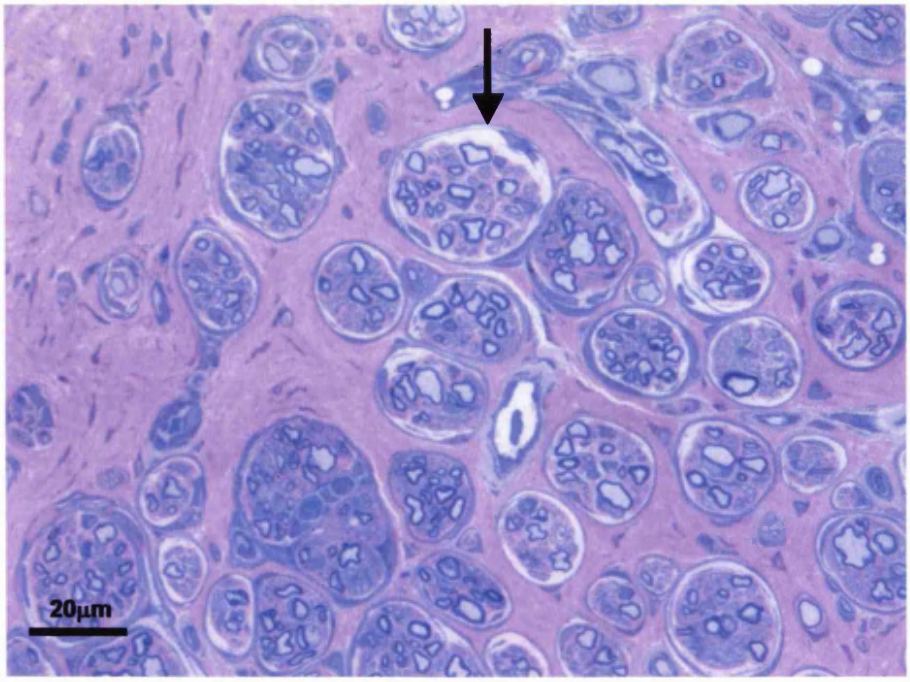

Figure 4.17a Transverse section through a 2cm PHB-GGF conduit showing minifascicle formation (arrow) typical of nerve regeneration (Humphrey's stain).

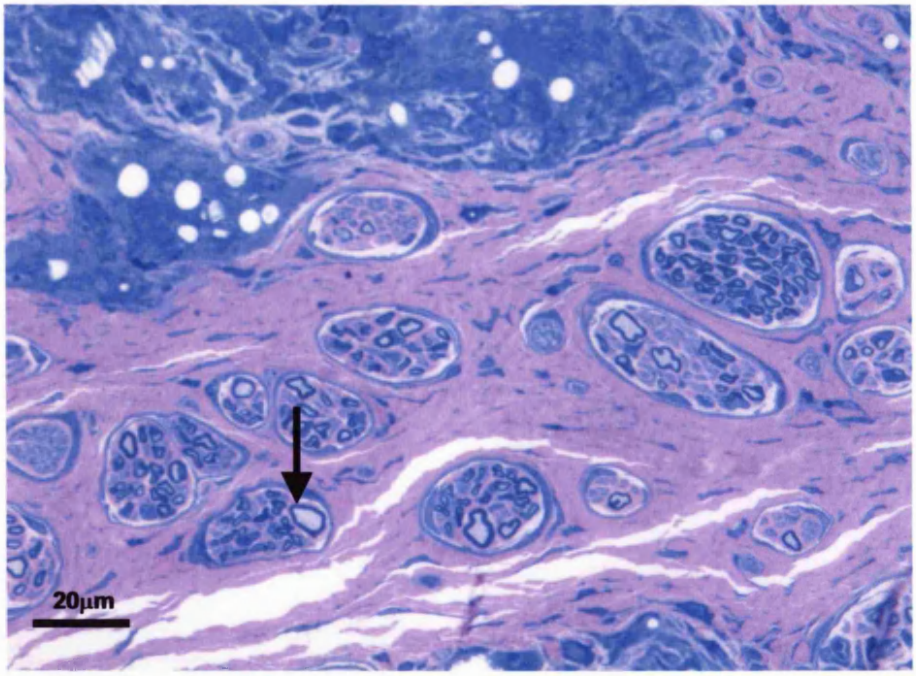

Figure 4.17b Transverse section through a 4cm PHB-GGF conduit with minifascicles containing a similar number of myelinated fibres (arrow) to the 2cm conduit (Humphrey's stain).

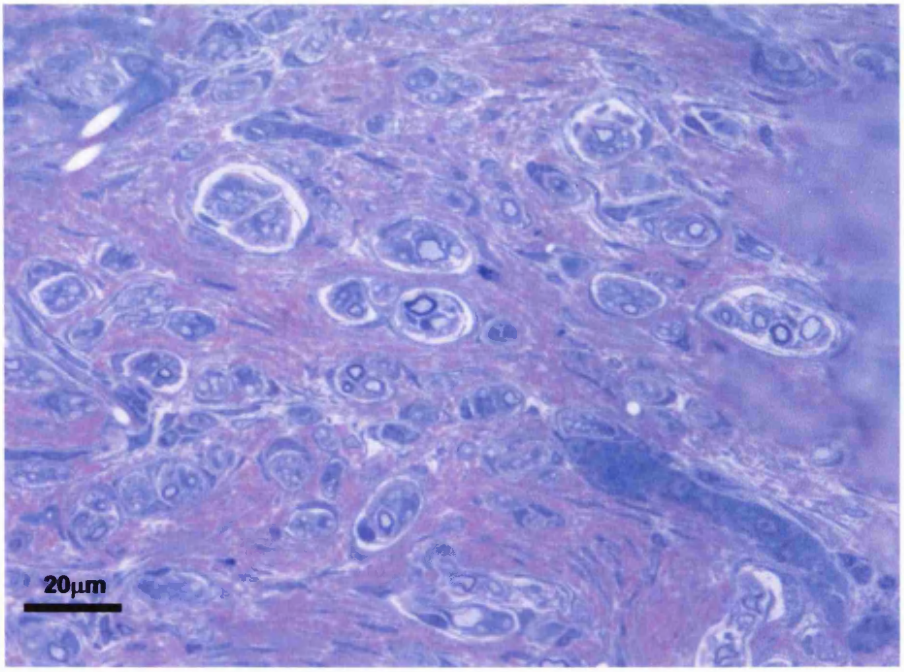

Figure 4.18a Transverse section through a 2cm PHB-ALG conduit showing fewer minifascicles containing less myelinated fibres than the 2cm PHB-GGF conduit (Humphrey's stain).

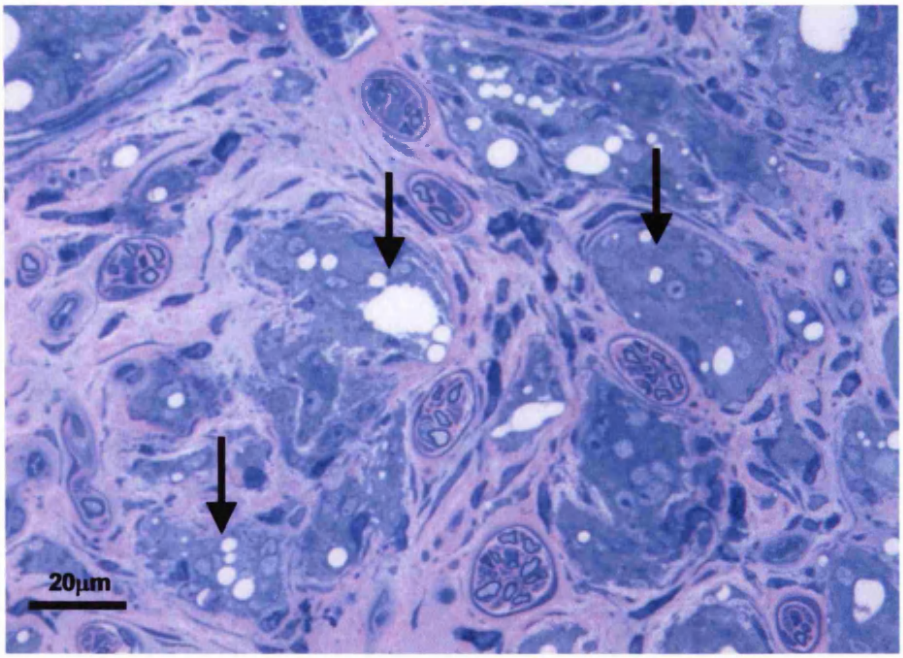

Figure 4.18b Transverse section through a 4cm PHB-ALG conduit. Macrophages are present in the vicinity of PHB fibre fragments (arrows) (Humphrey's stain).

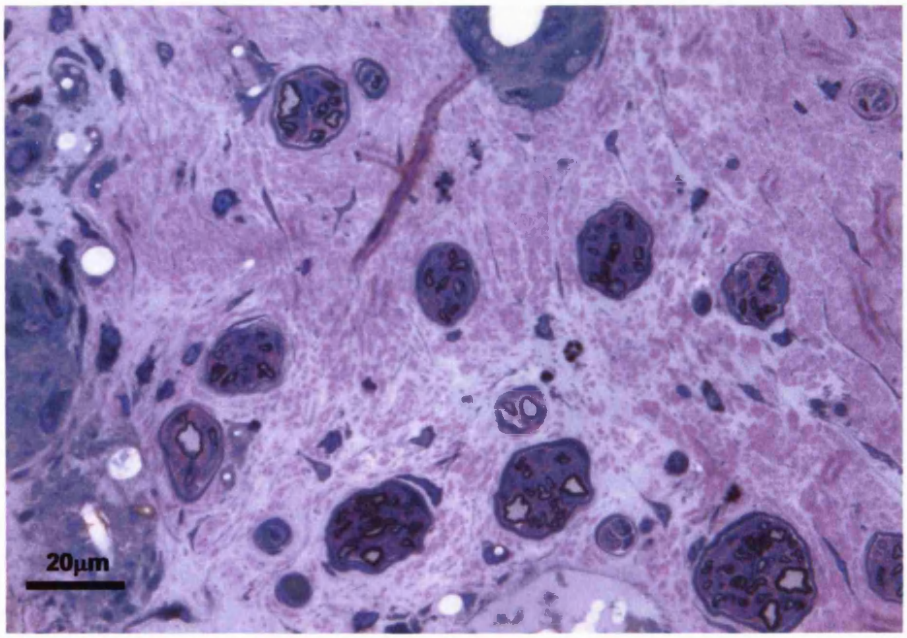

Figure 4.19a Transverse section through a 2cm E-PHB conduit showing a similar number of minifascicles to the 2cm PHB-ALG conduit (Humphrey's stain).

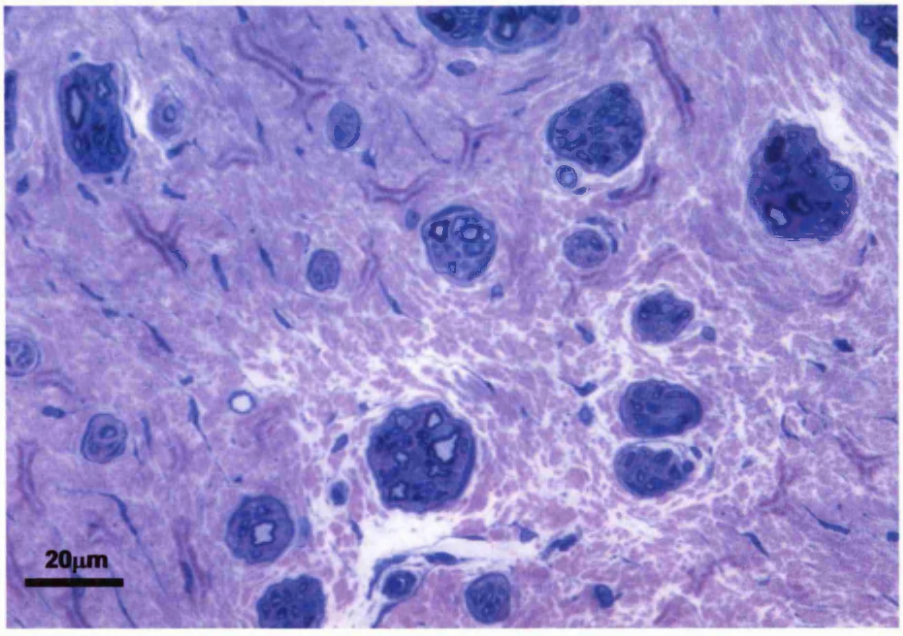

Figure 4.19b Transverse section through a 4cm E-PHB conduit showing minifascicles of varying sizes (Humphrey's stain).

4.4.4 PHB conduit transverse ultrathin sections

The electron micrographs of the conduits showed details of the minifascicles seen in the semithin sections. These demonstrated the presence of healthy regenerating myelinated fibres at both gap lengths for the PHB-GGF conduits (Figure 4.20). The PHB-ALG and E-PHB conduits contained fewer minifascicles with less myelinated fibres and a greater number of unmyelinated fibres (Figures 4.21 and 4.22). In addition to this macrophages were also present and were seen to aggregate around the PHB fibres (Figure 4.23).

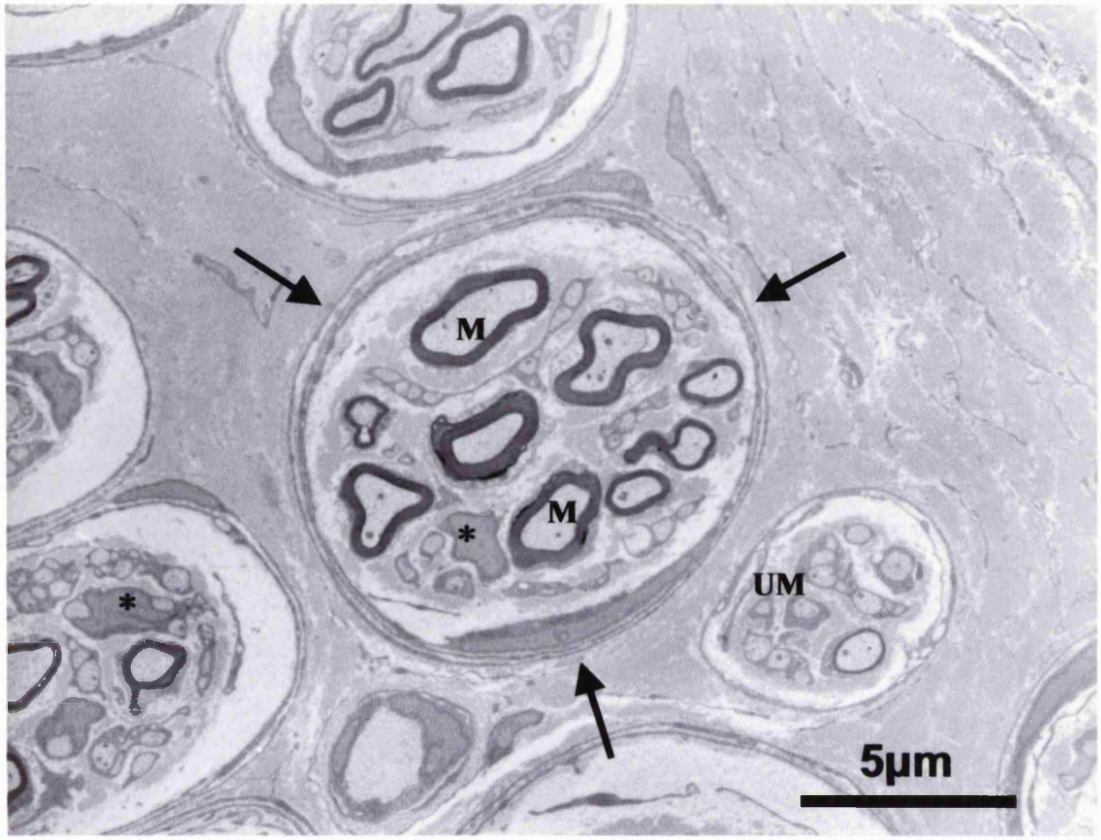

Figure 4.20 A transmission electron micrograph from a 4cm PHB-GGF conduit showing minifascicles containing myelinated (M) and unmyelinated (UM) nerve fibres surrounded by the basal lamina (arrows). SC nuclei are also visible (*).

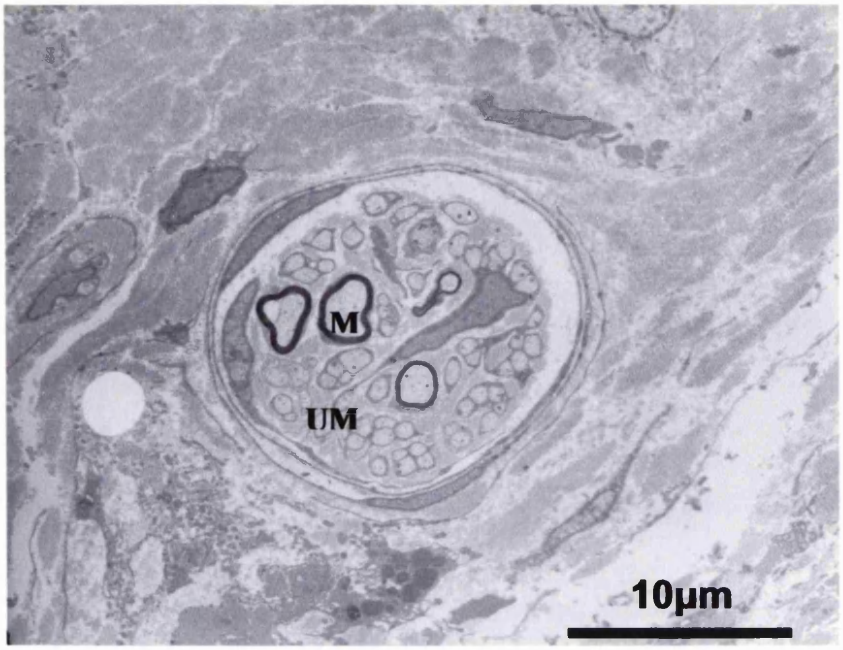

Figure 4.21 A transmission electron micrograph from a 4cm E-PHB conduit showing a minifascicle containing fewer myelinated (M) and more unmyelinated (UM) nerve fibres.

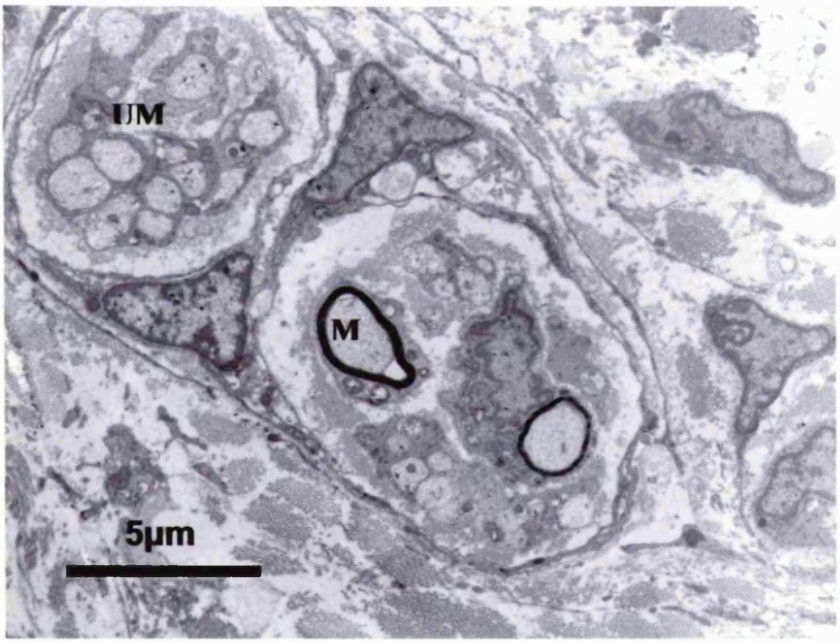

Figure 4.22 A transmission electron micrograph from a 4cm PHB-ALG conduit showing minifascicles containing fewer myelinated (M) and more unmyelinated (UM) nerve fibres.

