SYNTHETIC CONDUITS AND GROWTH FACTORS FOR IMPROVED PERIPHERAL NERVE REGENERATION

by

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ABSTRACT

Following trauma and loss of peripheral nerve tissue, an autologous nerve graft is the current gold standard for surgical repair. The alternative use of synthetic conduits has not yet offered suitable materials for wide clinical application. Two novel materials, poly-3-hydroxybutyrate (PHB) and polydioxanone (PDS), are bioresorbable and potentially useful as nerve conduits. The aim of this study was to examine peripheral nerve regeneration in vivo following grafting of PHB or PDS conduits, either alone or in combination with glial growth factor (GGF). Schwann cells are essential for nerve regeneration. GGF delivery to the injured nerve may increase their number within the graft and, indirectly enhance nerve regeneration. Nerve regeneration at the repair site was assessed by quantitative immunohistochemistry up to 1 year post-operatively. Centrally, primary sensory neuron survival and peripherally, target organ reinnervation were also studied morphologically following injury and repair.

The use of PHB wrap-around for primary nerve repair was compared with direct epineural suture in the cat superficial radial nerve model. The two methods showed comparable values of axonal counts, diameter, myelin thickness and g-ratio. The results demonstrated that PHB wrap-around is a suitable alternative to epineural suture in primary repair. PHB tubes tested for nerve gap repair in the rat sciatic nerve model exhibited axonal regeneration comparable to that in nerve autografts. GGF administration in PHB tubes produced progressive increase of axonal growth with time, secondary to GGF-induced Schwann cell proliferation. PDS tubes also supported axonal regeneration, although these presented problems of material fragmentation and intense inflammatory reaction. Microgeometry of the internal surface of PDS tubes did not significantly improve nerve regeneration, peripheral target organ reinnervation or sensory neuron survival.

The results of this study demonstrated PHB and PDS conduits sustain successful peripheral nerve regeneration, which can be further improved by targeted administration of growth factor.
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1.1 PERIPHERAL NERVE REPAIR

Inspite of advances in the field of peripheral nerve surgery using microsurgical techniques, the functional outcome is not always optimal. The odds are compounded when there is an irreducible gap between the nerve stumps. In clinical practice today, an autologous nerve graft is considered the surgical gold standard to bridge such gaps. The quest for the ideal material for a synthetic conduit has been on for more than a 100 years. Two new potential candidates for the ideal conduit material are polydioxanone (PDS) and poly-3-hydroxybutyrate (PHB). PDS has been widely used as a suture material in gastrointestinal and vascular surgery. PHB is a natural storage product of bacteria and occurs as discrete granules within the cell cytoplasm. As the concepts of 'trophism' and 'tropism' have evolved, several growth factors acting directly or indirectly on nerve regeneration have been identified. Schwann cells share an intimate relationship with neurons and are essential for their regeneration. An increase in Schwann cell numbers would be beneficial to axonal regeneration. They are directly affected by glial growth factor (GGF) and it could be hypothesised that delivery of GGF in a synthetic conduit would indirectly enhance nerve regeneration.

This study was carried out to examine the possibility of improving nerve regeneration by using synthetic nerve conduits, either alone or in combination with growth factors, namely GGF.

1.1.1 Historical aspects

The past history of peripheral nerve surgery to its present day status has been chequered, reflecting the progress of various doctrines through time. In the Hippocratic era, no distinctions were made between tendons and nerves and the belief that a severed nerve would not unite (Hippocrates, 1849, translation by F. Adams) held sway for
centuries to come. Certain beliefs, which now in retrospect seem more superstitious than the truth, were arduously believed, an example being that nerve wounds produce spasm and result in cruel death (Browne, 1951). After the Alexandrian School, Rufus distinguished nerves from tendons and described the motor and sensory functions of nerves (Browne, 1951). However, surgeons still hesitated to operate directly on nerves. It was proposed by Galen that a wounded nerve in continuity with the brain would induce convulsions and this led Oribasius to advise division of nerves to stop convulsions (Browne, 1951).

By the seventh century, new concepts were sporadically mentioned, some nerve wounds were sewed as advocated by Paul of Aegina, but whether direct coaptation was carried out is unclear. It is only in the thirteenth century, that direct nerve suture came to be practised, initially advocated by Giovanni of Saliceto, a professor at Bologna Medical School and then practised more widely by his pupils, the most well-known amongst them being Lanfrank (Lane, 1890; Browne, 1951). More general concepts of healing were applied by Guy de Chauliac consisting of removal of foreign bodies, apposition of the severed parts, preservation of the substance of the part and suture of the nerve in cases of severance (Browne, 1951). However, the first detailed description of a technique for nerve suture has to be credited to Gabriele Ferrara in the seventeenth century, whereby he directly coapted the ends with an aga needle bearing split tortoise tendons pre-soaked in hot red wine (Browne, 1951). None of these teachings were widely accepted, and only in the mid-nineteenth century were more serious attempts made.

At this time, knowledge regarding peripheral nerve regeneration was controversial. Two main schools of thought existed. The theory of continuity or monogeny, proposed by a
group of eminent histologists such as Waller, His, Ranvier, Vanlair and Stroebe believed that fibres of the peripheral stump of the sectioned nerve degenerate and die, and that the production of new fibres is a function of the central stump which maintains nutritive solidarity with the neurones in the spinal cord (Ramón Y Cajal, 1928). A group of physiologists based their opposed theory of discontinuity, or polygeny, on findings acquired from electrical stimulation experiments rather than micrographic observations and histological interpretation. The spirited champions of this doctrine were Philippeaux, Vulpian, Schiffe and Bethe and they claimed that the peripheral severed ends of nerves were able to regenerate without the help of the cells of origin, independent of the central stump, calling it ‘autogenous regeneration’ (Ramón Y Cajal, 1928).

This confusion in knowledge was reflected in the practise of peripheral nerve surgery at that time. The first documented success of the indirect nerve suture is by Von Lagenbeck in 1854, who repaired a median nerve injury with silk sutures and reported it a year later as having gained full functional recovery (Browne, 1951). The ‘centre suture’ technique was originated by Vulpian wherein a single linen thread was passed from the periphery of one nerve segment, through the centre of its nerve substance, and carried through the other nerve segment in a similar fashion resulting in apposition of some degree (Browne, 1951). Following a series of animal experiments, direct suture of divided nerves gained more rapid and widespread acceptance (Mitchell, 1872). In some cases, nerve ends were cut obliquely to increase the surface areas in contact (Markoe, 1885). Several variations in techniques then developed such as Rawa’s co-apted suture, the nerve ends being bound together side by side (Browne, 1951), Létiévant’s nerve flap (Stookey, 1919), wedge techniques of bulb suture and side to side nerve suture (Browne, 1951). This was followed by excision of contused nerve ends and the suture
of clean cut surfaces, provided the excision did not involve more than an inch of the nerve (Richardson, 1886).

With World War I, emphasis was placed on accurate coaptation of freshened nerve ends and it was recognised that a definite intra-neural funicular anatomy existed in peripheral nerves (Browne, 1951). It became accepted practise to mark corresponding points on the circumference of both the proximal and distal ends prior to resection and suture of a neuroma in continuity in order to minimise rotation. Inspite of this newfound knowledge, repair techniques did not improve due to lack of appropriate non-reactive suture materials, inadequate magnification and unavailability of microsurgical instruments.

1.1.2 Primary repair

Already in the seventh century, Paul of Aegina used agglutination to repair nerves and six centuries later, Roger of Parma used egg albumin to join severed nerves. A revival of interest in the sutureless technique of primary repair took place in the 20th century. Clotted plasma was used to secure union between the ends of a nerve graft in the facial canal and its use was advocated in the repair of intratemporal facial nerve injuries (Balance and Duel, 1932). Fibrin and fibrinogen clotting was also attempted (Young and Medawar, 1940) but was found to be of no value in repair under tension. A modification of the plasma clot technique using a tantalum sling suture to relieve tension at the nerve ends was then carried out (Tarlov, 1944). The use of cyanoacrylate glue was described in 1965 (Ferlic and Goldner, 1965). Although results following all such repairs were comparable to that of current microsurgical techniques, the use of adhesives to secure anastomosis failed due to poor tensile strength of the union (Millesi, 1984; Fields et al., 1989). Research into sutureless techniques is still continuing with the
use of carbon dioxide lasers to weld nerve tissue at the sites of injury (Richmond, 1986; Cerullo, 1988).

Through the centuries almost every material, ranging from the logical to the bizarre, has been applied as a suture material, including metallic sutures, silk, hair, tantalum and catgut (Baudens, 1836; von Lagenbeck, 1876; Browne, 1951). Catgut, popular in the 19th century, was shown in histological studies to result in a marked fibroblastic reaction which impeded the regeneration of nerve fibres (Sargent and Greenfield, 1919; Guttman, 1943).

With the advent of the operating microscope (Kurze, 1964; Smith, 1964), high magnification of the operating field, microsurgical techniques and fine suture materials allowed for the first time precise manipulation, dissection and coaptation of severed nerves with minimal surgical trauma. Fine and non-reactive synthetic suture materials such as 9(0) and 10(0) ethilon became available and are now routinely used in microsurgery. The anatomical differentiation of nerves according to their fascicular structure initiated the controversy between epineural and fascicular repair which to date has not been resolved. Millesi described four basic patterns: the monofascicular nerve where the nerve consists of one large fascicle, the oligofascicular nerve consisting of several large fascicles, the polyfascicular nerve segment without group arrangement in which the segment consists of several small fascicles without further arrangement and finally, the polyfascicular nerve segment with group arrangement where the nerve consists of many small fascicles of different sizes arranged in groups. Proponents of each type of repair have attempted to demonstrate the superiority of one type of repair over the other citing both experimental (Bora, 1967; Bora et al., 1976; Chabaud et al., 1976; Levinthal et al., 1977; Orgel and Terzis, 1977; Kline et al., 1981) and clinical
studies (Edsage, 1964; Hakstian, 1973; Lilla et al., 1979; Kutz et al., 1981; Snyder, 1981). Though Brushart demonstrated improved muscle reinnervation after fascicular repair in the rat sciatic nerve (Brushart and Mesulam, 1980; Brushart et al., 1980; Brushart et al., 1981), experimental studies in primates have shown no advantages in either of the techniques (Grabb et al., 1970; Chabaud et al., 1980). To date, no randomised study with adequate pre- and post-operative clinical and electrophysiologic testing parameters comparing the two techniques has been carried out. There is no real conclusive experimental or clinical evidence to suggest that one technique is better than the other. However, the general consensus is that both techniques should be used in the repair of severed nerves depending on the nature of the injury, location in the extremity, the timing of repair and the fascicular arrangement in the nerve to be repaired (Terzis & Smith, 1990).

1.1.3 Nerve grafting

Early in the history of peripheral nerve surgery, procedures were devised to overcome the problem of a gap between the ends of a severed nerve. The proximal and distal nerve stumps were stretched until approximation was established (Richardson, 1886). Nerve stretching was combined with limb positioning (Browne, 1951) inspite of the danger that it would cause interstitial haemorrhages and subsequent scarring (Stookey, 1922). Other methods predominantly practised during the World Wars included the shortening of bones with concomitant bony injuries (Browne, 1951), and combinations of radical dissection, gradual stretching and flexion or extension of neighbouring joints (Bristow, 1947). Browne, in his review of neurological surgery in 1951, classed surgical procedures to overcome large defects as nerve implants; Létiévant’s nerve flaps; Assaky’s suture à distance; Fluorens’s nerve crossing technique and finally tubulization (cf.1.3) (Browne, 1951). The first successful nerve
graft was carried out by Philipeaux and Vulpian (1870) in the hypoglossal nerve of dogs (Browne, 1951). The first nerve transplant in man is credited to Albert (1878), when he resected a sarcoma from the median nerve and repaired the defect with a nerve segment taken from an amputated limb (Browne, 1951). The first successful nerve graft in man was performed by Mayo Robson, when the posterior tibial nerve from a simultaneously amputated limb was used to bridge a 6 cm gap in the median nerve after removal of a tumour (Robson, 1889). From a series of 21 experiments resulting in 279 nerve grafts, it was concluded that the most favourable results were obtained with the use of autografts (Huber, 1919).

At this time, heterografts (xenografts), homografts (allografts) and autografts were used with indifference. Though heterogenous and homogenous grafts united, these always degenerated at a later stage (Verga, 1918). Failure of heterogenous transplants was attributed to necrosis of the transplanted portion (Ingebrigsten, 1916). Following World War I, nerve grafting fell into disrepute, and the widely held opinion that it should be used only as a last resort. However, between the wars, the procedure by Balance and Duel of grafting the facial nerve in the middle ear was generally accepted as standard. Good results were also reported for grafting of injured digital nerves (Bunnell, 1927).

Following a series of elaborate experiments in animals, a group of zoologists at Oxford, Young, Medawar, Sanders and Holmes, reached the conclusion that heterografts behaved as foreign bodies and were therefore completely useless, that homografts though capable of regeneration resulted in a brisk cellular reaction, and that autografts gave the most satisfactory results (Sanders, 1942). Encouraged by these results, Seddon set about grafting nerve gaps where no alternative form of repair was possible. Autografts were the first choice, although in 4 cases, homologous grafts were used due
to insufficient autograft material. The homologous grafts were a failure (Seddon & Holmes, 1944). Medawar's work, which ultimately earned him the Nobel prize, provided the explanation as the immune reaction provoked by the transplanted graft tissue destroyed it before the out-growing axons could reach the distal stump.