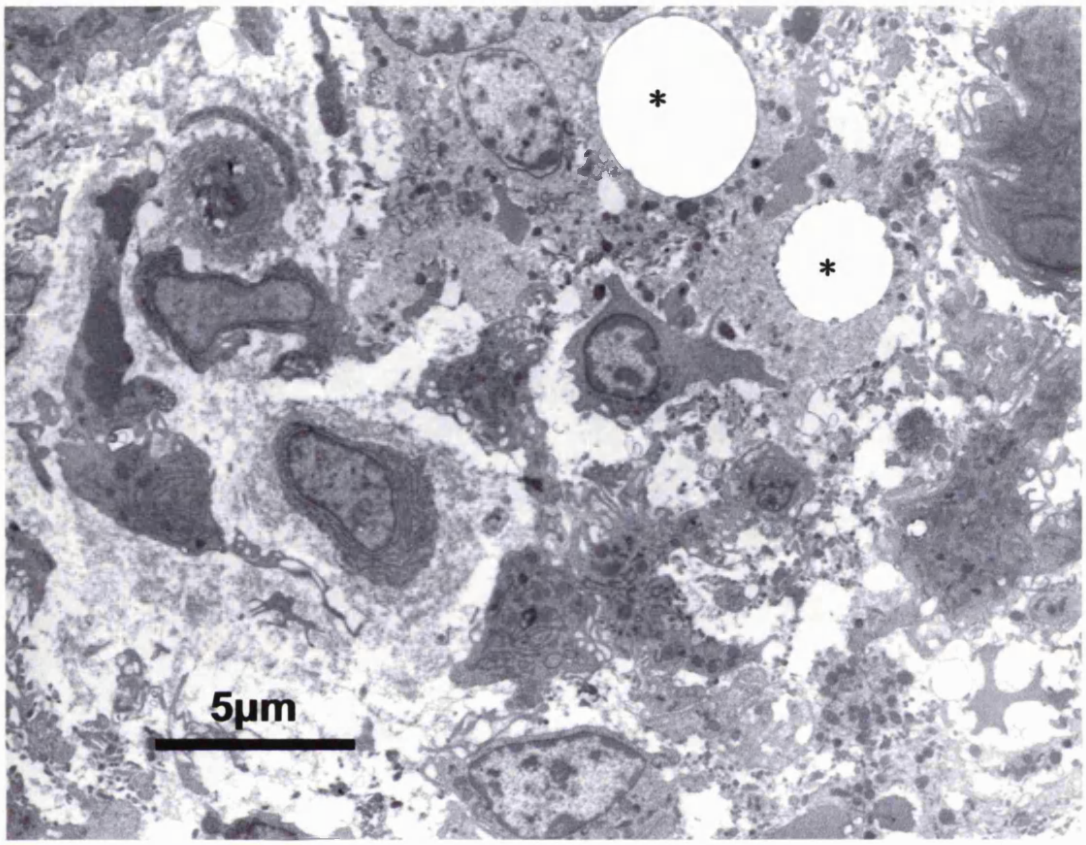

Figure 4.23 A transmission electron micrograph of a 4cm PHB-GGF conduit showing macrophages surrounding PHB fibres (*) 120 days after implantation.

4.4.5 Distal nerve stump semithin sections

The distal nerve stump transverse semithin sections for the PHB-GGF, PHB-ALG and E-PHB conduits were assessed for the presence of myelinated fibres. The transverse sections of the distal nerves did not demonstrate the usual circular configuration, probably as a result of fewer regenerating nerves being present to provide support. The regeneration in each group for both the 2 and 4cm repairs was comparable. The poorest regeneration was observed in the PHB-ALG conduits (Figures 4.24a and b), which showed numerous degenerating fibres with only isolated myelinated fibres present. The sections of the PHB-GGF conduits showed small fibres with features typical of regeneration, including small axon diameters, thin myelin sheaths and few fibres undergoing degeneration (Figures 4.25a and b). The regeneration observed in the distal stump of the E-PHB conduits was comparable to that of the PHB-GGF grafts, again with fewer areas of degeneration and more regenerative fibres (Figures 4.26a and b).

Within each group there was some variability in the degree of regeneration and myelination observed in the distal stump. Overall, when this was correlated with the percentage loss of muscle weight, it revealed that the animals with the greater degree of regeneration and myelination had an associated smaller percentage loss of muscle mass.

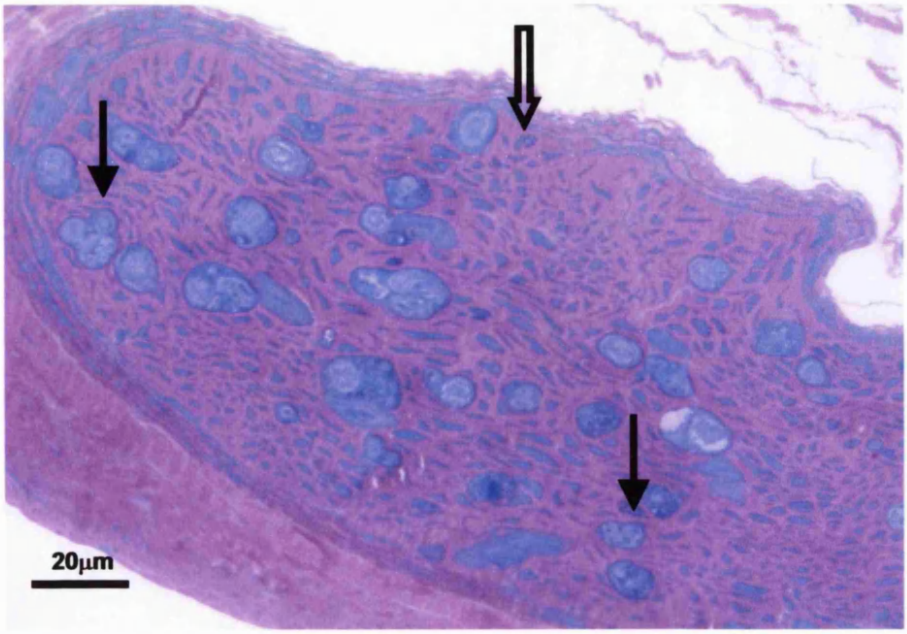

Figure 4.24a The nerve distal to a 2cm PHB-ALG conduit repair showing numerous degenerative fibres (solid arrows) and poor regeneration with few isolated myelinated fibres (open arrow) (Humphrey's stain).

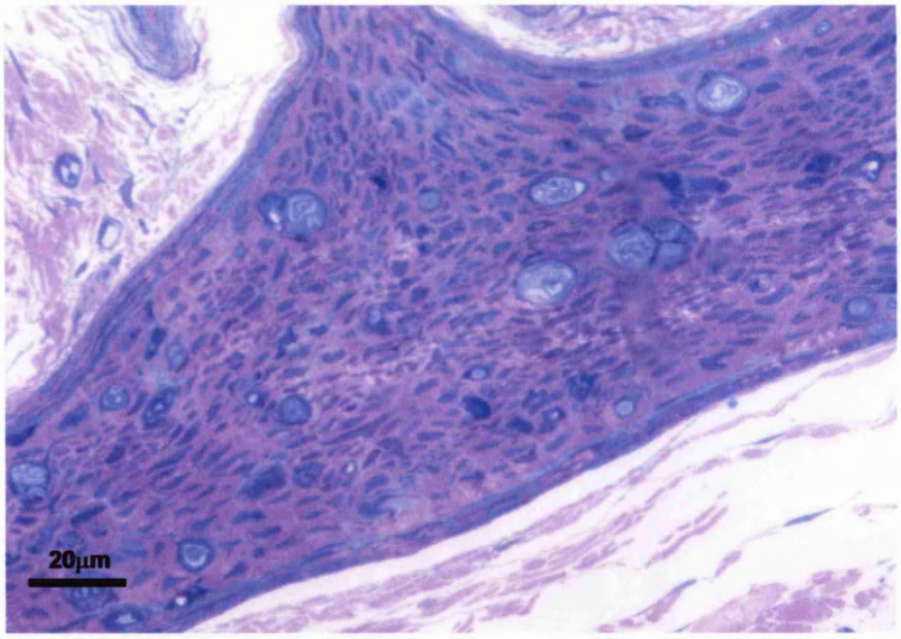

Figure 4.24b The nerve distal to a 4cm PHB-ALG conduit repair again showing numerous degenerative fibres (Humphrey's stain).

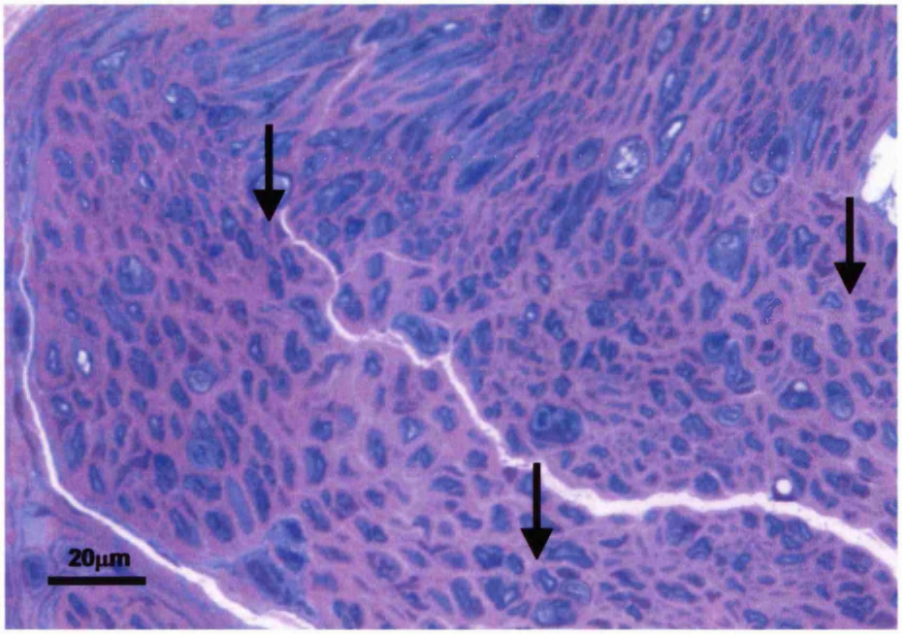

Figure 4.25a The nerve distal to a 2cm PHB-GGF conduit repair showing regenerative clusters (arrows) with fewer degenerative fibres (Humphrey's stain).

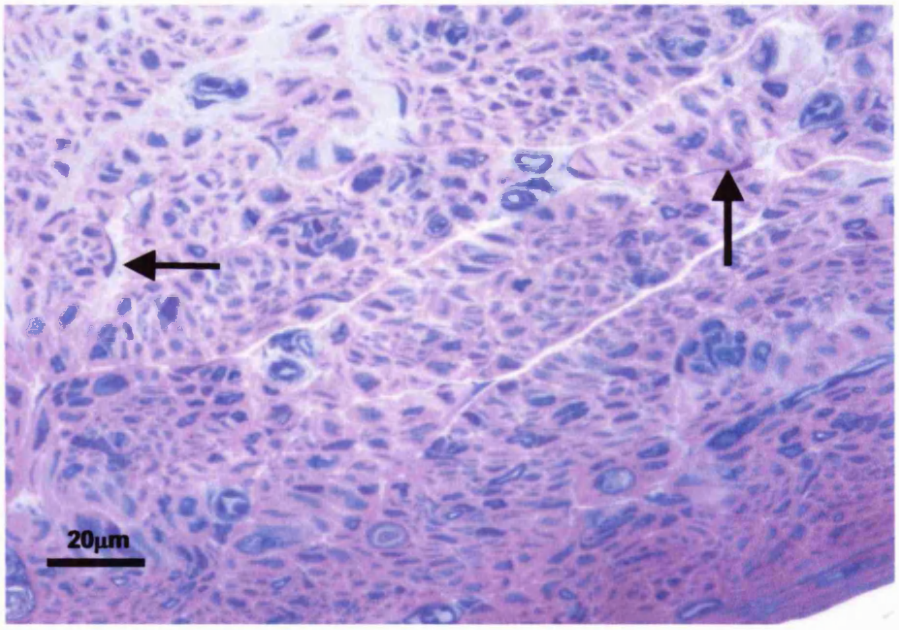

Figure 4.25b The nerve distal to a 4cm PHB-GGF conduit repair showing regenerative clusters and SC (arrows) (Humphrey's stain).

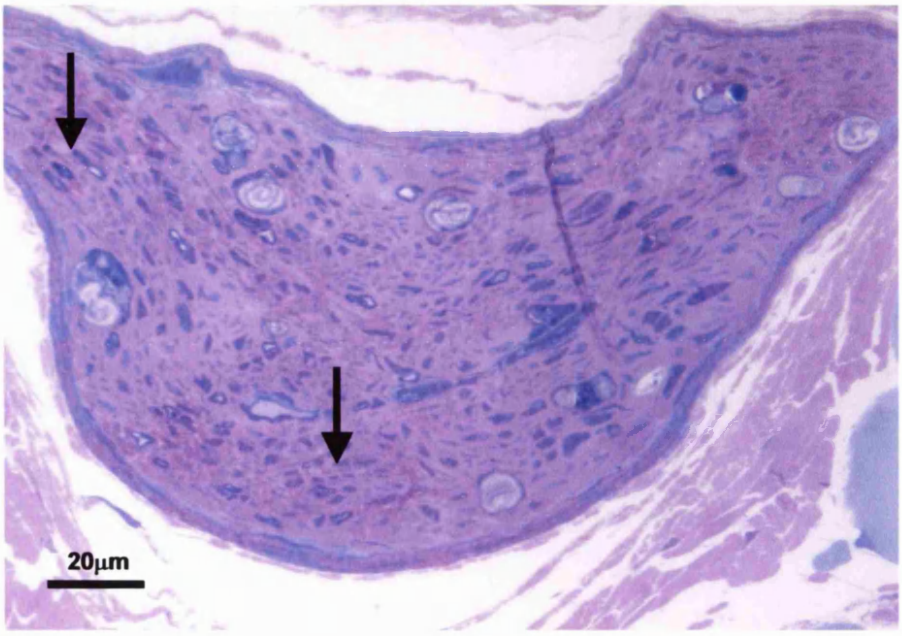

Figure 4.26a The nerve distal to a 2cm E-PHB conduit repair showing both degenerative fibres and regenerative clusters (arrows) (Humphrey's stain).

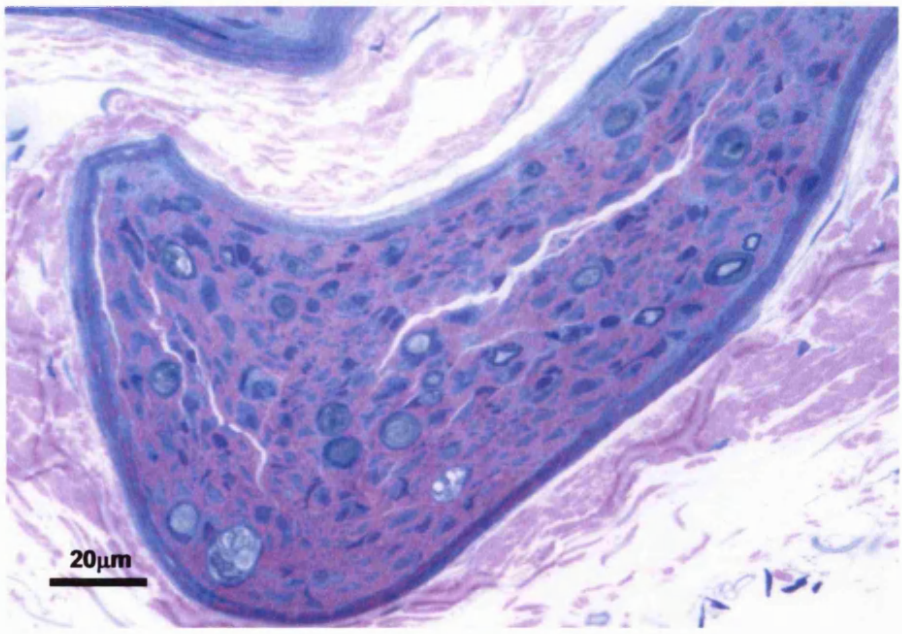

Figure 4.26b The nerve distal to a 4cm E-PHB conduit repair with features similar to the 2cm repair (Humphrey's stain).

4.4.6 Muscle reinnervation

The weights of the TA/EDL muscles on the left (operated) and right (non-operated) sides were measured to assess the degree of recovery. On macroscopic examination the left sided TA/EDL muscle complex in all three groups was smaller and paler than the contralateral side, but muscles of the PHB-GGF conduits showed a better appearance in comparison to those of the control groups (Figure 4.27). When the neurovascular pedicle to the TA/EDL muscles was tied prior to excision the muscles were noted to contract on the contralateral side but not on the operated side. In addition, the common peroneal nerve entering the posterior aspect of the muscles was noted to be thinner on the operated rather than the unoperated side.

To investigate the difference between the percentage loss of muscle mass between the three groups a one way ANOVA was performed for each gap length looking at the factor graft type (PHB-GGF, PHB-ALG and E-PHB). Following analysis of the initial results Bonferroni's multiple comparisons test was performed. In the PHB-GGF conduits the percentage loss of muscle mass was significantly less than the PHB-ALG and E-PHB tubes for either gap length repair ($p<0.05$, PHB-GGF vs PHB-ALG and E-PHB) (Figure 4.28). In addition, unpaired t-tests were carried out for each graft type looking at the factor gap length (2 and 4cm) and its effect on the percentage loss of muscle mass. There was no significant difference between percentage loss of muscle mass in each group, on increasing the gap repair length from 2 to 4 cm (Figure 4.28).

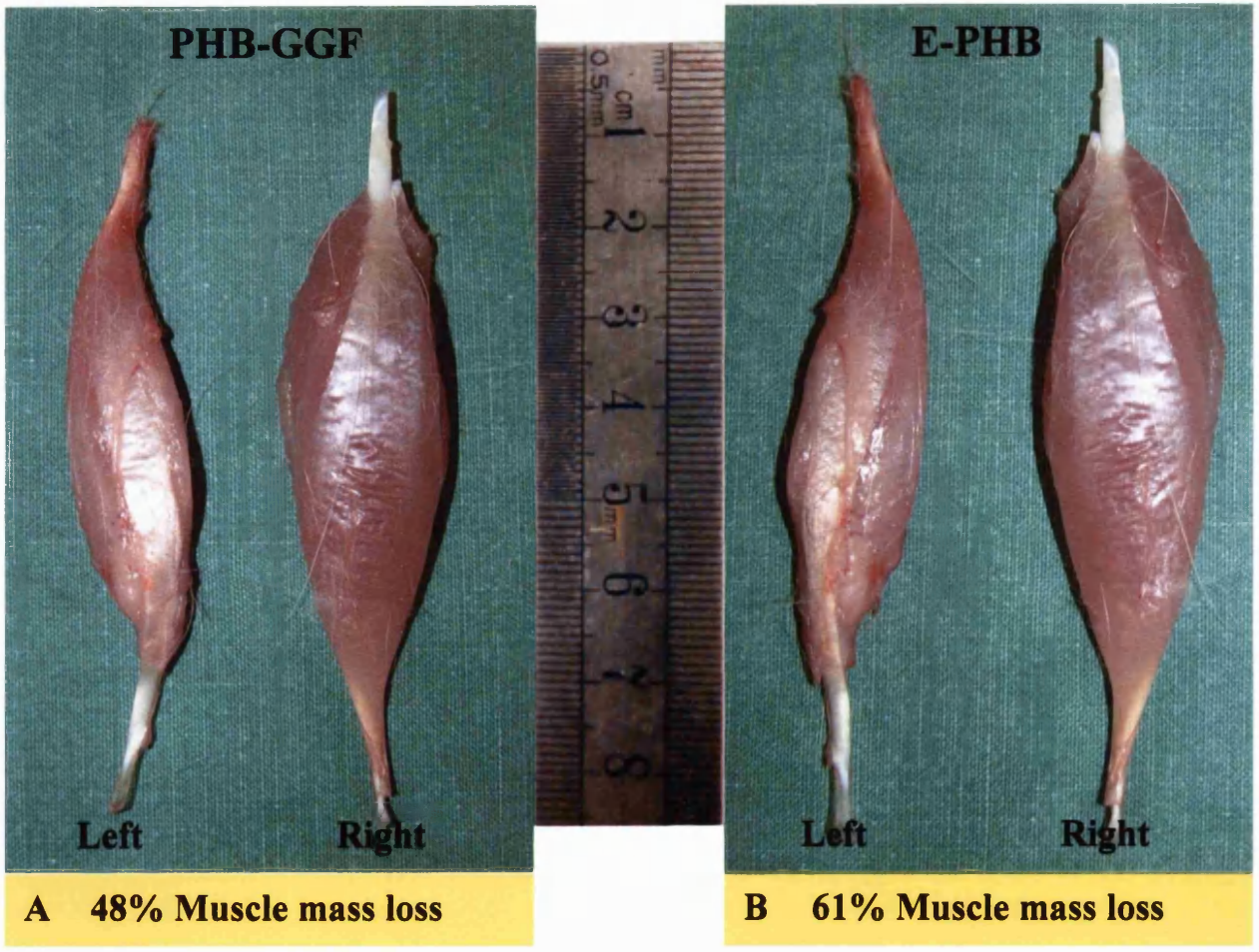

Figure 4.27 The tibialis anterior and extensor digitorum longus muscles from the left (operated) and right (non-operated) legs 120 days following a 4cm PHB-GGF (A) and E-PHB (B) conduit repair.