Inspite of Seddon's promising results, nerve grafting did not gain wide acceptance (Smith, 1966; Sunderland, 1968; Omer, 1974) until the introduction of microsurgical technique. Work pioneered by Hanno Millesi brought credibility to this technique (Millesi, 1977). Following his work, several reports appeared indicating satisfactory results (Millesi et al., 1976; Salvi, 1973; Tallis et al., 1978; Brunelli and Brunelli-Monini, 1979; Haase et al., 1980). Certain guidelines were laid down. Tension at the suture line in direct repair should be avoided and emphasis was placed on the need to graft in such cases. The importance in distinguishing between a nerve defect and a nerve gap was stressed, the former being the amount of actual nerve tissue that is lost whereas a gap refers to the distance between the two nerve ends (Millesi, 1987). If the two nerve stumps can be approximated easily after slight mobilisation without flexion of the adjacent joints, an end-to-end repair should be carried out. In all other situations, a nerve graft should be used (Millesi, 1982).

1.2 PATHOPHYSIOLOGY OF INJURY AND REGENERATION

Although results after surgical repair of severed nerves have improved with advances in microsurgical technique and a better understanding of the pathology involved, the return of function is never to the pre-injury level. In the last decade, there has been a shift of interest from surgical repair to the events that occur at a molecular and cellular level at the site of injury, the target organs and, centrally, in the dorsal root ganglia.
1.2.1 Nerve injury

Peripheral nerve pathology depends on the severity of injury and has lead to classifications based on disruption of the internal structures in the nerve (Seddon, 1948; Sunderland, 1978). Neurapraxia is the mildest form of injury resulting in a localised conduction block with maintenance of axonal continuity, with rapid and complete recovery. Axonotmesis shows disruption of axons associated with distal axonal degeneration but prognosis is good due to the continuity of the connective tissue. Neurotmesis is the most severe with complete severance of the nerve and lack of recovery without some form of surgical repair (Seddon, 1948). The classification devised by Sunderland (1978) is similar; however the status of the supporting structures is taken into account and the categories are expanded to five.

Nerve injury is characterised by a well defined series of events (Seckel, 1990; Terzis and Smith, 1990; Wong and Crumley, 1995). With disruption of the nerve structure and if neuronal death does not occur, changes reflecting the shift in the metabolism from maintenance of conduction to regeneration start to take place. Axoplasm begins to seep out of the cut ends, resulting in loss of turgor, collapse of axonal membranes and finally retraction of the severed stumps (Wong & Crumley, 1995). Calcium and sodium ions enter the axoplasm, activating various proteases and free radicals. Potassium ions and proteins leak out, and the injury has been compared to a 'chemical burn' (Medinaceli and Seaber, 1989). This rapid destruction extends to one or two internodes proximally and to the distal stump tip (de Koning et al., 1989). Macrophages invade the site of injury, actively scavenge the debris and degrade the distal stump (Perry and Brown, 1992a, b). Besides this phagocytic role, macrophages secrete mitogens and growth factors (cf. 1.4) which influence axonal regeneration. There is also secretion of factors
that aid the proliferation of Schwann cells and therefore indirectly influence regeneration and remyelination (Ignatius et al., 1987; Boyles et al., 1989).

In the distal stump, Wallerian degeneration follows 48 to 96 hours after the initial rapid damage in the distal stump (Dyck et al., 1984). This is characterised by degradation of myelin and proliferation of Schwann cells, which phagocytose the myelin debris leaving behind empty basal lamina tubes. The remainder of the proliferating Schwann cells line these tubes longitudinally to form the bands of Büngner (Sunderland and Bradley, 1952; Nathaniel and Pease, 1963; Thomas, 1963). Failure of regenerating axons to penetrate these tubes results in narrowing of the tubes by as much as 80% with concomitant deposition of collagen along both surfaces of the basal laminae.

In the neuronal body, the nucleus swells and is displaced to the periphery. The rough endoplasmic reticulum disintegrates and is marginated to form Nissl’s granules. This histological appearance is called chromatolysis, and it is followed by an increase in the synthesis of cytoskeletal proteins such as actin, tubulin and neurofilaments (Grafstein and McQuarrie, 1978). These proteins are transported by slow axonal transport at the rate of 5 - 6 mm per day, which indirectly correlates to the rate of axonal regeneration. Other growth-associated proteins such as GAP-43, an important component of growth cone membranes, are transported by fast axonal transport at 400 mm / day (Maier and McQuarrie, 1990).

1.2.2 Axonal regeneration

If cell death does not occur, regenerative changes commence as early as 24 hours after nerve injury. The tip of the proximal stump sprouts and the axon elongates through a process mediated by the growth cone, which has mobile filopodia extruding from a
flattened sheet of lamellipodia (Seckel, 1990). The growth cone changes form continuously by the extension and retraction of filopodia and lamellipodia (Harrison, 1990). Once in the bands of Büngner, it advances by contact guidance mediated by the filopodia responding to cues from laminin and fibronectin, both of which are components of Schwann cell basal laminae tubes (Kapfhammer and Raper, 1987; Chien et al., 1993). Growth cones also respond to tropic and trophic molecules which direct and sustain the advance of regenerating axons towards the target organ (Gundersen and Barrett, 1980). Generally each injured axon sprouts several regrowing fibres but only one sprout matures on establishing contact with a target organ while the other sprouts ‘die back’ and degenerate (Spencer, 1977; Bunge and Bunge, 1984). Regeneration of axons is a precise process with specific selection of pathways and is not a random process (Chien et al., 1993).

Following Wallerian degeneration, the behaviour of Schwann cells is dictated by the presence of regenerating axons, which stimulates a second phase of proliferation, accompanied by differentiation and production of myelin. The degree of remyelination is determined by the type of axon regenerating into the basal lamina (Hillarp and Olivecrona, 1946; Weinberg and Spencer, 1975). Axons and Schwann cells have a close interaction during regeneration, particularly as Schwann cells also provide a supportive and growth-promoting environment for the regenerating axons. Furthermore, without the presence of Schwann cells, regeneration is severely impaired (Hall, 1986, 1997).

1.2.3 Target organ reinnervation

Following axotomy, denervation leads to atrophy of target motor units and sensory end organs. After two years, if no adjunctive therapies such as physiotherapy and electrical stimulation are carried out, skeletal muscle is probably beyond satisfactory functional
recovery (Ducker, 1972). In a denervated muscle, the entire muscle membrane is responsive to acetylcholine in contrast with a normal muscle where only the motor endplates are excitable. Regenerating axons selectively reinnervate old motor endplates (Grinell, 1988). This is possibly due to a neurotropic influence exerted by the motor endplates on regenerating axons (Rosenthal, 1977; Brown et al., 1981). After transection of a mixed nerve, regenerating motor axons preferentially innervate distal motor branches, a process known as ‘preferential motor reinnervation’ (PMR) and though a motor axon may enter both sensory and motor endoneurial tubes in the distal stump, specificity of motor to motor is maintained by the pruning of collaterals from sensory pathways (Brushart, 1988, 1993).