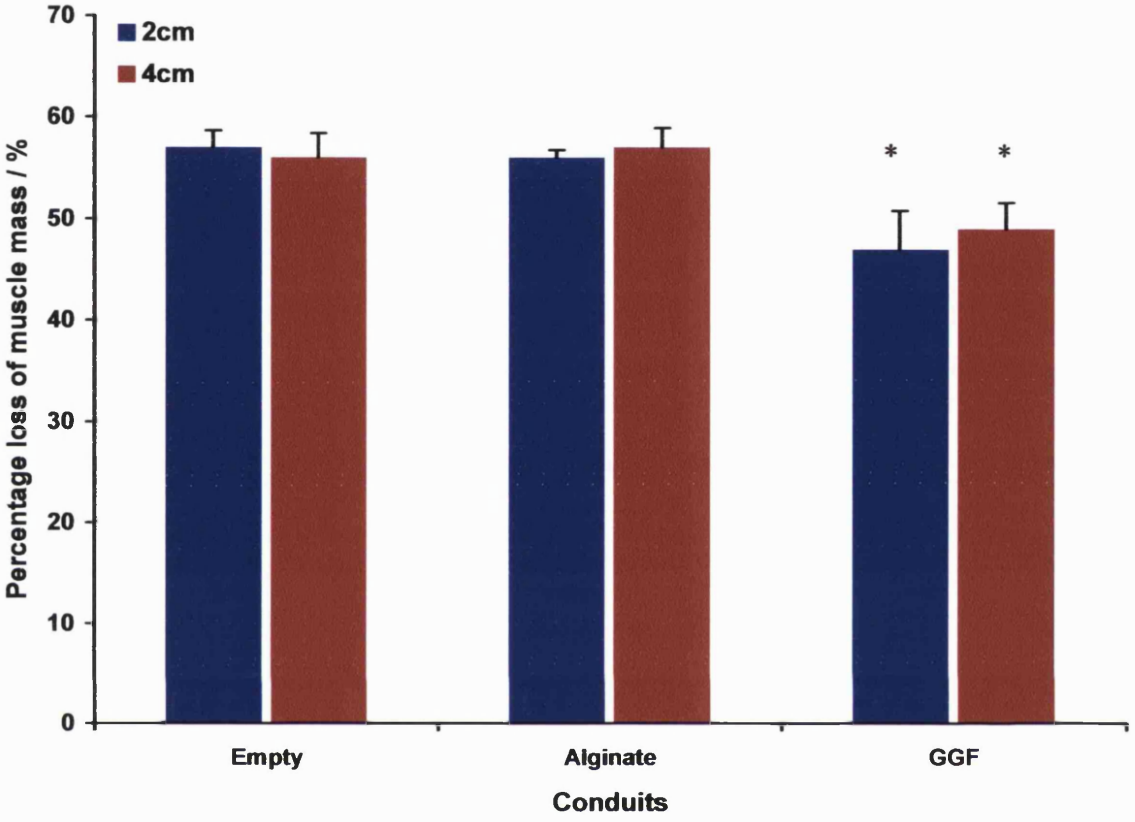

Figure 4.28 Percentage loss of muscle mass at 120 days after 2 and 4cm nerve gap repairs with Empty, Alginate and GGF conduits. Data expressed as group mean values with SEM. There was no significant difference in the percentage loss of muscle mass between the 2cm and 4cm gap repairs for each experimental group. *$p<0.05$ GGF vs Empty and Alginate for both the 2cm and 4cm grafts (post-hoc Bonferroni's test). N=5 for each group.

4.3 DISCUSSION

The presence of nerves within the conduit and in the distal stump is the first indication that regeneration has occurred, but the ultimate aim of nerve repair is to produce optimal functional recovery (Navarro *et al.* 1997). Even in nerve autografts the distal stump contains fewer fibres than the proximal stump following regeneration (Gutmann & Sanders 1943; Jenq & Coggeshall 1985), which is consistent with the acknowledged fact that functional recovery following nerve repair is suboptimal (Mackinnon & Dellon 1988a; Mackinnon & Dellon 1990b; Vanderhooft 2000).

In Chapter 3 the axons in the E-PHB conduits had bridged the 2 and 4cm nerve gaps by 42 days, and by 63 days the axons in the 2cm PHB-GGF tubes had entered the distal stump. Although the axons in the 4cm PHB-GGF grafts had not crossed the gap they were regenerating at a comparable rate. By 120 days PanNF positive fibres are observed in the distal stump of all three types of conduit used to repair nerve gaps of 2 and 4cm. Therefore despite the PHB-GGF and PHB-ALG conduits having a slower rate of axonal regeneration in comparison to the E-PHB conduits at the early time points, the regenerative capacity is sustained and the distal stump remains receptive to the arrival of the axons.

Interestingly the quantity of axonal and SC regeneration within the PHB-GGF conduits for both gap lengths is still significantly greater than in either the PHB-ALG or E-PHB grafts at 120 days. This is further supported by the greater number of minifascicles and

myelinated fibres observed in the transverse semithin sections of the PHB-GGF conduits compared to the controls. When the quantity of SC regeneration at 63 and 120 days are compared in the three graft types, it is evident that there is no significant change, indicating that a plateau has been reached in SC proliferation. This plateau is significantly higher in the PHB-GGF conduits compared to the controls. However, when the quantity of axonal regeneration is studied an interesting observation is made. For both gap lengths the quantity of axonal regeneration is significantly less for the PHB-ALG and the E-PHB conduits at 120 vs 63 days, whereas there is no significant difference in the PHB-GGF conduits between these two time points. There may be a number of explanations for the observed maintenance in the quantity of axonal regeneration within the PHB-GGF conduits and the significant reduction in the PHB-ALG and E-PHB grafts with time. Firstly, the greater quantity of SC regeneration in the PHB-GGF tubes may provide more neurotrophic support to the axons, thereby reducing the amount of axonal die back. Secondly, following axotomy the expression of c-erbB2 and c-erbB4 receptors on SC in the distal stump is rapidly upregulated (Li *et al.* 1997; Bryan *et al.* 2000). However, with chronic denervation downregulation of these receptors occurs and the SC lose their ability to proliferate in response to the GGF released by the regenerating axons (Li *et al.* 1997). It is possible that the release of exogenous GGF in the conduit prevents this downregulation and allows the SC in the distal stump of these conduits to remain receptive to the arrival of the regenerating axons and in turn the GGF they release, thereby providing further neurotrophic support. Although all three types of conduit have supported axonal regeneration across the nerve gaps, the regenerating axons must still make contact with the appropriate target organs

inorder to survive and continue to mature (Schroder 1972). The percentage loss of muscle mass with the PHB-GGF conduit repairs was significantly less, indicating a greater degree of successful target organ reinnervation and as a result improved neurotrophic support, reducing axonal pruning and die-back.

In general the short-term results of Chapter 3 demonstrated that the quantity of axonal and SC regeneration observed in the E-PHB conduits was significantly superior to that of the PHB-ALG grafts. By 120 days the quantity of axonal and SC regeneration is still greater in the E-PHB grafts and significantly so for axonal regeneration, indicating that alginate is still behaving as a physical barrier owing to its small pore size and very slow rate of degradation.

As the axons in the 4cm E-PHB conduits entered the distal stump at 42 days, by 120 days one would expect the axons in the distal stump to be further advanced in maturation, with more regenerative clusters and axons with thicker myelin walls. This however was not the case and the PHB-GGF conduits qualitatively demonstrated more regenerative fibres in their distal stump. Although the difference was not as dramatic as that observed with the quantity of axonal regeneration within the conduit, it is important to realize that the maturation of a regenerating nerve can take as long as 18 months or even more (Franklin *et al.* 1995) and these experiments may need to be repeated at longer time points to appreciate a noticeable difference.

When motor reinnervation was studied, it was observed that the nerve repairs carried out with PHB-GGF conduits, resulted in significantly less percentage atrophy of the EDL/TA muscles compared to the controls, implying a greater degree of accurate motor organ reinnervation. Therefore, despite the number of mature axons in the distal stump not being significantly greater than in the E-PHB or PHB-ALG tubes they were sufficient enough to reduce muscle atrophy. A possible explanation is the theory of preferential motor reinnervation, which was demonstrated by Brushart (Brushart 1993; Brushart & Seiler 1987; Brushart 1988). The mechanisms for this are still obscure but it was suggested that the presence of recognition molecules in motor SC tubes as opposed to sensory SC tubes may result in preferential motor reinnervation (Brushart 1993; Brushart & Seiler 1987; Brushart 1988). Possibly the presence of exogenous GGF may upregulate the expression of these molecules resulting in the regenerating axons observed in the distal stump of these conduits making successful motor organ connections. However, even in the PHB-GGF conduits 47% of the muscle mass had been lost, but it is possible that the end point of the experiment was too soon, thereby not permitting a greater degree of muscle reinnervation to be observed (Fu & Gordon 1997). This would be consistent with the slower rate of regeneration observed in the PHB-GGF conduits in comparison to the E-PHB tubes (cf Chapter 3) and a longer period of time being required for the regenerating nerves to reach the target organ.

For the majority of the outcome measures studied no significant difference was observed between the 2 and 4cm nerve gap repairs. However, when studying the total area and percentage area of PanNF staining in the PHB-GGF conduits there was a

significant difference, with the quantity being greater in the 4cm conduits. A possible explanation is that the amount of GGF present in the 4cm conduits is double that in the 2cm grafts, which may have a greater effect on the chronically denervated distal stump. Therefore the 4cm PHB-GGF grafts may, to a greater extent, prevent downregulation of the c-erb receptors on the SC in the distal stump, hence providing a greater degree of neurotrophic support to the regenerating axons. In the absence of GGF in the conduit, the nerve gap does not influence the neurotropic and neurotrophic effects produced by the distal stump on the proximal stump.

In conclusion, despite axons bridging the 2 and 4cm gaps repaired with E-PHB conduits by 42 days, in the long-term this did not confer an advantage with respect to successful target organ reinnervation. However, the presence of GGF within the conduits not only significantly increased the quantity of SC and axonal regeneration in the short-term, but also sustained this level of axonal regeneration at 120 days which resulted in a significant decrease in the loss of muscle mass compared to the controls. As a result of the improved target organ reinnervation further neurotrophic support was provided leading to maintenance of regeneration (Mackinnon *et al.* 1991). Longer term studies are required to appreciate a more significant difference in axonal maturation in the distal stumps of the PHB-GGF conduits as compared to the controls and as a result a greater improvement in the return of muscle mass. Therefore it is apparent that the trophic effect of GGF on SC, and the glial induced enhancement of axonal regeneration is not limited to the short-term time points but is maintained up to 120 days.

CHAPTER 5

SHORT-TERM ASSESSMENT OF A PERIPHERAL NERVE GAP REPAIR WITH A PHA CONDUIT

5.1 INTRODUCTION

5.2 AIMS

5.3 MATERIALS AND METHODS

5.4 RESULTS

5.4.1 Electron micrographs of the PHA material

5.4.2 Regeneration distance

5.4.3 Regeneration area

5.5 DISCUSSION

5.1 INTRODUCTION

The majority of the work in this thesis has focused on the use of poly-3-hydroxybutyrate (PHB) as a conduit material. Despite PHB fulfilling many of the properties of the ideal conduit, the delivery of glial growth factor (GGF) in alginate hydrogel loaded into the conduit lumen resulted in some shortcomings, which could potentially be overcome by bioengineering a new conduit thereby providing an alternative mode of GGF delivery. We therefore diverted our focus towards investigating an alternative conduit material, which had the potential to allow GGF to be loaded into the wall of the conduit. Since PHB has proved successful as a nerve graft material and was the first of the polyhydroxyalkatone family to be discovered, we decided to investigate another member of this polymer family, namely PHA4400.

A feature of the Polyhydroxyalkatones (PHA), which makes them an attractive candidate for use as a scaffold material in tissue engineering is their surface structure (Zinn *et al.* 2001). Their porous surface can be beneficial to peripheral nerve regeneration by permitting the influx of necessary nutrients and growth factors from the external environment. Additionally PHAs have successfully been used as drug carriers due to their biodegrability and biocompatibility and resulted in controlled release of various drugs from PHA microspheres, nanospheres and rods (Kassab *et al.* 1997; Yagmurlu *et al.* 1999; Zinn *et al.* 2001).

5.2 AIMS

The aim of this part of the study was to assess axonal regeneration through novel PHA conduits consisting of four different configurations, bridging 1cm gaps in the rat sciatic nerve. The study was conducted using immunohistochemical assessment of the distance and quantity of axonal regeneration within the PHA conduits and compared to nerve autografts of the same length.

5.3 MATERIALS AND METHODS

Thirty male Sprague-Dawley rats were used for this study (Table 5.1). The 5 groups consisted of empty PHA conduits prepared by four different methods (A-D) (cf 2.5):

A - TIPS (Thermally Induced Phase Separation) foam with water leaching

B - TIPS (Thermally Induced Phase Separation) foam with Tween80 leaching

C - Ethanol precipitate with water leaching

D - Ethanol precipitate with Tween80 leaching

and nerve autograft (AG). Twenty-four rats were allocated to the PHA groups (PHA A-D) and six to the Autograft (AG) group. Each group bridged a nerve gap of 1cm (cf 2.7) and was sacrificed at 10 and 20 days post-operatively. Therefore 3 animals underwent a 1cm nerve gap repair at each time point (Table 5.1).

At the designated time points the animals were sacrificed and the repair sites harvested (cf 2.8.2). After fixation the tissue was blocked, sectioned (cf 2.9) and then stained with polyclonal antibody to PGP and S100 (cf 2.10.1). The axonal and SC regeneration distance and axonal area of staining were then quantified (cf 2.11). In addition the architecture of the 4 configurations of PHA was assessed prior to conduit formation and implantation using scanning electron microscopy.

	10 days	20 days
PHA-A	3	3
PHA-B	3	3
PHA-C	3	3
PHA-D	3	3
AG	3	3

Table 5.1 The groups included in this study. N=3 in all groups

5.4 RESULTS

All 4 configurations of PHA handled well, were flexible, had a good tensile strength and held sutures. At the time of harvest there was no evidence of wound infections, no macroscopic evidence of inflammation and no anastomotic failures. At both harvest points the PHA tubes maintained their structure with no evidence of collapse. They were covered in a very thin pseudocapsule, which could be easily removed and the PHA tubes

explanted. The conduits had not become adherent to the underlying muscles. Macroscopically there appeared to be no difference between the 4 configurations of PHA. However on cryostat sectioning of the frozen blocks the PHA material was less stable than PHB. In particular PHA-C appeared the most friable, resulting in collapse of the conduit wall into the lumen of the tube.

5.4.1 Electron micrographs of PHA material

The electron micrographs of the 4 configurations of PHA showed in detail the porous architecture of the material. At both low and high power magnification it was apparent that in the PHA-A material (Figures 5.1a and b) and to a slightly lesser degree in the PHA-B material (Figures 5.2a and b), the pores were aligned in a longitudinal fashion. However, with the PHA-C and D (Figures 5.3a and b and 5.4a and b) materials the pores appeared to have a random arrangement with no particular alignment. On subjective assessment the PHA-C material demonstrated the largest variation in pore size, but the majority of the material consisted of pores at the smaller end of the range and these pores were the smallest of the 4 PHA configurations. The PHA-A (Figures 5.1a and b) material had the second smallest pores with very little variability in their size. The PHA-D (Figures 5.4a and b) had by far the largest pores whilst those of PHA-B (Figures 5.2a and b) were between that of PHA-A and D.

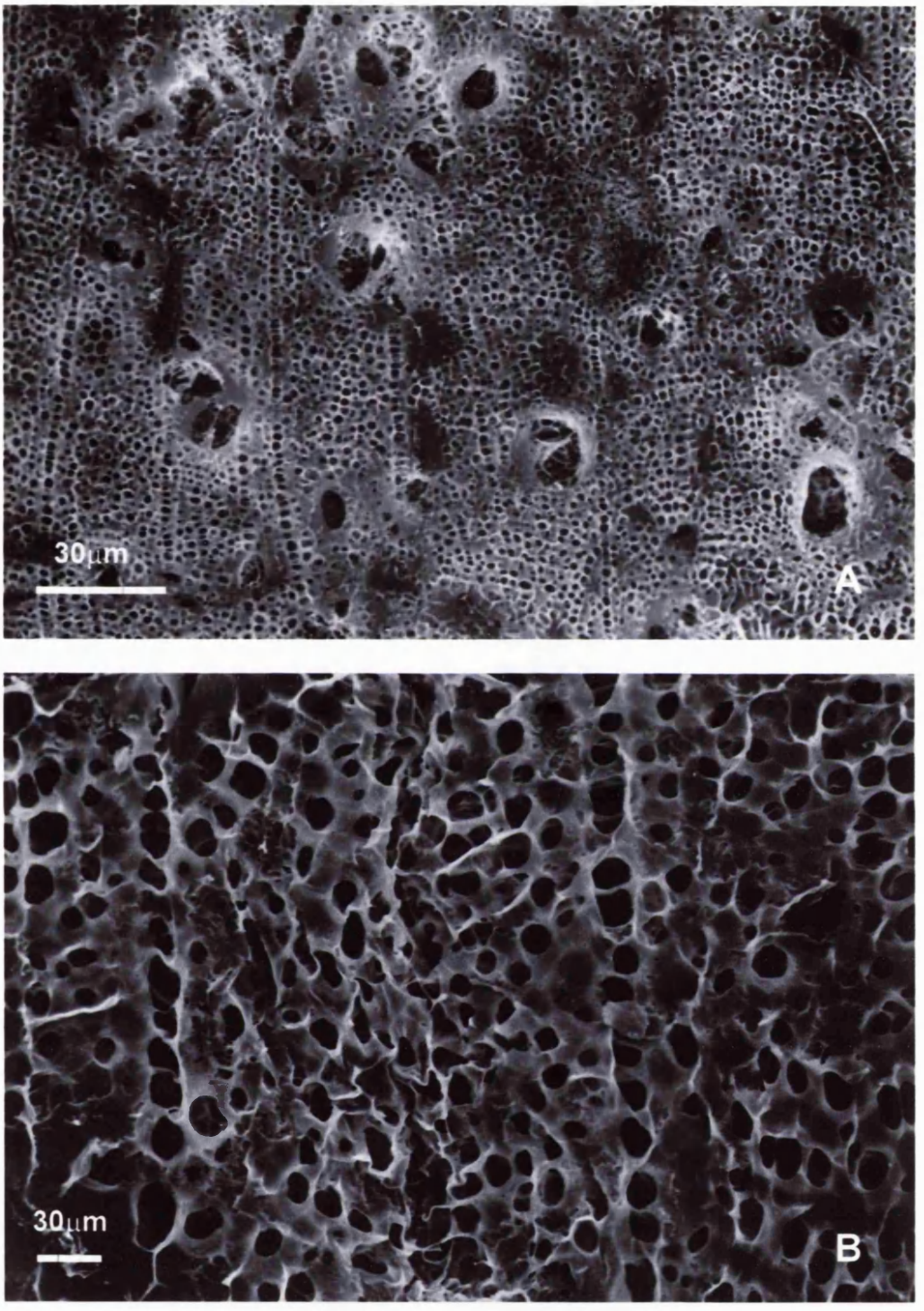

Figure 5.1 Low (A) and high (B) power electron micrographs of the PHA-A material.