Muscle fibres within an individual skeletal muscle do not exist as a homogenous population, but histochemically exhibit a ‘mosaic’ pattern with a heterogeneous mixture of slow and fast contracting muscle fibres (Bar and Pette, 1988). Muscle fibre phenotype is not an intrinsic property of the muscle fibre but is conferred upon the muscle by its innervating nerve. Changing the innervation alters the phenotype (Salmons and Sreter, 1975). Following denervation and reinnervation of a muscle, there is loss of the normal mosaic pattern distribution and the reinnervated fibres become grouped together (Karpatic and Engel, 1966; Sterne et al., 1997b), showing clusters of commonly innervated fibres and smaller motor units (Mira, 1987). The change in proportions of the fibre type subsequently is dictated by the reinnervating nerve (Pette and Vrbova, 1985). Recovery of motor function does not occur immediately upon connection to the neuromuscular junctions, and there is a delay of up to 18 days before nerve stimulation produces contraction and of a further 5 days before reflex activity takes place (Gutman & Young, 1944).
Regenerating axons grow into surviving denervated cutaneous sensory receptors and re-establish pre-existing connections (Ridley, 1970; Orgel et al., 1972; Dellon, 1981), with the exception of free nerve endings which are capable of regeneration (Terzis and Smith, 1990). The quality of regeneration depends on the condition of the denervated sensory end-organ. Merkel cell-neurite complexes degenerate fast with complete disappearance by 35 days post-transection (Burgess et al., 1975), Meissner’s corpuscles by 4-6 months (Dellon et al., 1975, Dellon 1981), while Pacinian corpuscles persist for over a year (Glees et al., 1949). As with motor target tissue, denervated sensory receptors exert a tropic effect on the appropriate regenerating axons; however preferential reinnervation and regeneration down sensory endoneural tubes has not been demonstrated (Rath et al., 1991). Morphology of reinnervated sensory receptors correlates well with the observed recovery of sensibility. Perceptions of pain and temperature innervated by Aδ and C fibre free nerve endings are the first to recover (Dellon, 1981), followed by touch (30 cycles per second) subserved by Meissner’s corpuscles, then constant touch supplied by Merkel cell-neurite complexes and finally moving touch and high frequency vibrations (256 cycles per second) innervated by Pacinian corpuscles (Dellon, 1981; Terzis, 1981).

1.2.4 Neuronal survival

After peripheral nerve injury, the nerve cell body may recover, or it may degenerate and die (Lieberman, 1974). Following transection, significant neuronal cell death occurs in the dorsal root ganglia (DRG) (Risling et al., 1983; Arvidsson et al., 1986). Even with optimal microsurgical repair, 20-40% of neurons within the DRG are still lost (Himes and Tessler, 1989; Liss, 1994), most probably due to apoptosis (Edström et al., 1996; Groves et al., 1997). Neurons that survive the initial insult respond to axotomy by a
reorganisation of the metabolism such that the synthesis of substances associated with neural transmission is reduced whereas the synthesis of substances associated with axonal outgrowth and cell survival is increased (Aldskogius et al., 1992). Cellular changes include an increase in cell-body size, chromatolysis, peripheral displacement of the nucleus, changes in protein synthesis and alterations in the synaptic connections of the primary afferent nerves (Aldskogius et al., 1985). A change also occurs in the phenotype (Hökfeldt et al., 1994) characterised by modification of neuropeptide expression. The mechanism that induces apoptosis in certain neuronal populations and survival in others after nerve transection and repair is still not known. However, improvement in neuronal survival has been reported with the use of nerve growth factor (NGF) (cf. 1.4), possibly due to trophic support exerted by exogenously supplied growth factor on the neuronal cell body (Rich et al., 1987).

1.3 CONDUITS FOR NERVE REGENERATION

The technique of using artificial tubes has risen from the need to bridge a nerve defect, while providing coaptation and strength of bond with minimal injury. Tubulization offers several advantages. It aids guidance of growing fibres along appropriate paths by mechanical orientation and confinement. It protects regenerating tissue by reducing invasion and scarring of the nerve.

It has also been used to administer adjuncts to promote regeneration, by confining and concentrating growth and trophic factors (cf. 1.4). It has served as an excellent tool as an in vivo experimental chamber for studying morphological, biochemical, and physiological aspects of nerve regeneration under controlled conditions.
The most comprehensive study investigating nerve ultrastructure and morphology in a silicon conduit has been carried out by Le Beau (Le Beau et al., 1988a), confirming the findings by Williams (Williams et al., 1988). At 7 days post-transection, the tube fills with fluid and a fragile tissue bridge consisting of fibrin and red cells connects the proximal and distal stumps. Fibroblasts extrude from both ends and migrate into the tube. At 14 days, axons and Schwann cells co-migrate through the proximal stump (Aguayo et al., 1973; Le Beau et al., 1988a). From 21 to 28 days, axons associated with Schwann cells cross the 5mm mark. By day 42, the axons invade the collagen-rich distal stump which is densely populated by Schwann cells and advance down endoneurial tubes. At this time, spontaneous degenerative changes occur in some axons which may be due to constrictive forces that act on the nerve, or are a reflection of axons that fail to reach their target organs; these changes have also been seen in developing nerves (Aguayo et al., 1973; Jenq et al., 1986; Le Beau et al., 1988a). In these studies, complete maturation of fibres as defined by an increase in axonal diameter and myelin sheath thickness back to normal was never achieved. The mean fibre diameter and myelin thickness of regenerated fibres was reported to be 47% and 38% of normal (Le Beau et al., 1988a).

1.3.1 Biological conduits

The technique of nerve tubulization can clearly be traced to 19th century practices. To a large extent, these pioneering techniques are distinguished from modern methods by the materials available at the time. These include decalcified bone (Gluck, 1880; Vanlair 1882a, b), fresh (von Büngner, 1893) and freeze-dried or chemically fixed arteries (Weiss, 1943; Moyer and Kimmel, 1948), veins used alone (Chiu et al., 1982, 1988; Rice and Bernstein, 1984; Suematsu et al., 1988) or in conjunction with muscle (Brunelli et al., 1993) and collagen (Wang et al., 1993); and many other materials such
as agar, peritoneum, casein, dura, fascia, fat, feather quills and epineurium (Fields *et al.*, 1989).

### 1.3.2 Synthetic conduits

The ideal material should have several properties. It should be inert, thin and flexible, translucent, bioresorbable, inhibitory to pathological processes such as scarring and oedema, and beneficial to processes of healing and regeneration.

Dimensions of an artificial tube affect nerve regeneration significantly. As tight fitting tubes constrict the anastomosis and loose fitting ones are ineffective (Ducker and Hayes, 1968), an ideal cross-sectional area is 2.5 to 3 times that of the nerve (1.5-1.7 times the diameter of the nerve); it is generally held that the diameter of the conduit should be greater than that of the nerve by at least 30%. (Buti *et al.*, 1996; Lundborg, 1997). Another aspect to consider is the wall thickness of the tube, as it has been shown that optimal regeneration is achieved with thickness between 0.125mm to 0.62mm (Ducker and Hayes, 1968). The length of the tube is also a limiting factor and at present the maximum length of a conduit that can be successfully bridged by a regenerating nerve has been estimated at 10mm in the rat sciatic nerve (Lundborg, 1991). The use of a ‘sandwich graft’ where synthetic conduit segments are interspersed with small nerve grafts which act as a source of Schwann cells, may be useful in lengthening this distance up to 5 cm (Calder *et al.*, 1995; Whitworth *et al.*, 1995). Surface microgeometry of guidance channels may also influence the outcome, potentially by affecting the early arrangement of the fibrin matrix and possibly inducing different cellular responses. A smooth internal surface supports better regeneration than a rough internal surface (Aeibscher *et al.*, 1990). Schwann cells have been shown to align themselves along etched grooves which act via contact guidance (Curtis and Wilkinson, 1990).
1997). This may indirectly influence axonal regeneration as Schwann cells and axons advance simultaneously (Le Beau et al., 1988a).