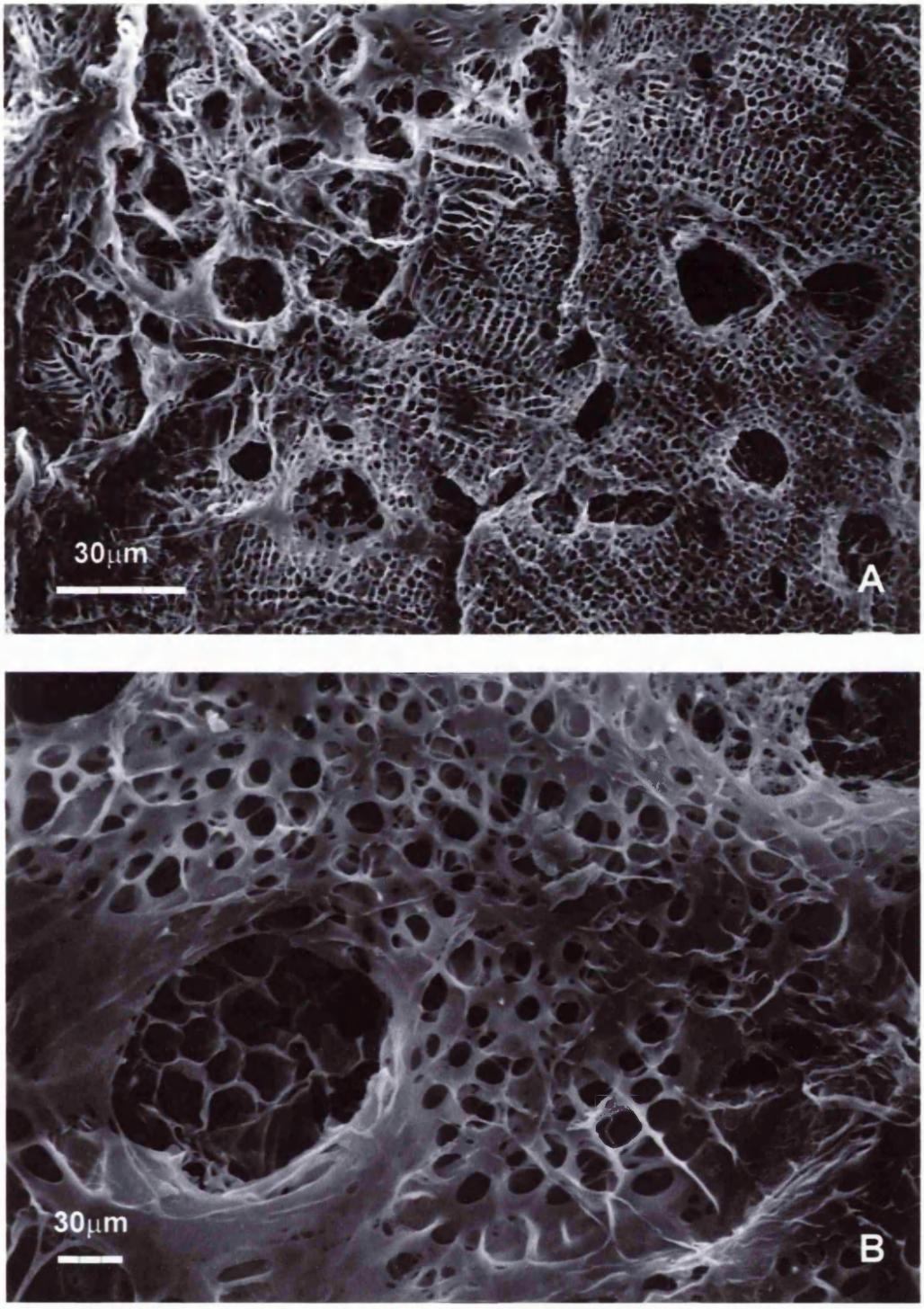

Figure 5.2 Low (A) and high (B) power electron micrographs of the PHA-B material.

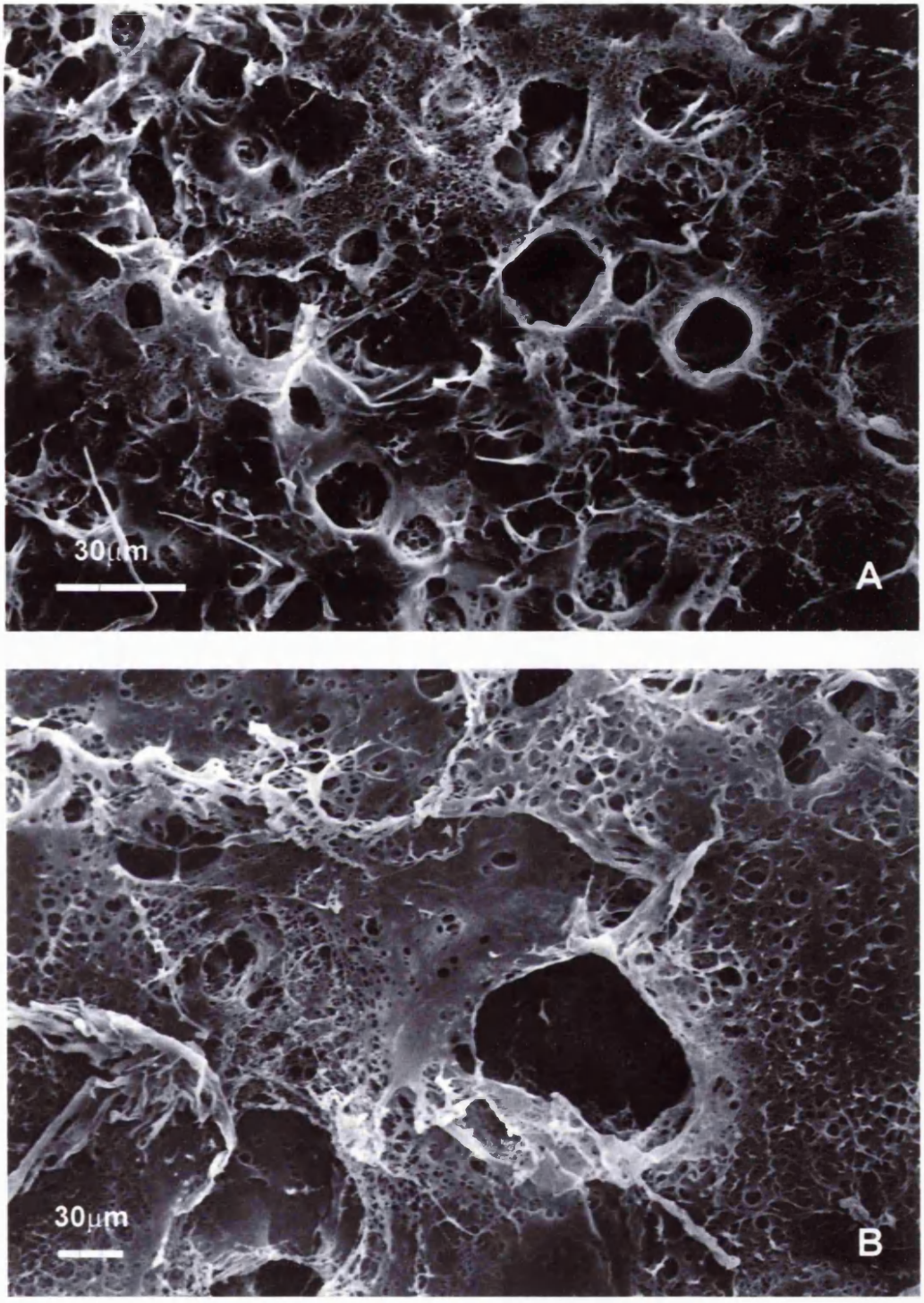

Figure 5.3 Low (A) and high (B) power electron micrographs of the PHA-C material.

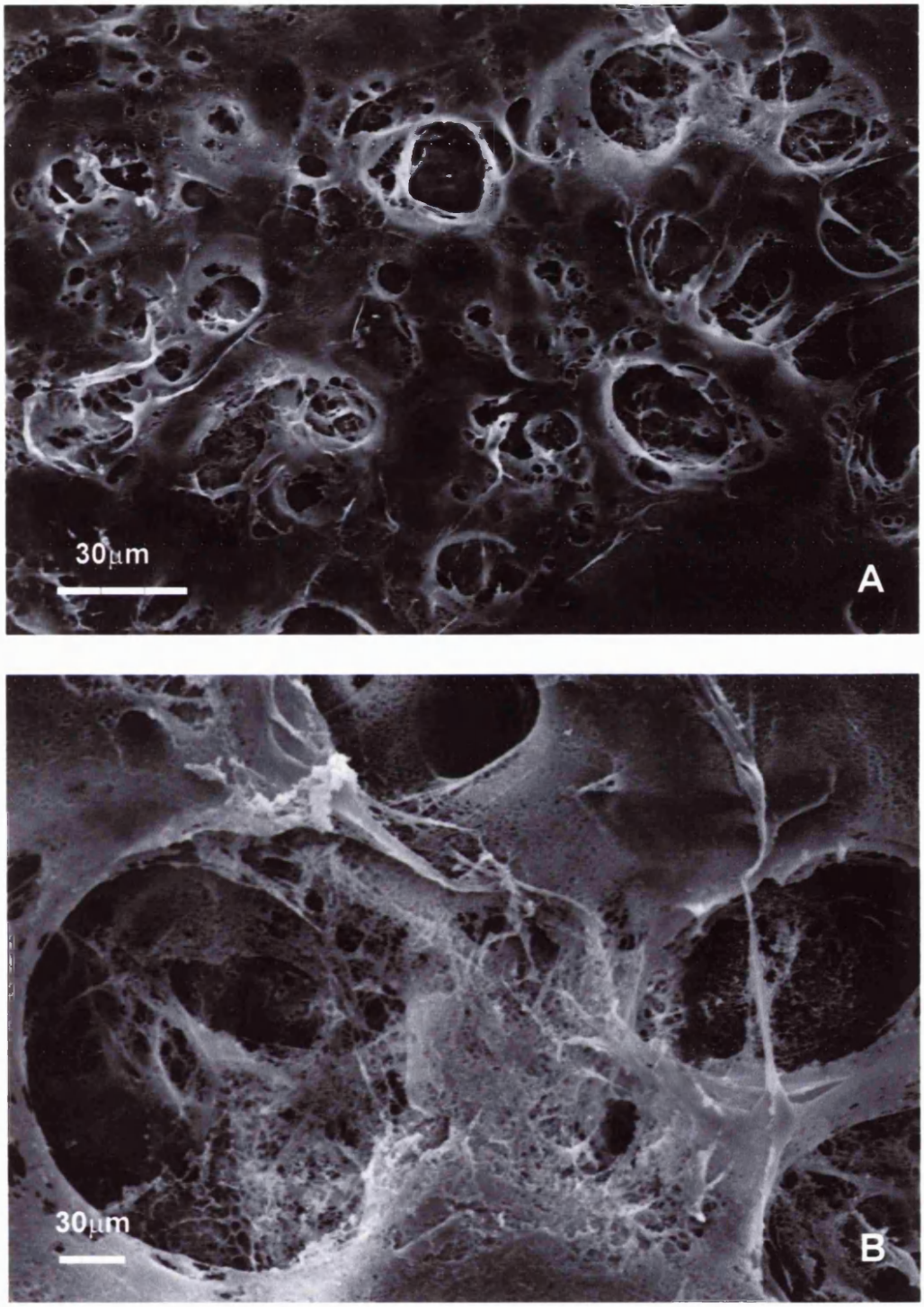

Figure 5.4 Low (A) and high (B) power electron micrographs of the PHA-D material.

5.4.2 Regeneration distance

The distance reached into the conduit by the furthermost PGP and S100 positively staining fibres and cells were measured at 10 and 20 days for each group. By 10 days PGP positive fibres were identified in the distal stump of all four types of PHA conduit indicating that the 1cm nerve gaps had been bridged. This gives an axonal regeneration rate of at least 1mm/day. This was accompanied by a continuous scaffold of S100 stained fibres across the gap. These results were sustained at 20 days.

By 10 days the SC and axons appeared to be regenerating in a straight line through the centre of the conduit. At 20 days the quantity of regeneration had increased such that the lumen of the graft, particularly in the proximal half was packed with PGP and S100 positive fibres. These fibres appeared to be restricted to the conduit lumen and did not traverse the porous walls of the graft out of the conduit (Figures 5.5 and 5.6).

Similarly in the nerve autograft repairs the 1cm nerve gaps had been bridged by 10 days giving an axonal regeneration rate of at least 1mm/day. This was accompanied by a continuous scaffold of S100 stained fibres across the gap. These results were sustained at 20 days.

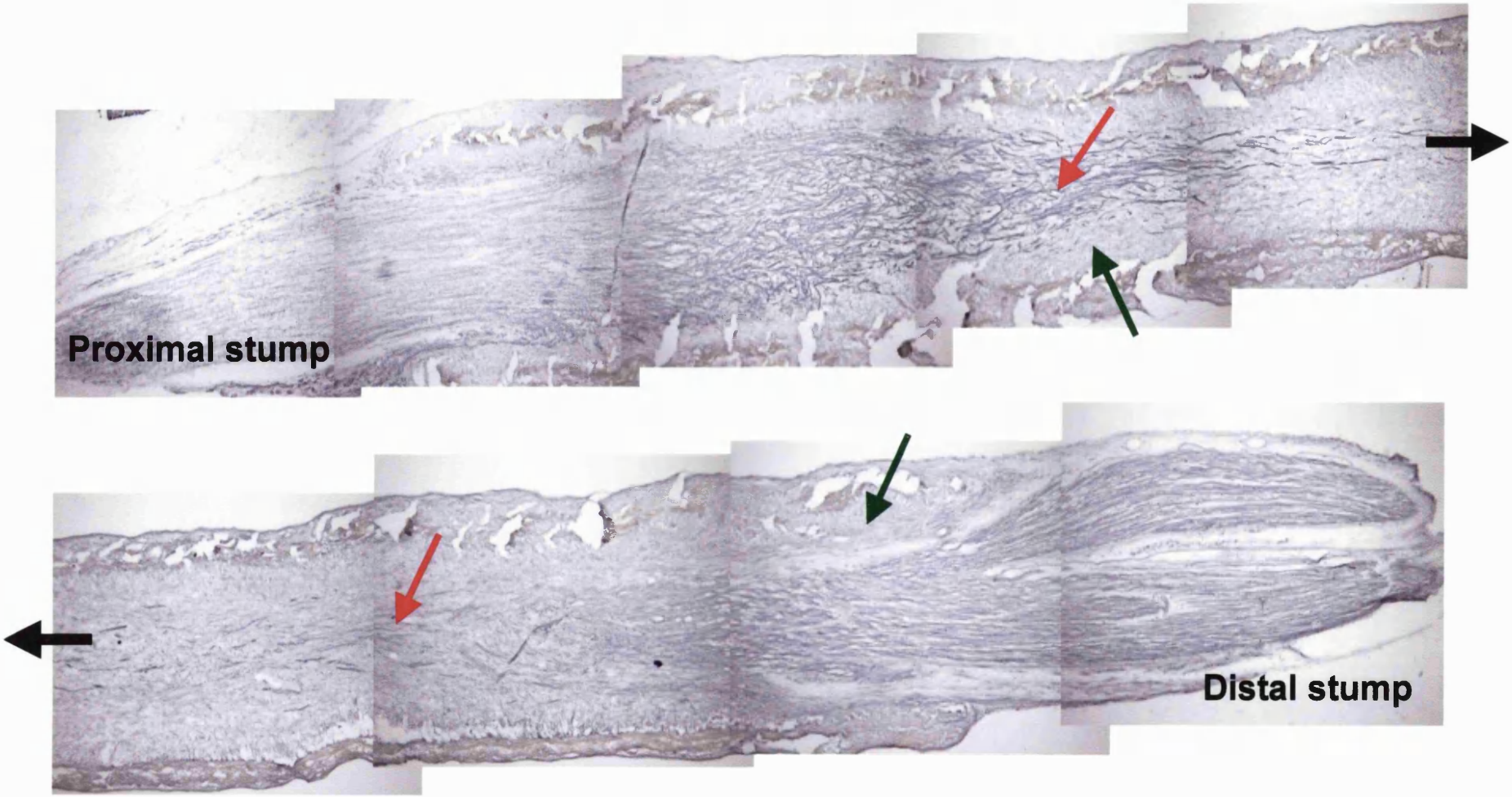

Figure 5.5 Composite photographs of a longitudinal section through the whole length of a PHA-D conduit showing axonal regeneration at 20 days. The image is divided into 2 sections and the black arrows indicate the continuation between the two images. (Red arrows = PGP staining and Green arrows = PHA-D conduit material).

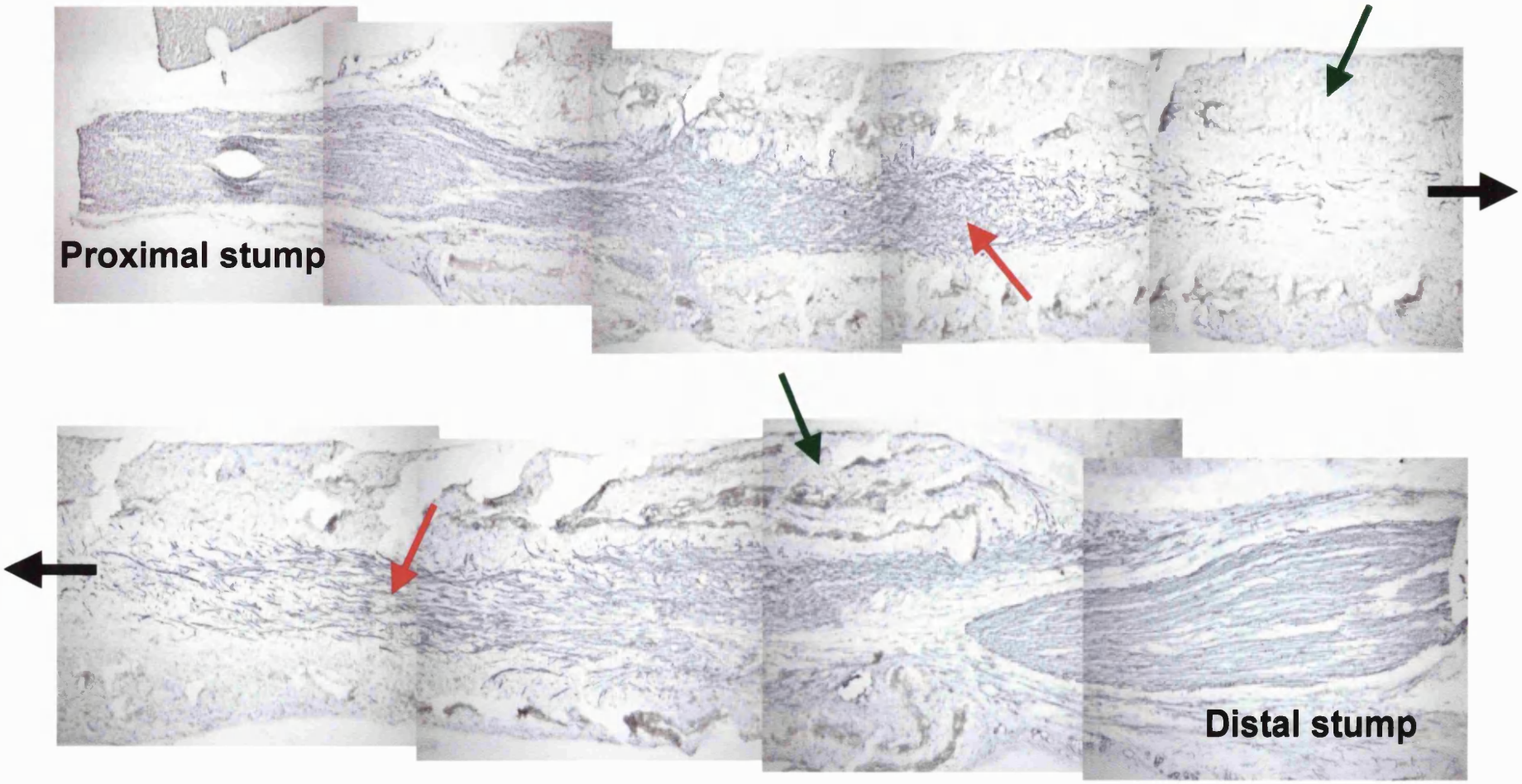

Figure 5.6 Composite photographs of a longitudinal section through the whole length of a PHA-D conduit showing SC regeneration at 20 days. The image is divided into 2 sections and the black arrows indicate the continuation between the two images. (Red arrows = S100 staining and Green arrows = PHA-D conduit material).

5.4.3 Regeneration area

As a result of the PHA-C conduit wall collapsing into the lumen of the tube on cryostat sectioning and consequently distorting axonal and SC staining in the lumen of these conduits, the PGP staining area and percentage staining area had to be quantified in the distal stump for all five groups at 10 and 20 days. Therefore quantification of S100 regeneration area was not carried out as this would also have identified resident SC in the distal stump, rather than just those regenerating and migrating into the conduit from the proximal stump. All 4 configurations of PHA conduits appeared to have comparable cross-sectional areas with no further change at the later time point of 20 days.

At 10 days the greatest percentage area of PGP staining was observed in the PHA-C conduits (39.8%) and the poorest in the autografts (14.7%) (Table 5.2 and Figure 5.7). By 20 days the PHA-D grafts supported the greatest percentage area of axonal regeneration in the distal stump (55.9%) (Table 5.2 and Figure 5.7). However, the greatest progression of regeneration area from 10 to 20 days was obtained in the PHA-B conduits with an 86% increase in the percentage area of axonal regeneration in the distal stump, followed by PHA-D tubes with a 82% increase (Table 5.2 and Figure 5.7). There was also progression of regeneration area in the nerve autografts with a 75% increase (Table 5.2 and Figure 5.7). No progression in the quantity of axonal regeneration was observed in the PHA-A and C grafts with time and in fact there was a small degree of regression of 14% and 10% respectively.

Graft	Days	% Regeneration area	% Increase in regeneration area with time
PHA-A	10	31.6%	-14%
	20	27.2%	
PHA-B	10	23.0%	86%
	20	42.8%	
PHA-C	10	39.8%	-10%
	20	35.8%	
PHA-D	10	32.5%	82%
	20	59%	
AG	10	14.7%	75%
	20	25.8%	

Table 5.2 Percentage axonal regeneration area and increase in axonal regeneration area with time in the distal stump at 10 and 20 days for the 4 configurations of PHA conduit (A-D) and autograft (AG) repairs.

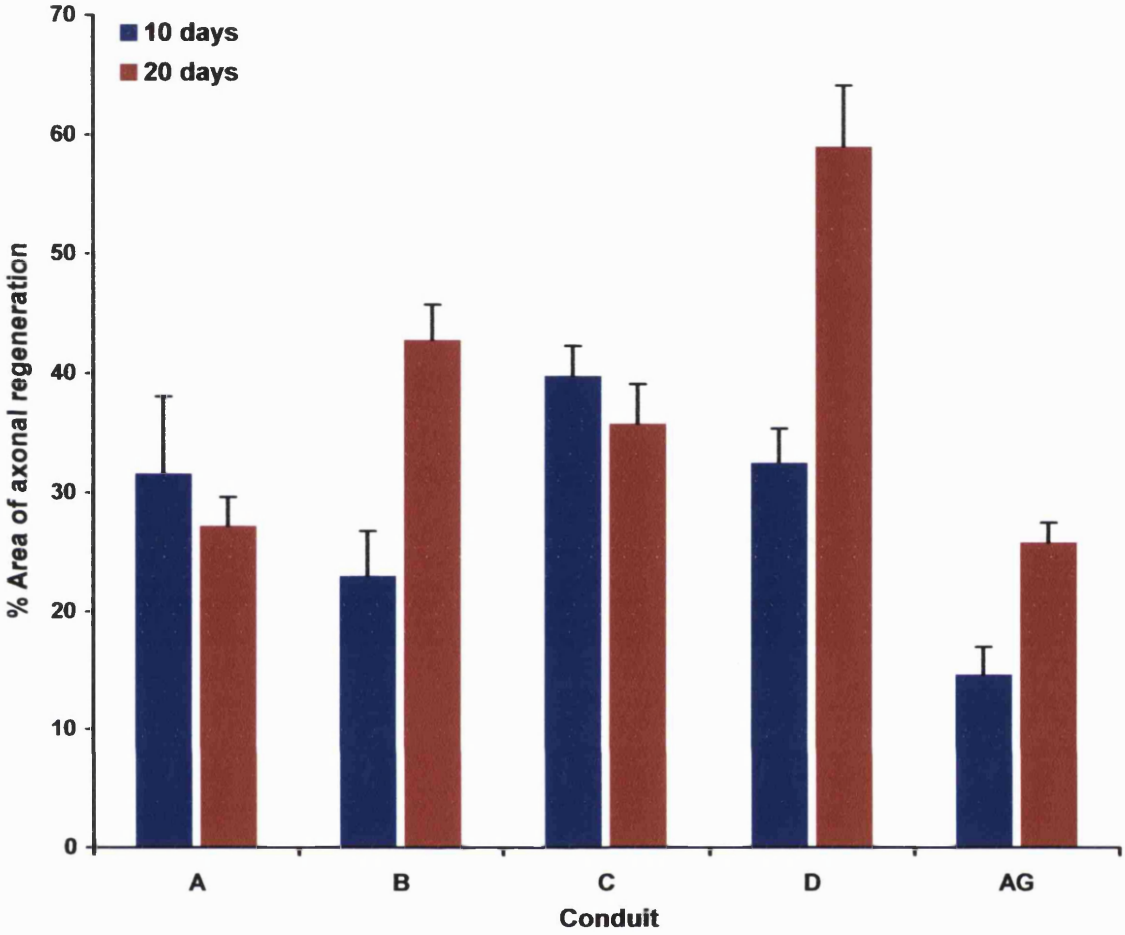

Figure 5.7 Percentage area of PGP staining in the distal stumps of the 4 configurations of PHA conduits (A-D) and nerve autografts (AG) at 10 and 20 days. Data expressed as group mean values with SEM. N=3 for each group.

5.5 DISCUSSION

Hazari *et al.* demonstrated that PHB conduits could be used effectively for both primary nerve repair (Hazari *et al.* 1999b) and short nerve gap repair (Hazari *et al.* 1999a). In addition, empty PHB conduits have also been shown to support axonal regeneration across long gaps (Young *et al.* 2002). In Chapter 3 the quantity of axonal and SC regeneration was enhanced by loading GGF into the lumen of the PHB conduit, but the method of growth factor delivery in alginate hydrogel had some drawbacks. The lack of mammalian enzymes for the digestion of alginate resulted in its presence behaving as a physical barrier to SC and axonal regeneration, and damping down the beneficial effects of GGF. An attractive alternative would be to bioengineer a conduit that would allow GGF to be released from its wall into the lumen, and the PHA family could possibly provide the answer. The PHAs have potential for numerous medical applications and have been investigated for use as scaffold materials in tissue engineering (Zinn *et al.* 2001) and as drug carriers (Kassab *et al.* 1997; Yagmurlu *et al.* 1999; Zinn *et al.* 2001). The appeal of PHAs lies partly in their biodegrability and biocompatibility as well as the potential to alter the chemical and mechanical properties of the material to suit the application.

Previous work carried out in our department demonstrated that empty PHB conduits supported axonal regeneration across a 1cm gap in the rat sciatic nerve by 14 days, inferring a regeneration rate of at least 0.7mm/day (Hazari *et al.* 1999a). In this study the PHA conduits appear to accelerate regeneration to at least 10mm/day, which is

comparable to the regeneration rate observed with the autograft nerve repairs. This may be as a result of the porous structure of PHA allowing the diffusion of nutrients and growth factors from the surroundings into the lumen of the conduit, which enhances axonal regeneration. However, further studies with earlier harvesting time points are necessary in order to conclude which configuration of PHA is optimal for axonal regeneration.

The pattern of axonal and SC regeneration seen in the PHA tubes was slightly different to that in the PHB conduits (cf Chapter 3). The PGP and S100 positive fibres were observed to be regenerating predominately through the centre of the PHA conduit lumen from the proximal to the distal stumps and to a lesser extent along the inner aspect of the conduit wall (Figures 5.5 and 5.6). In contrast, in the PHB grafts a trifurcation of regenerating fibres was seen at the proximal stump with axons and SC regenerating along the walls of the conduit and through the middle of the lumen. This occurred as a result of the PHB fibres gradually separating from each other and occupying a proportion of the original lumen, thus providing contact guidance and encouraging this pattern of regeneration. Although the PHA material is porous and not fibrous, it is possible that the longitudinally arranged pores in the PHA-A and PHA-B (Figures 5.1 and 5.2) may aid neuronal and glial regeneration through contact guidance, if during conduit preparation these pores are aligned along the length of the tube. This was demonstrated when fibronectin mats were used as conduits and the pores formed a trabecular structure providing contact guidance through their longitudinal alignment (Whitworth *et al.* 1996). Although only two of the four PHA configurations have the

potential to provide topographical and microgeometric cues, and these are not as specific as those provided by the PHB fibres, this does not appear to be detrimental to regeneration.

At both 10 and 20 days the percentage area of axonal regeneration in the distal stump of the autograft repairs was inferior to that observed in any of the four configurations of PHA conduit. Therefore despite the presence of the basal lamina tubes in the autografts providing a scaffold for regenerating axons (Thanos *et al.* 1998) and promoting the rate of regeneration, they are unable to enhance the quantity of regeneration to the level of the PHA conduits. The variability observed in the quantity of axonal regeneration in the four configurations of conduits is most likely a result of the variation in sample preparation and therefore pore size, which, according to the manufacturer, ranged from 25-150μm. At 10 days the PHA-C grafts supported the greatest percentage area of regeneration, but by 20 days the distal stumps of the PHA-B and PHA-D tubes contained the greatest quantity of axons and showed the greatest progression of axons penetrating the distal stump over time. Both PHA-B and D underwent salt leaching using Tween80 and water, whereas PHA-A and C were leached with water alone. It is possible that the addition of Tween80 affects the salt leaching process, thereby resulting in the Tween80 leached PHA (PHA-B and D) having larger pores than the water leached PHA (PHA-A and C) as was demonstrated in the electron micrographs. Hence, PHA-B and D may provide the optimal porosity to permit the diffusion of beneficial growth factors into the conduit whilst simultaneously excluding inhibitory molecules that are detrimental to regeneration ,whilst the pores in the PHA-A and C are too small

to allow the diffusion of beneficial growth factors into the conduit. Previous workers have shown that pore size can affect nerve regeneration. Aebischer *et al.* demonstrated that conduits which allowed molecules of up to 50kD to diffuse into the channel improved regeneration compared with impermeable silicone tubes, whereas conduits allowing diffusion of molecules of up to 100kD resulted in impaired nerve regeneration (Aebischer *et al.* 1989). In addition the manufacturers of PHA have demonstrated in their laboratories that Tween80 serves as a polymer wetting agent to facilitate cell seeding (personal communication) and it is possible that this may also make the polymer surface more conducive to regeneration. The lack of progression and the slight regression of axonal regeneration observed in the PHA-A and C conduits, may also reflect the start of axonal pruning due to lack of effective regeneration and poor neurotrophic support. Further work needs to be carried out to accurately measure and control the pore size of the four types of PHA material and the size of molecules they permit to pass through them, as it would be feasible to alter the porosity to an optimal size allowing more selective diffusion of molecules beneficial to regeneration.

The attraction of the PHA family is that the properties of these materials can be tailored to the specific application, they biodegrade to natural metabolites and they are already approved for certain human uses. We have demonstrated that they support axonal regeneration at a rate comparable to a nerve autograft repair, but result in a quantity of regeneration which is superior. With further research once a suitable type of PHA has been selected, alterations in the functional group may be performed to allow growth factors to be attached to the PHA conduit wall, in such a way as to allow their uptake by

the regenerating neurons over time in a bioactive form. In addition, the PHA scaffold has the potential to be seeded with cultured SC and may be used as a conduit to enhance nerve regeneration. If necessary, for longer gap lengths the chemical and mechanical properties of the scaffold may also be altered such that it provides adequate support for the regenerating and maturing axons for a sufficient period of time.

CHAPTER 6

IN VITRO AND *IN VIVO* ASSESSMENT OF ALGINATE FIBRES

6.1 INTRODUCTION

6.2 AIMS

6.3 MATERIALS AND METHODS

6.4 RESULTS

6.4.1 *IN VITRO*

6.4.2 *IN VIVO*

6.5 DISCUSSION

6.1 INTRODUCTION

In Chapters 3 and 4 alginate gel was used to deliver GGF into the lumen of a PHB conduit, but this form of alginate presented some disadvantages. However, alginate can be extruded into various physical forms, which have a range of applications. Alginate beads (Smidsrod & Skjak-Braek 1990) have been used *in vitro* as a vehicle for sustained release of endothelial cell growth factor (Ko *et al.* 1995). Alginate sponges have been used for *in vitro* cell cultures and they possess structural and morphological properties appropriate for cell growth, proliferation and neovascularisation (Shapiro & Cohen 1997). Beads, rods and tubes made from alginate hydrogel and loaded with leukaemic inhibitory factor (LIF) have been reported to release the growth factor at a rate of less than 1% per day for a period of several months (Austin *et al.* 1997).

Alginate beads have also been used to implant neuronal cells (Boisseau *et al.* 1993), genetically modified fibroblasts secreting nerve growth factor (Maysinger *et al.* 1994) and for controlled-release of various neurotrophic factors in central nervous system (Maysinger *et al.* 1996). Although alginate gel, beads and rods can all potentially provide a controlled and sustained release of a growth factor within the lumen of a conduit, they do not provide contact guidance to the regenerating axons. Researchers have demonstrated that the presence of either resorbable or non-resorbable filaments within the lumen of a conduit used to bridge a critical nerve gap in the rat sciatic nerve, support axonal regeneration when an empty conduit does not (Lundborg *et al.* 1982b; Lundborg & Kanje 1996; Lundborg *et al.* 1997b; Terada *et al.* 1997a; Terada *et al.*

1997b; Terada *et al.* 1997c; Arai *et al.* 2000). Alginate can also be extruded in the form of fibres, which could be longitudinally aligned in the lumen of a conduit and used to provide microgeometric and topographical cues to the regenerating axons. By loosely filling the PHB conduit with these fibres, the spaces or channels between the fibres could potentially provide a path for axonal regeneration, thereby overcoming the problems associated with the use of the alginate gel.

6.2 AIMS

The aims of this study were to assess the behaviour of different compositions of alginate fibres in culture medium with respect to swelling, maintenance of fibre structure, tensile strength and degradation profile. Also to study if the PHB conduits loaded with alginate fibres supported axonal and SC regeneration *in vivo*.

6.3 MATERIALS AND METHODS

Alginate fibres with an individual fibre diameter of between 10-20μm were supplied from commercial sources. Sorbasan, Comfeel and AMS fibres (cf 2.2) all had a high M content (M:G = 60:40), although they had varying molecular weights. Fibres were also manufactured specifically from an ultrapure mannuronic acid rich alginate (LVM) (M:G = 67:43) and ultra pure guluronic acid rich alginate (LVG) (M:G = 43:67) (cf 2.2).

For *in vitro* studies, six PHB conduits measuring 1cm in length were prepared (cf 2.4.1) for each of the 5 types of alginate fibre and similar quantities of the fibre were loaded into each conduit (cf 2.4.3). Each alginate loaded conduit was placed in the well of a 6 well tissue culture plate (Figure 2.9) and 3mls of culture medium was added (cf 2.4.4). The culture trays were incubated at 37°C, with the culture medium being changed every 2-3 days. Qualitative assessment of the maintenance of fibre structure, degree of fibre swelling, tensile strength on handling and degradation profile was carried out on days 2, 7 and 10 using a dissecting microscope (cf 2.4.4).

For *in vivo* studies, four PHB conduits measuring 2.4cm in length were prepared (cf 2.4.1). Two were loaded with ultrapure LVM fibres (PHB-LVM) and two with ultrapure LVG fibres (PHB-LVG) (cf 2.4.3) of similar quantities. Four female New Zealand White rabbits were used for this study, two in each group. The conduits were used to bridge a 2cm gap in the common peroneal nerve (cf 2.6) and the rabbits were sacrificed at 42 days. The conduit repair sites were harvested (cf 2.8.1.1), the tissue fixed, blocked, sectioned (cf 2.9) and then stained with monoclonal antibodies to PanNF and S100 (cf 2.10.1). The axonal and SC regeneration distance and area were then quantified (cf 2.11).

6.4 RESULTS

6.4.1 *In Vitro*

The following images have been captured using a digital camera mounted on the microscope eye-piece. The image on the left demonstrates the alginate tail leaving the PHB conduit and that on the right is the same conduit on cross section

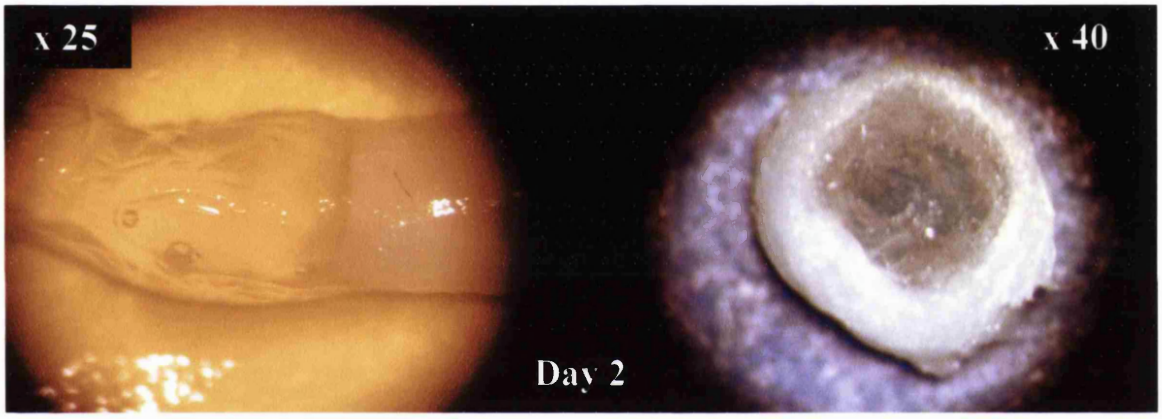

Figure 6.1 The Sorbasan fibre tail coming out of the PHB tube appeared gelatinous but its fibrous structure was still present. On cross-section the tube was loosely filled with the alginate and the fibres were more evident. The alginate had a good tensile strength on handling.

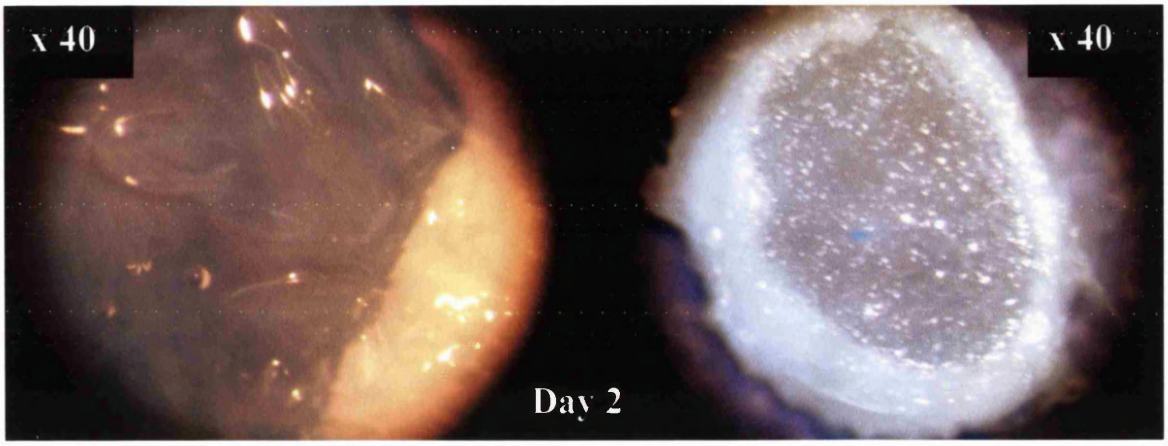

Figure 6.2 The Comfeel alginate appeared more gelatinous than the Sorbasan and the fibres were less evident. As a result on cross section the tube was filled more homogenously and on handling the alginate was more friable.