Tantalum metal cuffs (Weiss, 1944; Campbell and Luzio, 1964), magnesium (Payr, 1900), stainless steel mesh and tubes (Kuhn and Hall, 1977; Azzam and Brightman, 1985) have been used but all without lasting success. Silicone is inert and elastic (Ducker and Hayes, 1968) and over the last thirty years, silicone tubes have been the most widely used as an in vivo experimental chamber model in peripheral nerve regeneration (Ashur et al., 1987, Madison et al., 1988, Stenasaas et al., 1989, Spector et al., 1991, Lundborg et al., 1991, 1997). A silicone chamber provides an external guide for axonal regeneration while an intrinsic silicone filament network has been used to study the effects on nerve regeneration (Lundborg and Kanje, 1996). However, in clinical cases, nerve repair with a silicone polymer cuff resulted in deterioration of function necessitating the removal of the conduit (Merle et al., 1989). These changes were due to chronic nerve compression proceeding to Wallerian degeneration. In a recent study, silicone entubulation was compared to conventional microsurgical repair in 18 patients and clinical follow up carried out for one year. In certain cases, removal of the tube at a second operation was necessary. Clinical evaluation showed no difference between the techniques except the sensation of touch, which was significantly better with tubulization at 3 months (Lundborg et al., 1997). However, the non-degradable nature of silicone is its main drawback, and the use of bioresorbable tubes is preferable.

Regeneration is optimal if a conduit is in situ for an appropriate length of time so as to support axonal guidance but is reabsorbed before effects of chronic constriction take place. Several materials have been used to construct such tubes: copolymers of glycolic
and lactic acids (Molander et al., 1982; Nyilas et al., 1983; Seckel et al., 1984; Madison et al., 1985; da Silva et al., 1985); collagen alone (Archibald and Madison, 1984; Colin and Donoff, 1984; Toyota et al., 1988) and in conjunction with other materials (Yannas et al., 1985; Kiyotani et al., 1995; Ellis and Yannas, 1996); copolymers of lactic acid and caprolactone (den Dunnen et al., 1993, 1995 and 1996); maxon (glycolide trimethylene carbonate) (Mackinnon and Dellon, 1990); poly (organo)phosphazene compounds (PEIP) (Langone et al., 1995) and polytetrafluoroethylene (PTFE) (Zetti et al., 1991).

More recently solidified fibronectin has been used for nerve regeneration. Fibronectin is a high molecular weight glycoprotein produced by fibroblasts forming an abundant constituent of basement membrane. It is also found in the plasma from which it has been concentrated and aggregated into mats (Egim et al., 1993). Fibronectin mats provide an acellular scaffold of orientated fibres, which act by contact guidance in directing regenerating axons and migrating Schwann cells towards the distal stump. It also allows revascularisation and infiltration of macrophages (Hobson et al., 1998). Fibronectin is completely reabsorbed and has the additional advantage of binding to and releasing growth factors (Whitworth et al., 1996; Sterne et al., 1997). Though fibronectin is a good experimental tool and supports nerve regeneration in vivo, the clinical application of this conduit type may be difficult as it is reabsorbed rapidly within 3 weeks and therefore may not be able to support regeneration over longer gaps. It also presents with problems inherent to pooled plasma from which it is manufactured (Brown et al., 1994).
Two new biodegradable materials which have been analysed in this research project as candidates for use in nerve regeneration are poly-3-hydroxybutyrate (PHB) and polydioxanone (PDS).

1.3.2.1 Poly-3-hydroxybutyrate (PHB)

Micro-organisms are capable of producing a wide range of polymers generated from 3-hydroxypropionic acid, which is widely present in nature. One such homopolymer, poly-3-hydroxybutyrate (PHB), is a natural storage product of bacteria and algae and occurs as discrete granules within the cell cytoplasm. PHB granules are produced from bacterial cultures using a fermentation process followed by solvent extraction. PHB can also be produced from carbon substrates as diverse as glucose, ethanol, acetone, methane and gaseous mixtures of carbon dioxide and oxygen (Anderson et al., 1990). PHB is non-antigenic, biocompatible, easy to handle, has good tensile strength and is completely resorbed within 24-30 months by hydrolytic degradation. Non-specific esterases and lysozymal enzymes secreted by leucocytes and macrophages in the body catalyse the process, increasing the rate of degradation. The final biodegradation product, (R)-3-hydroxybutanoic acid, is a normal metabolite in human blood (Holmes, 1988; Gogolewski et al., 1993; Malm et al., 1992, 1994). Sheets of PHB (Astra Tech, Gothenberg, Sweden) have been used experimentally in cardiovascular surgery (Malm et al., 1992, 1994; Duvernoy et al., 1995).

In a sheet, the PHB fibres are orientated along one direction which, when a sheet is rolled into a tube, can be orientated according to need. Based on previous experience (Whitworth et al., 1995; Curtis and Wilkinson, 1997), we hypothesised that for application as a conduit in this study, longitudinally placed fibres may aid neuronal and glial cell growth by contact guidance and mechanical orientation. Schwann cells will
align themselves along the longitudinal fibres, preferentially propagate in the longitudinal axis (Mahanthappa et al., 1996) and facilitate the regrowth of axons through the conduit.

1.3.2.2 Polydioxanone (PDS)

Polydioxanone is a polymer of the polyester monomer paradioxanone. It was introduced in 1981 as a synthetic bioresorbable suture, widely used in cataract, vascular, plastic and abdominal surgery (Bartholomew, 1981; Berry et al., 1981; Blaydes and Werblin, 1982; Chusak and Dibbell, 1983; Hoile, 1983; Tuchmann and Dinstl, 1984; Leaper et al., 1985; Myers et al., 1986). Being synthetic, the tissue response found only at the site of implantation, is predominantly mononuclear in composition. Collagen deposition took place with time. PDS undergoes non-enzymatic hydrolytic degradation in the body to 2-hydroxyethoxyacetic acids and is excreted in urine, faeces and expired carbon dioxide (Ray et al., 1981). Mass absorption is completed by 180 days (Ray et al., 1981; Lerwick, 1983).

Two types of PDS conduits (Ethicon, Edinburgh, UK) have been assessed in this study: 'smooth' - with a smooth internal surface and, 'grooved' - with longitudinal grooves along the internal surface. Preliminary in vitro experiments with monolayer Schwann cell cultures using longitudinally grooved PDS sheets showed promising results. The Schwann cells aligned themselves along the grooves in the PDS sheet and preferentially propagated in the longitudinal axis (unpublished data, Curtis and Wilkinson). It was hypothesised that the longitudinal grooves aid growth by contact guidance and mechanical orientation (Curtis and Wilkinson, 1997).
1.4 GROWTH FACTORS

According to current knowledge and the neurotrophic hypothesis, target tissue provides trophic support, by means of various growth factors, for the survival of the developing neuron (Levi-Montalcini and Angeletti, 1968; Purves, 1986; Oppenheim, 1991; Davies, 1996). In an adult neuron, a steady maintenance state exists between the neuron and the target organ, the latter producing neurotrophic factors, which are taken up by the axon terminals and retrogradely transported to the nerve cell soma. A nerve injury interrupts the retrograde flow and deprives the neuron of its trophic support. The neuron must both repair its damaged axon and re-establish the flow, or face atrophy and death (Seniuk, 1992).

The first neuronal growth factor to be discovered, the prototypical neurotrophic factor, is nerve growth factor (NGF) (Levi-Montalcino and Hamburger, 1953), which specifically acts on a subset of primary sensory and sympathetic neurons (Levi-Montalcino, 1987). NGF belongs to the neurotrophin family which also includes brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5). The neurotrophin family and several other growth factors such as glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF) show specific and well defined action on neuronal subpopulations, and they have been comprehensively reviewed (Snider and Johnson, 1989; Senuik, 1992; Lindsay, 1996; Terenghi 1998,).

Some of these growth factors have been administered at sites of nerve injury via artificial conduits. NGF and NT-3, in particular, have been studied in detail and their effects on regeneration and reinnervation of motor and sensory target organs have been documented (Whitworth et al., 1996; Sterne et al., 1997). Another such growth factor is
glial growth factor (GGF) which promotes Schwann cell proliferation \textit{in vitro}. Schwann cells share an intimate relationship with neurons and are essential for their regeneration (cf. 1.2.2). An increase in Schwann cell numbers would be beneficial to axonal regeneration. Therefore, it could be hypothesised that delivery of GGF in a synthetic conduit would indirectly enhance nerve regeneration.

1.4.1 The neuregulins and GGF

In a search for Schwann cell mitogens, a 31 kD protein was isolated from bovine pituitary and named glial growth factor (GGF) (Raff \textit{et al.}, 1978). This molecule was purified into different molecular masses and subdivided as GGF-I (34 kD), GGF-II (59 kD) and GGF-III (45 kD) (Goodearl \textit{et al.}, 1993). Subsequently it was shown that GGF is the product of differential gene splicing and the spliced proteins were collectively named 'neuregulins' (Marchionni \textit{et al.}, 1993). The neuregulins act through heterodimer receptors of erbB2, erbB3 and erbB4 which are also expressed on Schwann cells (Carraway and Burden, 1995). GGF-mRNA is expressed in both somatic and visceral motor neurons, primary sensory neurons in the dorsal root ganglions and sympathetic neurons (Marchionni \textit{et al.}, 1993; Chen \textit{et al.}, 1994).

GGF is trophic for Schwann cell precursors and at higher concentrations drives proliferation and maturation (Dong \textit{et al.}, 1995). GGF is also critical for the survival of terminal Schwann cells in developing neuromuscular junctions (Trachtenberg and Thompson, 1996). Dose-dependant effects of progressively increasing concentrations of human recombinant GGF-II (rhGGF-II) have been demonstrated \textit{in vitro}. At submaximal levels, rhGGF-II increases the motility of cultured Schwann cells. When applied focally, it causes directed migration in monolayer cultures and at higher doses causes proliferation (Mahanthappa \textit{et al.}, 1996).
Schwann cells are vital for axonal regeneration (Hall, 1986 and 1997). They are an important source of neurotrophic factors (Heumann et al., 1987; Le Beau et al., 1988b; Acheson et al., 1991; Sendtner et al., 1992), exert a trophic influence on neuronal cells and affect their survival (Reynolds and Woolf, 1993). Conversely, regenerating axons stimulate proliferation of Schwann cells, an effect which is mediated by GGF (Li et al., 1997). A reciprocal interaction thus exists between neurons and Schwann cells, and it is now accepted that GGF may be the major component of this reciprocity and an important mediator of these events. It is also likely that GGF secreted by regenerating neurons establishes a concentration gradient, highest at the growth cones and less toward the periphery. Schwann cells furthest away would be recruited to the site of injury by low concentration via chemotaxis, begin to proliferate at a closer distance and then at the site of injury, on exposure to supra-mitotic concentrations, provide trophic support to the regenerating neurons (Mahanthappa et al., 1996).

In spite of the beneficial effects of GGF seen in vitro and an upregulation of erbB receptors on Schwann cells in the distal stump, correlating with an increased secretion of GGF in response to axotomy (Carroll et al., 1997; Li et al., 1997), no study is available showing an effect in vivo and it is not known whether GGF may indirectly benefit axonal regeneration.

1.5 HYPOTHESIS

It is clear that despite widespread experimental work, the search for a conduit for nerve gap repair continues. The available evidence also indicates that addition of growth factor(s) may be beneficial to the regeneration process. However, it is still unclear which of the growth factors would be the ideal candidate for future clinical application.
1. It is proposed that the morphological and functional results of nerve repair will be improved by the use of synthetic (PHB or PDS) tubes to surround the repair or bridge a nerve gap.

2. It is also proposed that the addition of GGF may improve the structural and functional results of nerve gap repair.

1.6 AIMS

1. It is the aim of this study to investigate the use of PHB and PDS as conduits for nerve gap repair using quantitative immunohistochemistry on regenerating nerves, target organs and neuronal cell bodies, as well as analysis of myelinated axons in rat sciatic nerves.

2. As either of these conduits used alone may not give results which would be required for clinical application, it is a further aim to determine whether the addition of GGF will improve the overall nerve regeneration achievable with these conduits.
CHAPTER II

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2.1.1 Conduit formation

The conduits were formed from PHB sheets (5 cm x 10 cm PHB patch, Astra Tech, Gothenberg, Sweden) (Fig.2.1). Rectangular pieces measuring 8 mm x 14 mm were cut, ensuring that the unidirectional orientation of the PHB polymer fibres was along the longitudinal axis of the conduit as this aids neuronal and glial growth by contact guidance (Whitworth et al., 1996; Curtis and Wilkinson, 1997). These were then rolled around a 16G intravenous cannula (16G Abbocath®-T, Abbott Ireland, Sligo, Republic of Ireland), thus standardising the internal diameter of the conduits at 1.6 mm (Fig.2.2), leaving adequate space for post-injury swelling of the 1 mm-diameter rat sciatic nerve. The rolled sheets were sealed longitudinally with cyanoacrylate glue (Histoacryl ®, Braun Melsungen AG, Germany). The conduits, still rolled around the template, were pre-soaked in normal saline to saturate the polymer and ensure maximum expansion of the fibres without a reduction in the internal diameter of the conduit.