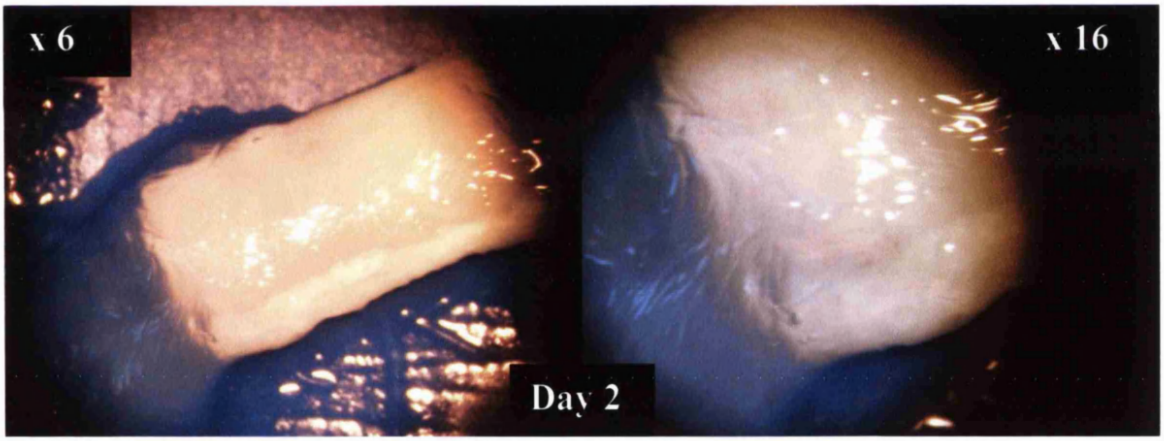

Figure 6.3 All 6 of the AMS fibre filled tubes had unravelled by this time point despite the quantity of fibres in the PHB tubes being similar to that of the Comfeel and Sorbasan filled tubes. This was most likely secondary to the excessive swelling of the fibres. As a result the fibrous skeleton was less obvious and the tensile strength was reduced.

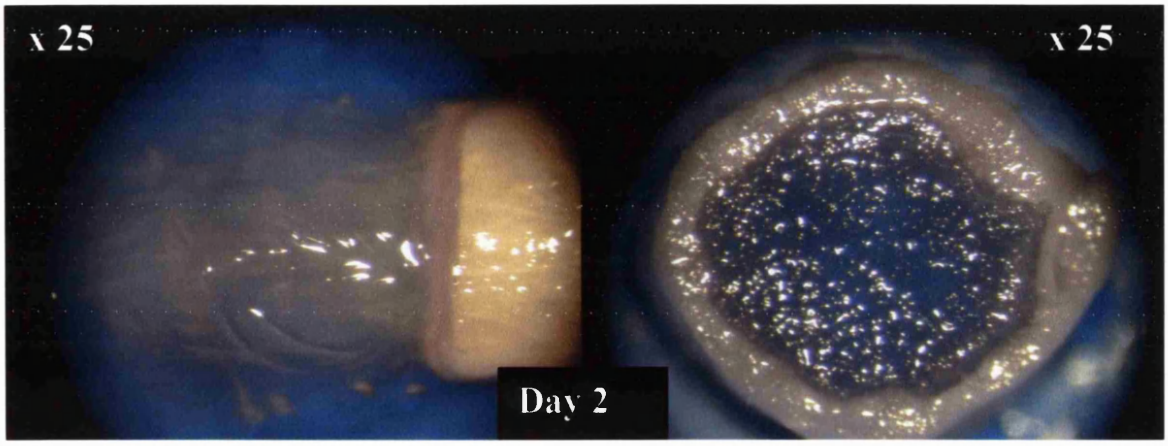

Figure 6.4 Two of the PHB-LVM alginate tubes had unravelled due to swelling of the fibres. The tail was already beginning to lose its fibrous structure but still maintained a good tensile strength. On cross section the tube was homogenously filled with a gelatinous form of alginate.

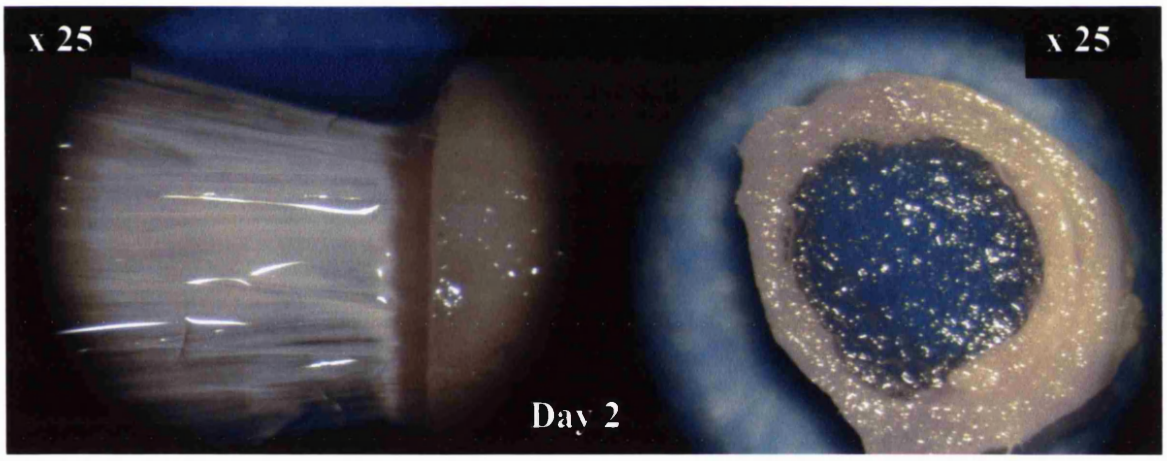

Figure 6.5 None of the PHB-LVG conduits had unravelled and the alginate maintained a very fibrous tail, which was difficult to cut. On cross section the alginate fibres were still apparent.

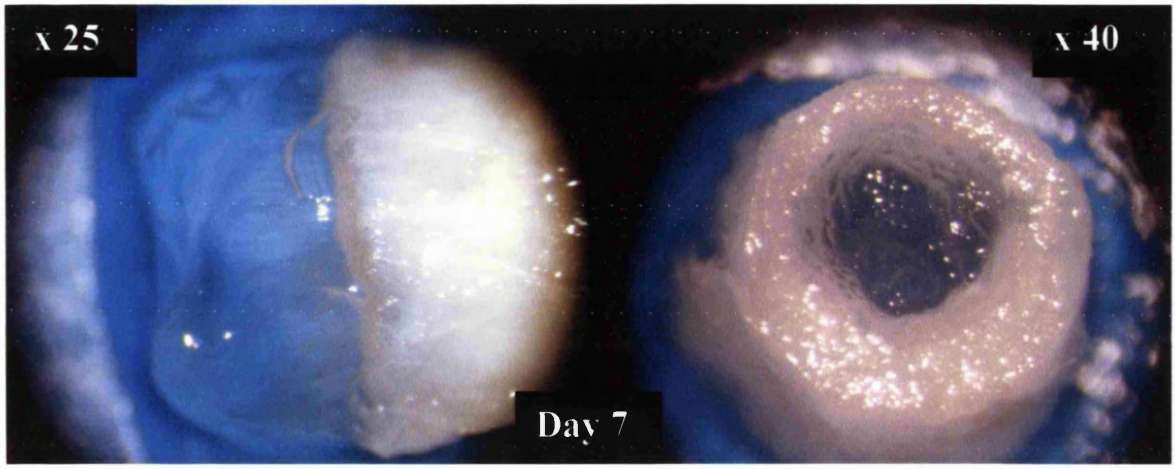

Figure 6.6 By day 7 the Sorbasan had became more gelatinous but still maintained a good tensile strength. The fibrous structure was now less evident on cross section.

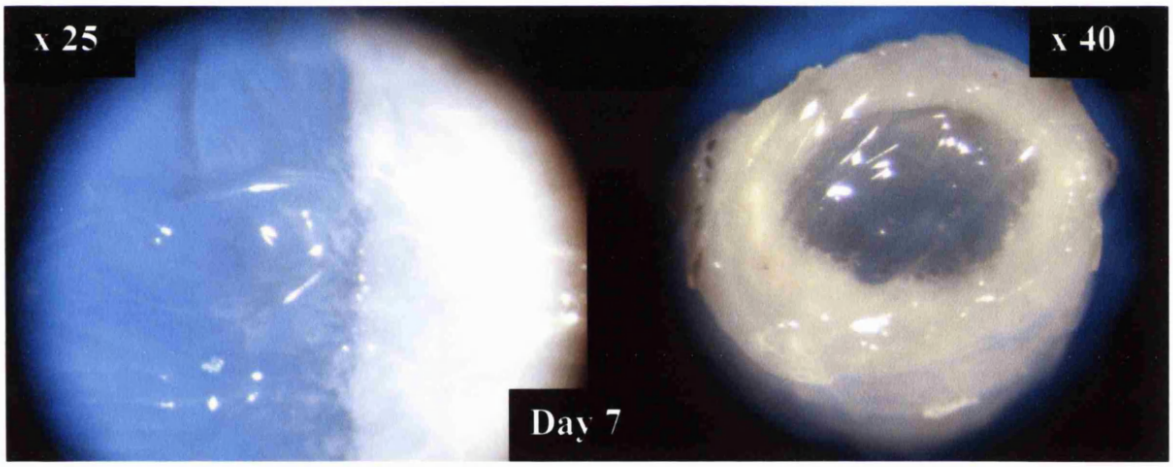

Figure 6.7 The swelling of the Comfeel fibres increased by day 7 resulting in unravelling of 2 out of the 6 tubes. The fibres were now less apparent and the alginate tensile strength was reduced

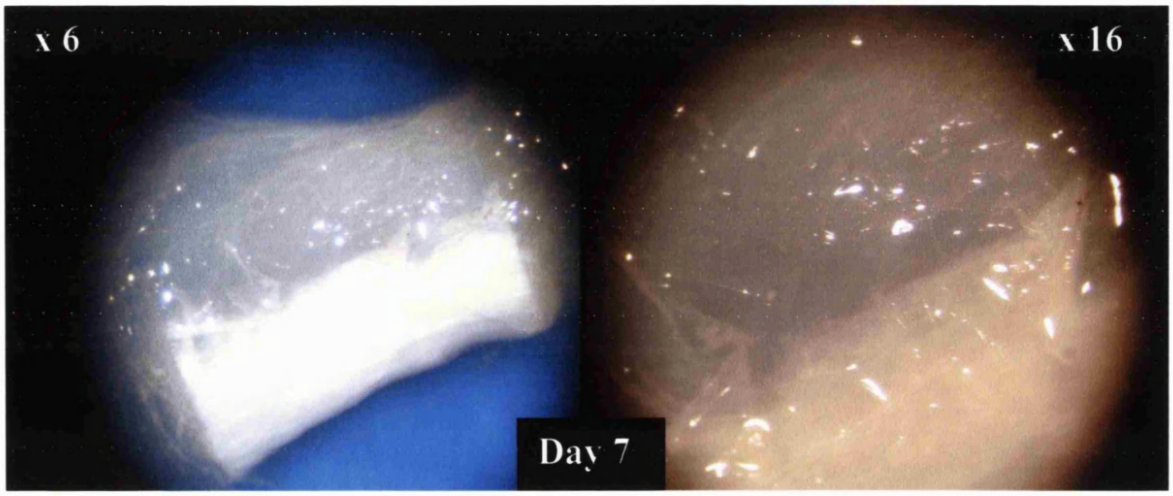

Figure 6.8 The AMS by day 7 appeared unchanged.

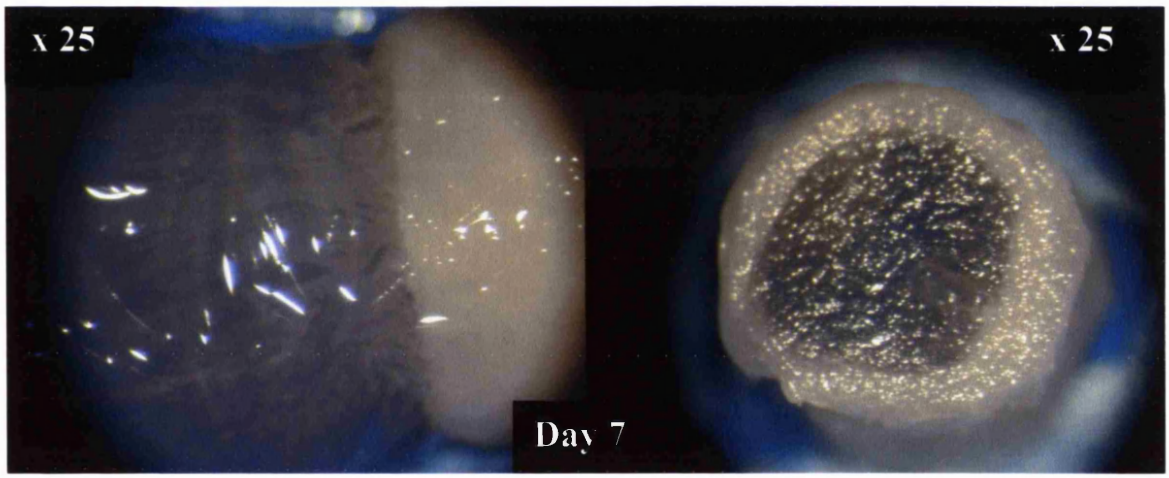

Figure 6.9 The LVM alginate appeared even more gelatinous, further losing its fibrous structure and tensile strength. No further tubes had unravelled.

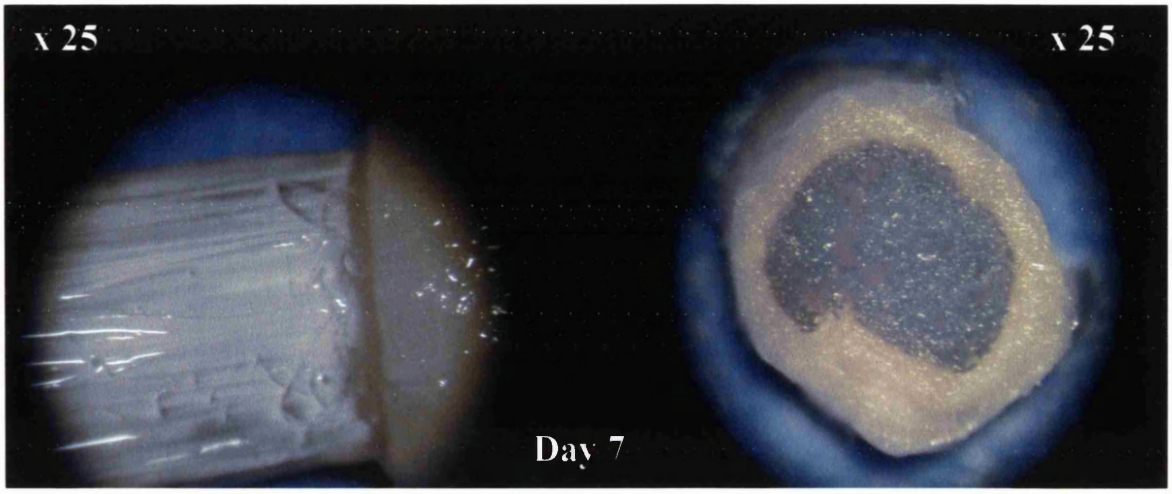

Figure 6.10 There was no real change in the appearance or tensile strength of the LVG alginate.

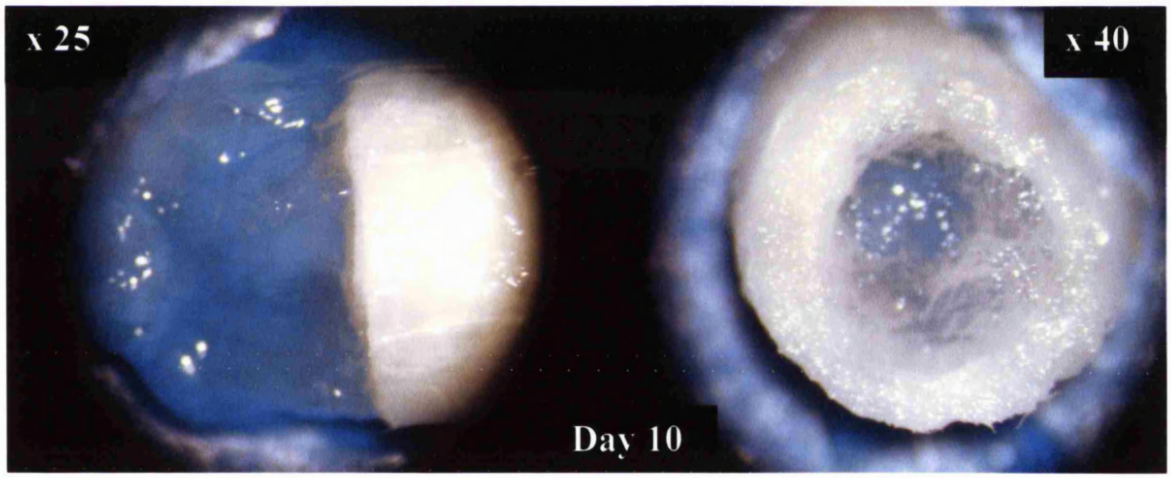

Figure 6.11 By the 10th day no further changes of the Sorbasan fibres were observed. In cross section the tube appeared to be under filled, but this was the result of some alginate fibres exuding out on transversely cutting the conduit.

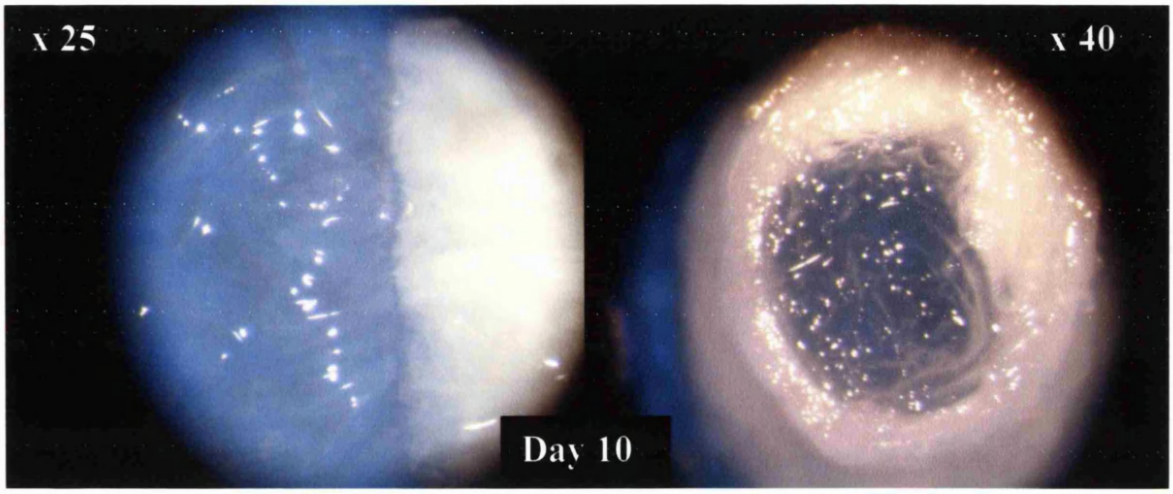

Figure 6.12 Two more of the Comfeel alginate tubes had unravelled, indicating that further swelling of the fibres must have occurred. The fibrous skeleton was as poorly visible as on day 7, with no alteration of the tensile strength.

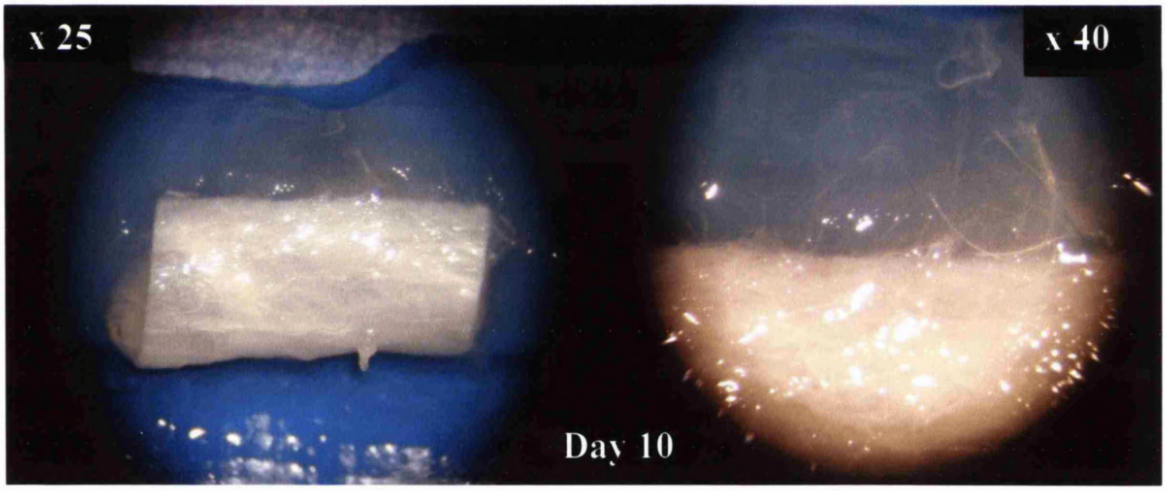

Figure 6.13 The AMS fibres appeared more gelatinous, with a far less obvious fibrous skeleton and a reduced tensile strength.