PDS conduits were supplied in the form of tubes measuring 14 mm in length and with an internal diameter of 1.6mm in sterilised packs (Ethicon, UK). The tubes were either ‘plain’ with a smooth internal surface, or ‘grooved’ with longitudinally etched grooves of 6 - 10 μm depth and 12.5 μm width (pitch = twice width = 25 μm).

2.1.2 Preparation of rhGGF-augmented growth factor-reduced Matrigel

RhGGF2 was supplied in stock solution at a concentration of 1.29mg/ml (batch 4/23/97, Cambridge NeuroScience, Massachusetts, USA). To achieve a final concentration of 500ng/ml in rhGGF2 augmented Matrigel, the rhGGF2 was diluted to 1.7μl/ml test
Fig. 2.1  Electron microscopy scanning of a PHB sheet showing the unidirectional orientation of polymer fibres.

Fig. 2.2  PHB sheet (8 x 14 mm) wrapped around a 16G cannula to form a PHB conduit 14 mm long and with an internal diameter of 1.6 mm. The polymer fibres are orientated along the longitudinal axis of the conduit.
solution (dilution 1:760) in phosphate buffered saline (PBS) with bovine serum albumin (1mg/ml) and added to 70% GFR Matrigel. Care was taken not to cause foaming.

Matrigel is a soluble basement membrane extract of the Engelbreth-Holm-Swarm (EHS) tumour comprising of laminin, collagen IV, entactin and heparan proteoglycan. Growth factor-reduced (GFR) Matrigel (Becton Dickinson, Massachusetts, USA) contains reduced levels of a variety of growth factors except for TGF-β, and a 40-50% decrease in the level of heparan proteoglycan. The matrix is liquid at 4°C and gels rapidly at 22°C - 35°C. GFR-Matrigel was thawed overnight in a refrigerator and all apparatus to be used for the preparation of rhGGF2 augmented GFR-Matrigel was pre-cooled on ice. RhGGF2 augmented GFR-Matrigel consisted of 30% rhGGF2 test solution and 70% GFR-Matrigel. The rhGGF2 test solution was added to GFR-Matrigel under sterile conditions and on ice, maintaining the temperature of Matrigel at 4°C. The final solution was stored on ice until its injection into an implanted PHB conduit.

2.1.3 Silanisation

As the rhGGF2 protein tends to adhere to surfaces by electrostatic interaction, all glassware and plasticware, including polypropylene pipette tips and eppendorfs for handling and storage of rhGGF2 dilutions, were silanised to minimise rhGGF2 adsorption. The silanisation was carried out by a published method (Molecular Cloning: A Laboratory Manual, eds: Sambrook, Fritsch and Manniatis). 1 ml of dichlorodimethylsilane was added to a small beaker placed inside a large dessicator with the items to be siliconised. The dessicator was then placed under an extraction hood and was attached to a vacuum pump through a trap. Suction was applied until the dichlorodimethylsilane started to boil, at which point the connection between the
vacuum pump and the dessicator was clamped and the pump then switched off. It is essential to turn off the vacuum pump as soon as the dichloromethylsilane begins to boil, as it causes irreparable damage to the vacuum seals if sucked into the pump. The dichloromethylsilane was left to evaporate completely for two hours after which the dessicator was opened under an extraction hood and the fumes were allowed to disperse. The items were removed and rinsed three times in distilled water, dried overnight and sterilised by gamma irradiation.

2.2 CAT SUPERFICIAL RADIAL NERVE MODEL

The aim was to test PHB in direct nerve repair i.e. primary epineural repair vs. PHB wrap-around, by assessing the inflammatory response to PHB and the level of axonal regeneration at 6 and 12 months. The study was carried out in collaboration with the Department of Hand and Plastic Surgery, Umeå University, Umeå, Sweden and Astra Tech, Gothenberg, Sweden. One-year old adult female cats weighing 2.6 – 3.7 kg used in the study were housed together in the animal department at Astra Hässle, Mölnndal, Sweden. Surgery was performed by Professor M Wiberg (Department of Hand and Plastic Surgery, Umeå University) in the operating theatres at Astra Hässle under aseptic conditions and with continuous monitoring. The experiments were approved by the Regional Committee for Ethics in Animal Experiments, Gothenberg and were performed according to the European Communities Council Directive (86/609/EEC).

2.2.1 Anaesthesia

Anaesthesia was induced with medetomidin (0.15 mg/kg) (Orion Pharma, Finland) injected subcutaneously. The animal was then intubated using lidocaine gel to diminish laryngo-pharyngeal reflexes and anaesthesia maintained by isoflurane/oxygen
inhalation. On completion of the surgical procedure, the effect of medetomidin was reversed by atipamezol (0.4 mg/kg) (Orion Pharma, Finland) and a single peri-operative dose of 150 mg benzylpenicillin (Boehringer Ingelheim, Germany) was administered intramuscularly. The animals were kept pain-free by subcutaneous buprenorphine (0.01 mg/kg) (Reckitt & Coleman, UK) for 3-5 days.

2.2.2 Operative procedure
Using an operating microscope, the superficial radial nerve was exposed bilaterally in the mid-forelimb and transected. In the first group of animals (n=10), end-to-end nerve repair was carried out on one side with epineural sutures using 9(0) polyamide (Ethicon, Germany) (Fig.2.3). The contralateral nerve was apposed and a pre-soaked PHB sheet was wrapped around it, leaving a gap of approximately 2-3 mm between the transected nerve ends. The sheet was sealed longitudinally with fibrin glue (Tisseel® glue) to form a tube which was anchored to the epinerium with one 9(0) suture at each end of the conduit (Fig.2.4).

For each group, ten cats were sacrificed at 6 and 12 months. The animals were premedicated with medetomidin as in 2.2.1, deeply anaesthetised with intravenous thiopental (1.25 mg/kg) (Abbott, USA), intubated and then perfused transcardially with heparinised buffer followed by 1.5% glutaraldehyde + 1% paraformaldehyde or 4% paraformaldehyde. The anastomosis site was approached through the previous operative
Fig. 2.3 Intra-operative photograph of primary epineural repair in the cat superficial radial nerve.

Fig. 2.4 Intra-operative photograph of the contralateral superficial radial nerve where the transected nerve stumps were repaired by PHB wrap-around technique.
incision to harvest the conduit / epineural repair site for assessing the inflammatory response and was followed by excision of the distal nerve segment for assessment of the level of axonal regeneration.

2.3 RAT SCIATIC NERVE MODEL

The rat sciatic nerve model was used to assess peripheral nerve regeneration in gap repair with a synthetic conduit, with or without a growth factor. The conduit materials tested were PHB and PDS. In PHB conduit gap repair, the rate and quality of regeneration was assessed in comparison with an autologous nerve graft, i.e. PHB conduit vs. autograft (n=6) at 7, 14 and 30 days. In the long-term, the level of axonal regeneration was assessed by myelinated axons counts in the distal nerve at 80 and 120 days. Similar short-term time-points (7, 14) were used to test the effect of glial growth factor (GGF) i.e. GGF-augmented Matrigel vs. Matrigel only (n=6), on peripheral nerve regeneration.