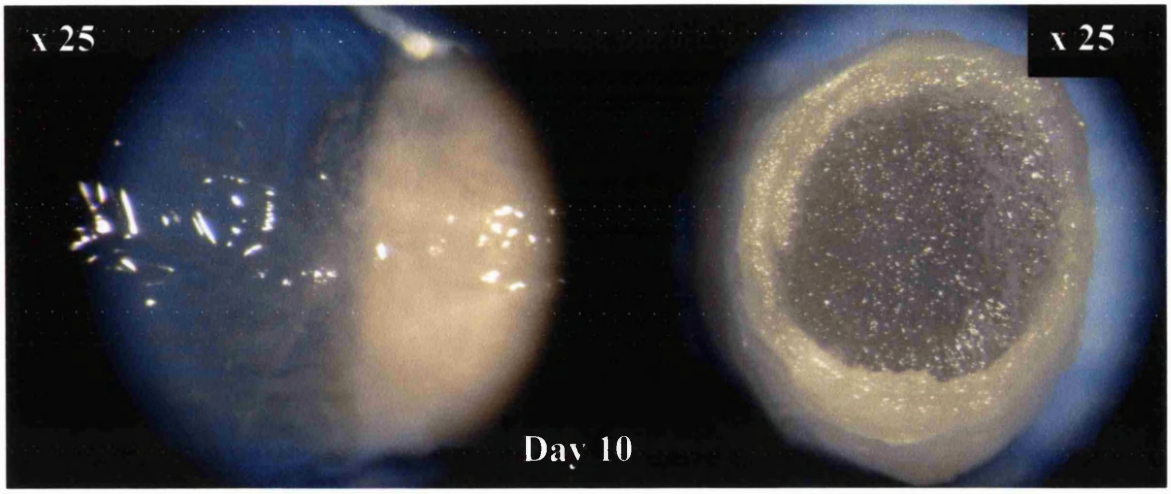

Figure 6.14 There appeared to be no major changes in the LVM fibres between day 7 and 10.

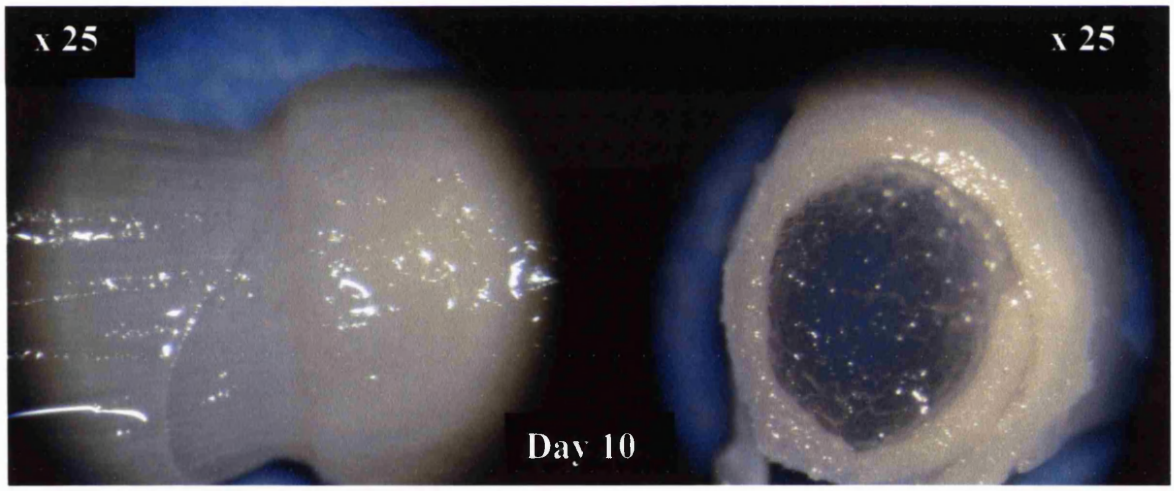

Figure 6.15 By day 10 the LVG alginate fibres were a little more gelatinous, particularly on cross section, but still maintained an obvious fibrous structure and tensile strength.

In summary, out of the 3 commercially available alginates the AMS fibres underwent the greatest degree of swelling within 24 hours, independent of the amount of fibres loaded into the tubes. In addition, these fibres almost completely lost the fibrous structure and tensile strength within the first two days. The Comfeel fibres became more swollen losing their fibrous appearance and becoming more friable. The Sorbasan fibres appeared the most stable, causing no unravelling of the tubes and maintaining their fibrous structure and tensile strength throughout. Of the specifically manufactured alginate fibres, the M-rich fibres underwent more swelling particularly in the first two days, becoming more gelatinous and in turn losing their tensile strength.

Of the five types of alginate studied *in vitro* only the LVM and LVG fibres were studied *in vivo*, because their exact chemical compositions were known, they were ultra pure, of low endotoxicity and sterile.

6.4.2 *IN VIVO*

In the post operative period there was no evidence of wound infection or wound dehiscence. At the time of harvesting all nerve repairs were intact with no anastomotic failures and there was no evidence of ulceration in the distribution of the common peroneal nerve. The PHB tubes had become well vascularised and were noted to be covered in a very thin pseudocapsule, which could be easily removed and the PHB tube explanted. The PHB tubes had not become adherent to underlying muscles.

In vivo the LVM and LVG alginate fibres behaved in a similar manner. At 42 days the results of axonal and SC regeneration were very disappointing for both the PHB-LVM and PHB-LVG conduits. The regenerating axons and SC from the proximal stump did not appear to grow through the centre of the conduit, as was observed with the alginate gel tubes (cf 3.4), but were regenerating along the PHB fibres, which made up the wall of the conduit (Figures 6.16 and 6.17). There was also some regeneration in the small space between the inner aspect of the conduit wall and the outer aspect of the alginate fibres. At the distal stump the S100 staining was a mirror image of the regeneration pattern observed at the proximal stump, with the SC regenerating into the wall of the conduit. For both the PHB-LVM and PHB-LVG conduits the mean distance of SC and axonal regeneration was 4.5mm and 4.0mm respectively. This was approximately 50% less than the mean distance of SC and axonal regeneration (9.7mm and 9.05mm respectively) observed in the 2cm PHB-ALG conduits at 42 days (cf 3.4.1). Microscopically there was evidence that the LVM and LVG fibres were still present and occupied the majority of the conduit lumen (Figures 6.16 and 6.17).

The quantity of SC and axonal regeneration could not be assessed as there was no S100 or PanNF staining 5mm distal to the proximal end of the conduit, where quantification is carried out (cf 2.11). However, subjectively the quantity of SC and axonal regeneration in the proximal part of the conduit was significantly less than that seen in the 2cm PHB-ALG conduits at 42 days.

In summary, neither the LVM or LVG alginate fibres supported successful axonal or SC regeneration through the lumen of the PHB conduit by 42 days.

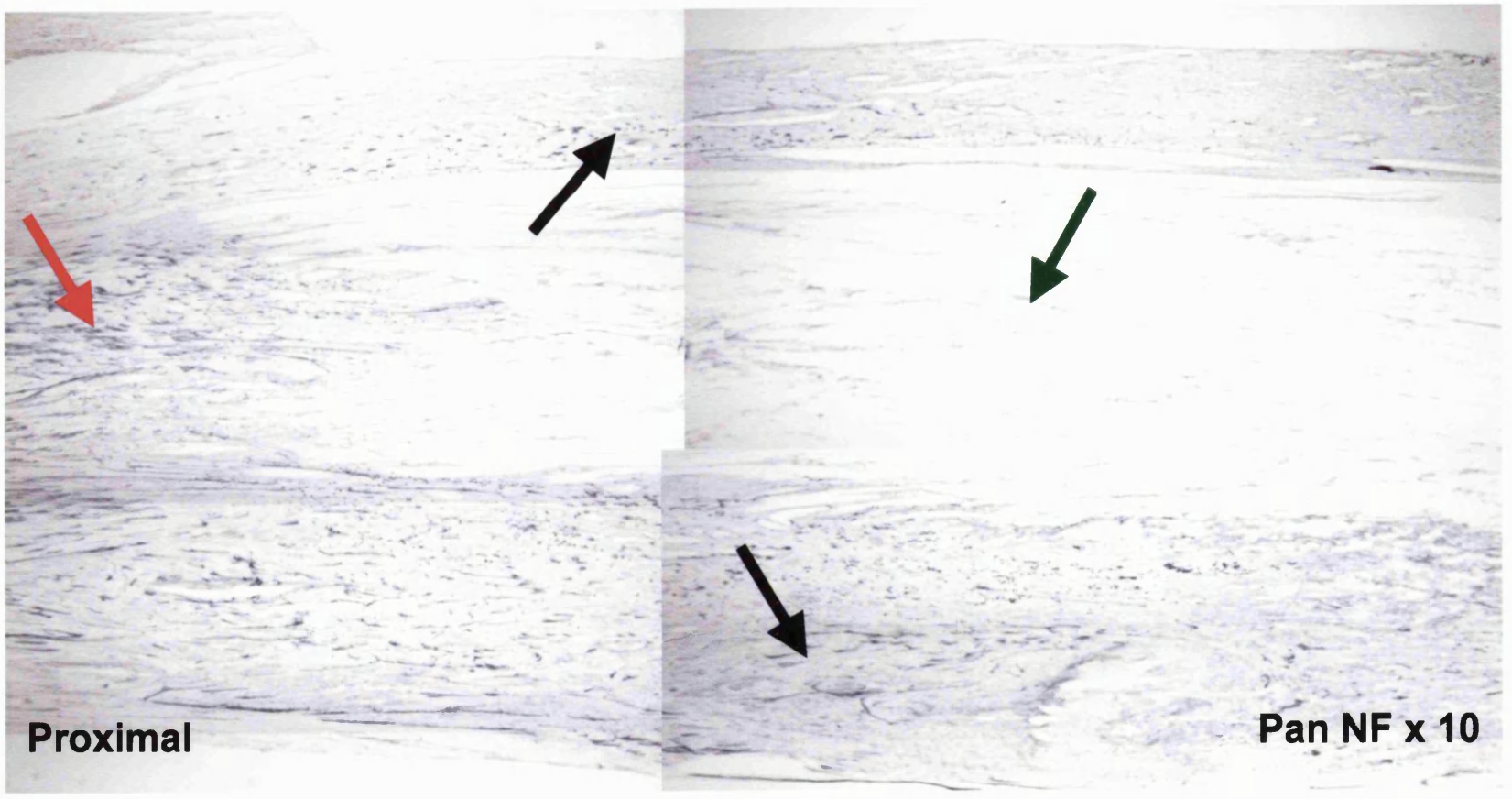

Figure 6.16 The image consists of 8 photographic frames captured through the proximal part of a longitudinal section of the PHB-LVG conduit. PanNF positive staining is seen in the proximal stump (red arrow) and in the upper and lower edges of the conduit (black arrows). Only LVG fibres are present in the lumen of the conduit (green arrow).

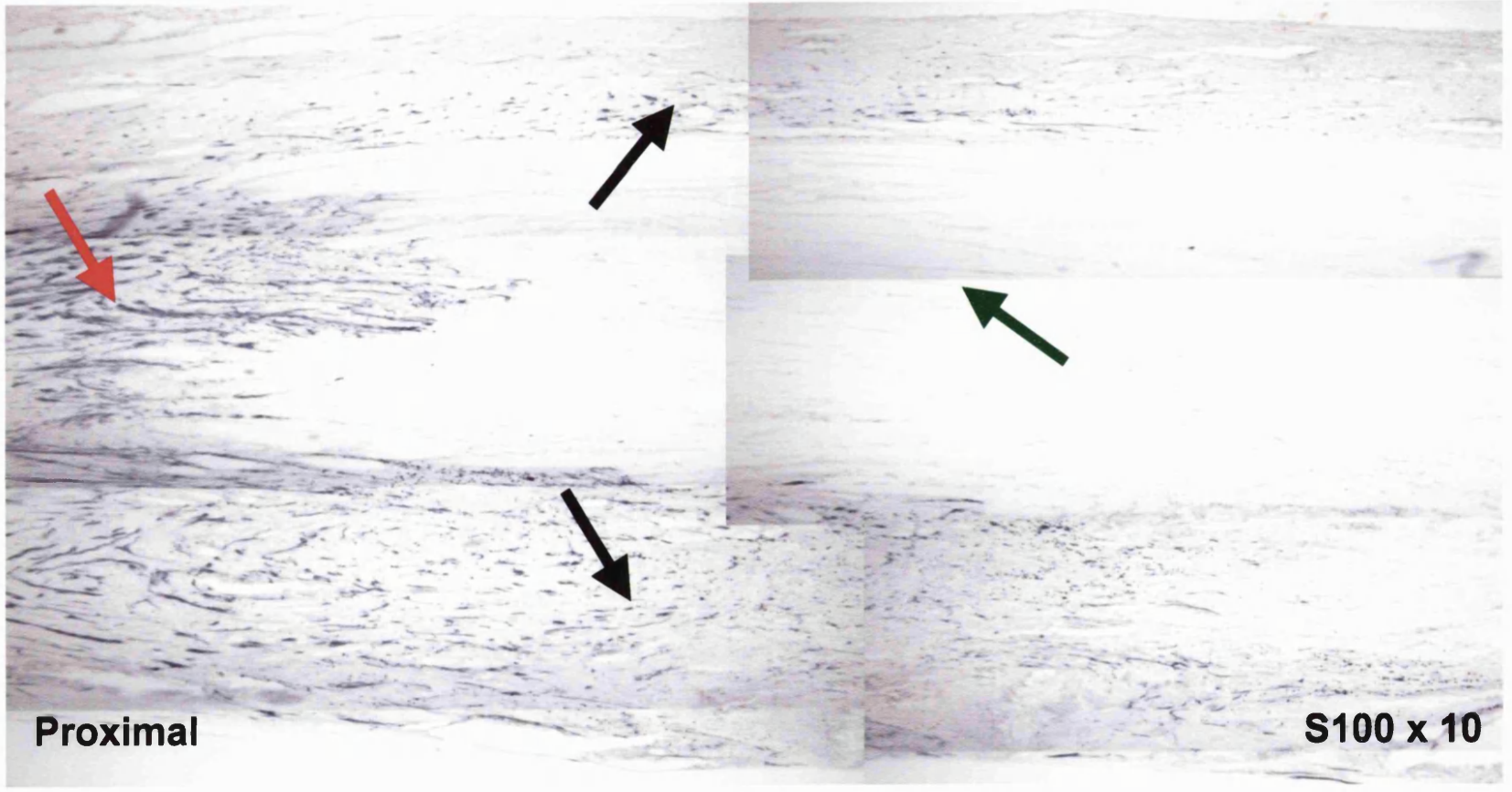

Figure 6.17 The image consists of 8 photographic frames captured through the proximal part of a longitudinal section of the PHB-LVG conduit. S100 positive staining reflects that of PanNF staining (Figure 6.16), and is seen in the proximal stump (red arrow) and in the upper and lower edges of the conduit (black arrows). Only LVG fibres are present in the lumen of the conduit (green arrow).

6.5 DISCUSSION

Alginates constitute a family of unbranched binary co-polymers of 1,4-linked β-D-mannuronic acid (M) and 1,4-linked α-L-guluronic acid (G), of widely varying composition and sequence depending on the algae of origin. As previously mentioned it is the proportion and distribution of these blocks (MM, GG, MG) which determines the chemical and physical properties of the alginate (Draget *et al.* 1997; Smidsrod & Skjak-Braek 1990).

In this study despite all the commercially available alginate fibres being from the same algae of origin and having the same M:G ratio of 60:40, they appeared to behave differently *in vitro*. This may have been as a result of the alginate fibres having different molecular weights. The flexibility of alginate molecules is dependant on the proportion of M blocks. The links in the M chain are more flexible than those in the G chain, therefore the intrinsic flexibility of the alginate molecules increases in the order GG < MM < MG, and the more flexible the alginate the more it swells (Draget *et al.* 1997; Smidsrod & Skjak-Braek 1990). The AMS fibres had the highest molecular weight indicating that the alginate was composed of a greater number of M and G blocks compared to the Sorbasan and Comfeel fibres. It is possible that as a result of this the fibres underwent more swelling in the culture medium resulting in unravelling of all six conduits within the first 48 hours. This could not be attributed to the number of fibres in the conduits because the weights of the fibres in the six tubes were comparable to those of the Sorbasan and Comfeel loaded conduits. In addition, as these were commercially

available alginate fibres they had been coated to maintain their fibrous structure. It is likely that the type of coating varied between the fibre types, possibly contributing to the differential behaviour of the fibres in the culture medium.

As the alginate fibres swelled in the culture medium, they lost their fibrous structure and in turn became more gelatinous. Consequently the tensile strength appeared to be proportional to the degree in which the fibrous skeleton of the alginate was maintained. In addition, both the rate of swelling and fibre disruption are likely to determine the degradation profile and the rate of alginate resorption. Although the timing of the *in vitro* studies was too short to assess these parameters, from the current results one would expect the AMS to have the fastest degradation profile and the Sorbasan the slowest.

In vitro the ultra pure LVM and LVG fibres, which had been produced specifically for this study, behaved as expected. The LVM fibres swelled more readily, particularly in the first 48 hours, resulting in the unravelling of two out of the six PHB conduits. Again as the LVM fibres became more swollen they lost their fibrous skeleton and tensile strength becoming more gelatinous. As a result of these observations one would expect the LVM fibres to have a faster degradation than the LVG fibres. These findings are in concordance with previous work, which has demonstrated that M rich alginates are less rigid and have a faster resorption as a result of the calcium ion cross links being weaker than in the G rich alginates (Draget *et al.* 1997; Smidsrod & Skjak-Braek 1990). Therefore calcium and sodium ion exchange occurs more easily, resulting in the loss of structure and slow renal excretion (Draget *et al.* 1997; Smidsrod & Skjak-Braek 1990).

The *in vitro* results suggest that an alginate, which maintains its fibrous structure such as LVG, does so at the expense of a slower degradation profile, therefore making it more likely to behave as a physical barrier. Hence, a more suitable candidate for the *in vivo* delivery of neurotrophic factors would be an ultra pure mannuronic rich alginate such as LVM, which loses its fibrous structure as it becomes gelatinous. However, this loss of fibrous structure and hence faster degradation, will occur at the expense of the loss of contact guidance, for which the alginate fibres were selected in preference to the alginate gel.

In vivo the LVM and LVG loaded PHB conduits behaved in a similar manner. By 42 days both the LVM and LVG fibres were still recognizable in the conduits. They appeared to 'repel' the regenerating SC and axons such that none regenerated within and along the alginate fibres. Instead a minimal amount of regeneration was seen to occur in the wall of the conduit and in the space between the outer aspect of the alginate fibres and the inner aspect of the conduit wall. The SC and axonal regeneration distance and area were inferior to that observed in the PHB conduits filled with alginate gel, for the same gap length and time point (cf 3.4). Despite the alginate fibres having the potential to provide contact guidance to the regeneration front, they were unable to provide an environment, which was conducive to regeneration. It is possible that the fibres were too tightly packed in the conduit presenting a path of high resistance to the regenerating growth cone and forcing it to grow down paths of lower resistance. Also the presence of the alginate fibres in the lumen may have impeded the neurotropic effect of the growth factors released from the distal stump. Similar observations have been made when

semipermeable guidance channels have been filled with either collagen or laminin-containing gels and regeneration have been hindered (Valentini *et al.* 1987).

However, previous researchers have demonstrated the advantages of using either biological or synthetic filaments to enhance nerve regeneration. In some instances these have been used instead of a tube conduit. Yoshii *et al.* used both laminin-coated filaments and collagen filaments to make nerve grafts, both of which effectively guided regenerating axons across a 2cm gap in the rat sciatic nerve (Yoshii *et al.* 1987; Yoshii & Oka 2001). Filaments have also been placed inside the lumen of a conduit for nerve gap repairs. Bioartificial nerve grafts composed of a silicone tube and synthetic filaments inserted longitudinally into the tube have been used to bridge a 15mm defect in the rat sciatic nerve. Whereas the filament containing conduits supported regeneration across the nerve gaps the empty silicone tubes did not. Interestingly no regenerating axons were seen in contact with the filaments and it was suggested that the filaments stabilised the initial fibrin matrix, thereby promoting regeneration rather than behaving as a scaffold (Lundborg *et al.* 1982b; Lundborg & Kanje 1996; Lundborg *et al.* 1997a; Arai *et al.* 2000). Matsumoto *et al.* used a conduit made of polyglycolic acid mesh coated with collagen and filled with laminin-coated collagen fibres to bridge an 80mm gap in the peroneal nerve of a dog. Electrophysiological and histological restoration was obtained by 12 months (Matsumoto *et al.* 2000). It is possible that in our study the conduits were overfilled with alginate fibres, which presented a barrier to the formation of the fibrin matrix within the conduit, with minimal or no subsequent invasion by capillaries, SC and axons.

We hypothesised that an alginate, which maintained its fibrous structure would be beneficial because in addition to providing a mechanism for growth factor delivery, it would also provide contact guidance to the regenerating axons. However, these results demonstrate that this would be at the expense of a slower degradation profile and therefore the likelihood of the alginate again behaving as a barrier and physically impeding the diffusion of critical molecules, the migration of cells or the elongation of axons. This is in keeping with the observations of Terada *et al* who demonstrated that when resorbable filaments were used to bridge a 10 mm nerve gap, improved axonal regeneration occurred compared to the use of non-resorbable filaments (Terada *et al.* 1997a;Terada *et al.* 1997b;Terada *et al.* 1997c).

Hence for alginate fibres to have a possible application further work is needed. Ultra pure LVM fibres with an accelerated and predefined resorption profile need to be studied. This type of alginate fibre may be present long enough to provide contact guidance and possible delivery of growth factors, but also degrades sufficiently quickly so as to provide a path of low resistance to the regenerating front. In addition the quantity of fibres loaded into the conduit requires optimisation such that suitably sized channels are present between the alginate fibres to accommodate and promote regeneration.

CHAPTER 7

GENERAL DISCUSSION

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Peripheral nerve injuries remain a major cause of functional morbidity since they are common, involve major nerve trunks in ~35% of cases (Allan 2000), and their clinical outcome remains disappointingly poor (Allan 2000). Despite major advances in surgical technique outcome has not dramatically improved over the last 25 years (Lundborg 2000). Hence there is a need to shift focus away from surgical technique and towards therapeutic modulation of the neurobiological events integral to peripheral nerve regeneration

In the quest to identify an alternative method for peripheral nerve repair, encouraging results were obtained in studies using PHB as an alternative to direct epineural nerve repair in the cat superficial radial nerve model (Hazari *et al.* 1999b). As a result of this work a multicentre prospective randomised control clinical trial has been recently established by our unit comparing the use of PHB as a wrap around repair with the current gold standard of primary nerve repair for median and or ulnar nerve injuries at the level of the wrist. In addition, PHB has been successfully used as a nerve conduit in the repair of short gaps in the rat sciatic nerve model (Hazari *et al.* 1999a). When used to bridge long nerve gaps of up to 4cm in the rabbit common peroneal nerve model, it supported spontaneous axonal regeneration into the distal stump by 42 days but did not result in functional target organ reinnervation in the long term (Young *et al.* 2002).

SC are pivotal to peripheral nerve regeneration (Hall 1986a) and in effect become indigenous neurotrophic factories after peripheral nerve injury producing a host of neurotrophic agents (Fu & Gordon 1997; Hall 1997; Terenghi 1999). The key to the mutually beneficial axon-SC interaction during regeneration is the action of GGF released by regenerating axons (Fu & Gordon 1997; Li *et al.* 1997). Therefore instead of administering individual neurotrophic factors, the function of the endogenous SC population can be enhanced by GGF resulting in indirect promotion of axonal regeneration in a more physiological manner. Therefore we manipulated the microenvironment by delivering GGF in an alginate hydrogel matrix, thereby increasing the rate and quantity of regeneration through the PHB conduits and ultimately improving functional target organ reinnervation.

Chapter 3 demonstrated that GGF significantly increased the quantity of SC and axonal regeneration over both short and long gaps when compared to empty and alginate filled conduits. This effect was most dramatic at 21 days although it was sustained upto 63 days. In addition the axonal regeneration distance in the GGF conduits was significantly greater than controls for both gap lengths at 21 days, which may provide further evidence that the peak of GGF release occurs in the first 3 weeks. It would be interesting to carry out further studies to analyse the release profile of GGF from alginate *in vivo* both with respect to the rate and duration of release and correlate this with the enhancement of regeneration that was observed at particular time points.

Despite the axonal regeneration distance being significantly greater in the PHB-GGF conduits for both gap lengths at 21 days compared to the controls, by 42 days the empty conduits have supported axonal bridging of both gap lengths whilst the GGF conduits have not. What has resulted in the sudden acceleration in axonal regeneration in these empty conduits? Possibly dissociation of PHB fibres following hydration and their resultant migration into the conduit lumen provides contact guidance and the presence of alginate in the GGF tubes may be impeding this. Perhaps the fact that SC tend to bridge nerve gaps ahead of axons results in accelerated axonal regeneration, by the provision of a continuous scaffold and chemical messengers. These questions maybe better answered by repeating the experiments and assessing regeneration distance in the conduits at a time point midway between 21 and 42 days.

Optimal functional recovery relies on a maximum number of regenerating axons reaching the distal stump in the fastest time before resident SC have undergone atrophy and phenotypical changes combined with endoneurial fibrosis (Li *et al.* 1997). In addition the regenerating axons must demonstrate fascicular, target and topographical specificity. In chapter 4 it became evident that the trophic effect of GGF on SC and the glial induced enhancement of axonal regeneration was not limited to the short-term time points, but was sustained upto 120 days, with the GGF conduits supporting a significantly greater quantity of axonal and SC regeneration compared to controls. Possibly of greater importance was the observation that the quantity of axonal regeneration at 120 vs 63 days was not significantly different in the GGF conduits whereas it was significantly less in the controls. In the GGF conduits this resulted in an

improvement of motor organ reinnervation demonstrated by significantly less muscle atrophy. Despite the GGF conduits having a slower rate of axonal regeneration compared to the empty conduits at 42 and 63 days for both gap lengths this did not appear to be detrimental to GGFs long-term effects. Possibly, in the GGF group the SC maintain the phenotypic expression, which renders them conducive and responsive to regeneration for a longer period. In addition the presence of GGF maybe preventing the down regulation of c-erbB2 and B4 receptors on the SC in the distal stump (Li *et al.* 1997), which permits them to support neurite ingrowth even if it occurs at a later time point. All these factors in combination may increase endogenous neurotrophic support and thereby improve axonal survival and maturation. More evidence may be provided for these theories by studying the phenotypic expression of the SC in the distal stump and the c-erbB2 and B4 receptors on these SC at 120 days in all 3 conduit groups.

A constant observation for both the short and long–term studies was that the presence of alginate in the conduits resulted in regeneration inferior to the empty tubes for both gap lengths and all time points, clearly implying that it maybe damping down the maximal effects of GGF as well as the endogenous neurotropic effects of the distal stump. Similar observations were made by Valentini *et al.* who demonstrated that semipermeable conduits loaded with collagen and laminin gels impeded peripheral nerve regeneration, by possibly preventing diffusion of critical molecules from the distal stump and migration of cells from the external environment (Valentini *et al.* 1987).

To overcome these problems further studies were undertaken to potentially identify novel methods of growth factor delivery. Further work was carried out using alginate for a number of reasons. Firstly, previous workers have successfully used alginate *in vivo* for the controlled-release of various neurotrophic factors (Austin *et al.* 1997; Maysinger *et al.* 1996a). Secondly, the composition and configuration of alginate can be manipulated, giving scope for the identification of a more appropriate matrix. Lastly, alginate has been extensively used clinically for the microencapsulation and transplantation of cells and has been shown to be clinically compatible.

Therefore an alternative configuration of the alginate matrix was studied to improve the regenerative potential of the bioengineered nerve conduit. Alginate fibres of varying mannuronic and guluronic acid compositions were considered and it was hypothesised that they could ideally provide both contact guidance as well as a means for growth factor delivery. However, the *in vivo* observations were very disappointing, as the alginate fibres appeared to repel SC and axonal regeneration from the proximal stump producing results inferior to the alginate hydrogel. Previous workers have demonstrated that alginate beads are a successful mode of growth factor delivery *in vivo* (Austin *et al.* 1997; Ko *et al.* 1995; Smidsrod & Skjak-Braek 1990). Currently work is being undertaken in our laboratories looking at the possibility of using alginate hydrogel beads of varying compositions as a mode of GGF delivery. Alginate microbeads of 400μm diameter loaded with GGF are being manufactured using a co-axial flow bead generator. *In vitro* studies are being carried out initially to look at the release profile of GGF from the beads. These will be followed by *in vivo* work using PHB conduits loaded with

GGF-alginate microbeads for the repair of nerve gaps in the rat sciatic nerve model. In addition work is underway to assess the potential of dissociated PHB fibres as a delivery vehicle. Firstly their ability to support neurite outgrowth *in vitro* from chick embryo DRG is being assessed followed by *in vivo* studies to assess their potential to enhance nerve regeneration when loaded into PHB conduits with and without GGF. If this proves to be successful it maybe possible to further promote regeneration by coating the fibres with components of the extracellular matrix such as laminin, collagen and fibronectin.

In addition to studying alternative configurations and compositions of alginate for a suitable mode of growth factor delivery, PHAs were also evaluated. These polymers have the potential to bind growth factors and release them at a controlled rate, thereby providing a mode of delivery directly from the conduit wall. Like alginate PHA has been extensively used clinically for a number of medical applications, thereby making it also clinically compatible. The PHA conduits supported axonal regeneration *in vivo* at a rate comparable to nerve autografts but resulted in a quantity of regeneration which was superior. The success of the PHA material as a conduit appeared to be related to pore size, with the larger pore sizes supporting the greatest progression of axonal regeneration with time. Therefore PHA presents an exciting possibility both as a material for nerve conduit formation and growth factor delivery as it is a biologically compatible biopolymer which can undergo a number of manufacturing processes in order to produce a form and structure that is suitable for the desired application.

Collaborative work is presently ongoing between our laboratories and the manufacturers of PHA (Tepha) to identify the optimal pore size of this polymer, which results in maximal peripheral nerve regeneration. In addition modifications to the biomaterial are being undertaken to produce a bioactive polymer with alterations in the functional groups allowing growth factors to be attached to the PHA (Cannizzaro *et al.* 1998; Shakesheff *et al.* 1998). This will permit the controlled release of bioactive growth factors from conduit wall, into the regenerative milieu thereby enhancing axonal regeneration. In addition, this tissue-engineered scaffold could have the potential to be seeded with cultured SC, which are known to enhance nerve regeneration (Mosahebi *et al.* 2002). For clinical applications, longer gap lengths may need to be bridged, and the chemical and mechanical properties of these modified scaffolds would provide adequate support for the regenerating and maturing axons, lasting a sufficiently long period of time.

In conclusion these studies have demonstrated that by modifying the conduit microenvironment through the addition of GGF, peripheral nerve regeneration could be enhanced resulting in an improvement of motor organ reinnervation. However, the need for a more successful and clinically applicable mode of growth factor delivery has been highlighted and many new strategies are currently being evaluated which may hold the answer and bring us closer to our goal of a clinically useful bioartificial nerve graft.

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APPENDIX

GENERAL LABORATORY PROTOCOLS

Silanisation

Growth factors tend to adhere to surfaces by electrostatic interaction, all glassware and plasticware including polypropylene pipette tips and eppendorfs for handling and storage were silanised to minimise adsorption. The silanisation was carried out by a published method (Molecular Cloning: A Laboratory Manual, eds: Sambrook, Fritsch and Manniatis).

Put 1ml of dichlorodimethylsilane in a small bowl inside a large dessicator with the items to be silanised.

In an extraction hood attach a vacuum pump to the dessicator and apply suction until the dichlorodimethylsilane starts to boil then clamp and switch off the pump.

Leave for two hours and open the dessicator under an extraction hood.

Rinse the items three times with distilled water and dry overnight.

Sterilise by gamma irradiation.

Vectabond coated slides

The slides were treated to enhance adhesion of sections and prevent loss of tissue as a result of washes required for immunostaining procedures.

Place clean slides in metal slide racks.

Immerse slides in acetone for 5 min, then remove and drain well.

Prepare Vectabond™ reagent treatment solution by adding 7ml of reagent to 350ml acetone and mix well.

Immerse slides in Vectabond™ reagent treatment solution for 5 min, then remove and drain well.

Rinse slides by dipping rack several times into distilled water over 30 seconds. Avoid the creation of bubbles during this process.

Tap slide rack before drying to remove excess water droplets.

Dry slides at 37°C.

GENERAL LABORATORY SOLUTIONS

Antibody diluent

0.03% Triton X-100

0.1% bovine serum albumin (BSA)

0.1% sodium azide

For 100ml

Dissolve the following in 100ml of PBS:

BSA	0.1g
Sodium azide	0.1g
Triton X- 100	30µl

Glutaraldehyde

20mls of 20% Paraformaldhyde + 16mls of 25% Glutaraldehyde + 59mls of Phosphate buffered saline.

0.05% Hydrogen Peroxide

Mix 0.5ml of Hydrogen peroxide (30% Hydrogen peroxide, BDH Laboratories, UK) with 299.5ml of distilled water and stir well.

Phosphate buffered saline (PBS)

For 10 litres

Dissolve the following in 5 litres of distilled water:

NaCl	87.9g
KH_2PO_4	2.72g
$Na_2HP0_4 (H_20)_{12}$	3.9g
or Na_2HP0_4	11.35g

Stir and leave for a few hours, then add another 5 litres of distilled water.

Check pH and adjust to pH 7.3.

Mix and allow to stand for at least 8 hours.

0.1M Sodium Acetate

Dissolve 82.03g of sodium acetate in 700ml of distilled water.

Make up solution to 1 litre and adjust pH to 6 using NaOH or Acetic acid.

Sucrose-PBS

15% Sucrose + 0.1% Sodium azide in PBS

For 1 litre

Dissolve the following in 700ml of PBS:

Sucrose 150g

Sodium azide 1g

When dissolved make up to 1 litre.

Triton X-PBS

0.2% Triton X-100

Mix 2ml with 1 litre of PBS.

Zamboni's solution

85ml of 2% Paraformaldehyde in PBS

15ml of saturated Picric acid

Dissolve 20g of Paraformaldehyde in 800ml of PBS at 60°C max and stir until solution is clear.

Adjust volume to 1 litre and allow to cool.

Add appropriate amount of saturated Picric acid and store at 4°C until use.

STAINING

Heamatoxylin and Eosin Staining

Place slide in haematoxylin for 30 seconds.

Wash the sections in running tap water for 5-10 mins to 'blue' the haematoxylin.

Place the slides in 0.15% eosin for 6 minutes.

Wash the sections in tap water until water runs clear.

Dehydrate the sections by placing them first in 70% and then 100% ethanol (1 minute each step).

Clear the section by immersion in Xylene, 1 minute and then mount in DPX.

Indirect ABC Peroxidase Nickel Enhancement Technique

Day 1

Thaw tissue sections at room temperature for at least 2 hours if previously frozen for storage.

Dilute primary antibodies in antibody diluent and store on ice until required.

Draw around sections with DAKO pen to avoid the antisera spreading beyond the tissue on the slide.

Permeabilize the tissue by placing in Triton X/PBS for 1 hour.

Rinse briefly in PBS.

Inhibit endogenous peroxidase by placing slides in H_2O_2/PBS for 20 minutes (299.5ml PBS + 0.5ml 30% vol. H_2O_2).

Wash slides in PBS 3 x 5 minutes

Block background by applying to the sections normal horse serum (for monoclonal antibodies) or normal goat serum (for polyclonal antibodies) dilution 1:100 in PBS, and incubate at room temperature for 15 minutes in humid chamber.

Drain slides (do not rinse).

Apply diluted primary antibodies to the sections.

Incubate overnight at 4°C in humid chamber

Day 2

Dilute secondary biotinylated antibodies in horse anti-mouse for monoclonal or goat anti-rabbit for polyclonal antibodies (dilution 1:100 in PBS).

Wash sections in Tween/PBS (1:4000) 2 x 30 seconds.

Wash sections in PBS 3 x 3 minutes

Apply biotinylated antibodies to the sections.

Incubate for 1 hour at room temperature in humid chamber.

Make up ABC complex by diluting 5µl of solution A and 5µl of solution B (Vectastain® ABC kit, Vector Laboratories, UK) in 990µl of PBS. Adjust volumes according to the number of slides.

Mix and leave on ice for no more than 30 minutes.

Wash the sections in Tween/PBS for 30 seconds x 2.

Wash in PBS 3 x 3 minutes.

Apply diluted ABC complex to the sections.

Incubate for 1 hour at room temperature in humid chamber.

Mean while prepare the DAB solution by dissolving 7.25g ammonium nickel sulphate in 300ml of 0.1M sodium acetate. Filter the diluted DAB (5ml solution=125mg) into ammonium nickel sulphate solution. Add 600mg α D-glucose and mix.

Wash the sections in Tween/PBS 2 x 30 seconds.

Wash in PBS 3 x 3 minutes.

Wash in 0.1M sodium acetate 2 x 5 minutes.

Activate the DAB solution by adding 6mg glucose oxidase and mix.

Immerse the slides in the DAB solution for 4-6 minutes (check under microscope for blue/black staining keeping rest of slides in PBS, then place all slides back in DAB to stain further, if necessary).

Wash the sections in 0.1M sodium acetate 2 x 5 minutes then wash in PBS 2 x 5 minutes.

Dehydrate the sections in ascending concentrations of ethanol (30%, 70%, 100%, 100%) 3 minutes for each step.

Clear in Xylene for at least 1 minute.

Mount sections in DPX and allow to dry.

ELECTRON MICROSCOPY PROTOCOL

Processing and sectioning

The specimens were fixed in 1% paraformaldehyde/1.5% glutaraldehyde in phosphate buffer overnight and were then processed using a Lynx tissue processor.

The processing schedule was as follows:

60 minutes PBS + 2% sucrose

12 hours osmium tetroxide + 3% sodium iodide

10 minutes 15 % ethanol

20 minutes 30% ethanol

30 minutes 50% ethanol

60 minutes 70 % ethanol

3 hours 100% ethanol

5 hours 50/50 Lemix resin ethanol mixture

7 hours 75/25 Lemix resin ethanol mixture

The specimens were then infiltrated with 100% Lemix resin for a minimum of 48 hours and were finally embedded in fresh Lemix Resin and polymerised at 700°C overnight.

Semithin (1μm) sections were then cut using glass knives on a Reichert-Jung ultracut microtome, collected on glass microscope slides and left on a hotplate for approximately

one hour. The slides were then flamed with an alcohol lamp before staining to assist the section in adhering to the glass slide.

Ultrathin sections were cut using a diamond knife (Diatome) and collected on 200HS, 3.05mm copper grids (Gilder). The ultrathin sections were stained with saturated uranyl acetate in 50% ethanol (TAAB) for 5 minutes followed by Reynold's lead citrate, also for 5 minutes.

The ultrathin sections were viewed and photographed using a Philips 201 transmission electron microscope or Philips CM 120 TEM.

Humphrey's Stain

Place the slide on a hotplate (80-90°C) and cover with Methylene blue/Azure II (diluted in PBS 1:400) for 10 minutes. The staining solution is topped up as necessary to avoid drying of the sections.

Wash the sections in distilled water and then blot dry.

Immerse the sections in Basic fuchsin (diluted in distilled water 1:400) for 1 minute.

Wash in distilled water and then blot dry

Air dry the sections and mount in DPX