PDS conduits, in two forms 'plain' or 'grooved' (n=6), were tested to assess the effect of surface microgeometry on nerve regeneration in nerve gap repair. The rate and quality of regeneration were quantified at 7, 14 and 30 days. At 180 days (6months), the quality of long-term axonal regeneration was assessed by axon counts in the distal nerve, and cell survival was estimated by neuronal counts in the dorsal root ganglia. Finally, motor and sensory target-organ reinnervation were assessed in the gastrocnemius muscle and footpad skin respectively.
2.3.1 Anaesthesia

Eight week-old inbred male Lewis rats were used in the study. The animals were anaesthetised with 0.3ml/kg of intramuscular Hypnorm (fentanyl citrate, 0.315 mg/ml; fluanisone, 10 mg/ml; Janssen Pharmaceuticals) and 2.5 mg/kg of intraperitoneal diazepam (Phoenix Pharmaceuticals, Gloucester, UK). All procedures were carried out in compliance with UK Home Office regulations.

2.3.2 Operative procedure

Using an operating microscope (Wild, Heerbrugg, Germany), the left sciatic nerve was exposed 5mm distal to the sciatic notch via a gluteal muscle splitting incision. In the PHB group, a 5 mm nerve segment was resected using sharp microsurgery scissors to produce a 10 mm nerve gap after retraction of the transected nerve ends. The gap was bridged using a 14 mm PHB or PDS conduit (cf. 2.1.1), entubulating 2 mm of the nerve stump at each end, producing a final gap of 10mm between the nerve stumps. Two 10/0 ethilon sutures (Ethicon, Edinburgh, UK) were used to anchor the conduit to the epineurium at each anastomosis (Figs.2.5, 2.6). In the autograft group, a 10 mm nerve segment was excised, reversed in a proximo-distal direction and re-anastomosed in the gap with three to four 10/0 ethilon sutures at each anastomosis (Fig.2.7). Tension was avoided and atraumatic handling and correct rotational alignment were employed throughout all procedures. In the groups where rhGGF2 augmented GFR Matrigel or GFR Matrigel were used, the solutions were stored on ice until use to maintain liquidity (cf 2.1.2). 200μl were injected into the PHB conduit using a 1 ml silanised syringe from the distal end of the conduit under direct vision (Fig.2.8). The solution was allowed to gel, excess gel extruding from the proximal and distal ends of the conduit was carefully removed (Fig.2.9) and the wound closed. Every effort was made to minimise animal numbers and
Fig. 2.5 (a) Pre-formed PHB tube around 16 G cannula
(b) PHB conduit placed in a 10mm gap in the rat sciatic nerve.
Fig. 2.6 PDS conduit bridging a gap of 10mm in the rat sciatic nerve model.

Fig. 2.7 Reversed nerve autograft, the surgical gold standard, was used as the control.
Fig. 2.8  Liquid GGF-augmented Matrigel in pre-cooled syringe ready to be injected into a PHB conduit.

Fig. 2.9  Excess gelled GGF-augmented Matrigel has been wiped away to leave a small blob at the proximal end of the conduit.
distress. Animals received post-operative analgesia for 24 hours and were caged separately, kept warm till fully recovered from the anaesthetic, with free access to food and water. On the first post-operative day, the animals were colonised in groups of six in large cages and returned to the holding room.

At various time-points (cf.2.3), the animals in each group (n=6) were killed by an overdose of inhaled enflurane (Abbot Laboratories, UK) and fracture dislocation of the cervical spine. Only the 180 days PDS ‘plain’ and ‘grooved’ groups were anaesthetised (cf.2.3.1) and the previous incision extended to allow adequate access to harvest the nerve, muscle and footpad skin specimens. This was followed by intracardiac perfusion with PBS and fixation with 4% paraformaldehyde prior to harvesting the spinal cord and dorsal root ganglia.

2.4 TISSUE COLLECTION

2.4.1 Nerve conduits

The nerve at the repair site (primary repair / nerve autograft / conduit repair) and a short length of proximal and distal nerve were harvested en bloc and pinned onto a card to avoid shrinkage and deformation (Fig.2.10). The suture material was then removed to prevent tissue damage during cryostat cutting. For immunohistochemistry, fresh tissue (less than 0.5cm thick) was immersed in Zamboni’s fixative (Appendix I) (minimum ratio, fixative : tissue, 10:1) for six hours at room temperature followed by rinsing in 0.01M PBS containing 15% (w/v) sucrose and 0.1% (w/v) sodium azide and maintained at 4°C in a refrigerator. The washing solution was changed three times over the following two days or until the solution no longer was discoloured by excess Zamboni’s fixative and the tissue had sunk to the bottom of the container. Using a perspex template
Fig. 2.10 (a) PHB conduit harvested at 30 days with a segment of the proximal and distal nerve pinned *en bloc* on cardboard prior to fixation.

(b) PDS conduit was removed to prevent crushing of nerve tissue during cutting, and exposed the fragile regenerated nerve bridging proximal and distal nerve stumps at 30 days.
the proximal and distal nerve stumps were trimmed to 2mm from the anastomosis sites using the sutures in a nerve graft or the ends of the PHB or PDS conduits as markers. This allowed measurements of regeneration to be made from a fixed point in each specimen. The specimens were then blocked in OCT compound (Tissue-tek, Sakura, Japan) and orientation of each specimen was identified by placing a piece of rat liver next to the proximal nerve end.

2.4.2 Distal nerve

A five mm length of the regenerated nerve, 5 mm distal to the distal end of the conduit or nerve graft was collected. A similar segment corresponding to the same level was harvested from normal uninjured nerves to provide a ‘normal’ control sample. For correct orientation, a single 9(0) ethilon suture was placed at the nerve end opposite to where the serial semithin sections were to be cut. The samples were fixed in 2.5% electron microscopy grade glutaraldehyde in 0.1M PBS overnight at 4°C and subsequently washed twice in PBS (0.1 M, pH 7.4) after fixation. The nerve segments were post-fixed with 1% osmium tetraoxide (Agar Scientific, UK) in 0.1 M phosphate buffer for 1 hour at room temperature, washed with phosphate buffer and dehydrated serially through increasing concentrations of acetone. Infiltration of the specimen was initially carried out overnight with acetone: araldite CY212 resin (1:1) (Agar Scientific, UK), followed by two changes of fresh resin and finally embedded in araldite CY212 resin. The blocks were polymerised at 60°C for 18 hours. Care was taken to ensure correct tissue orientation to allow transverse semithin sections of the nerve to be cut.

2.4.3 Gastrocnemius muscle

The calf muscles in the rat were approached by extending the lateral thigh incision beyond the ankle joint. Gastrocnemius muscles were harvested bilaterally from each
animal (Fig.2.11). Using an operating microscope, the entire muscle was carefully cleaned and dissected, dividing its two tendinous origins and insertion flush with the bone. The muscle from each side was weighed immediately after dissection to avoid dehydration and the percentage reduction in muscle mass following denervation and repair (experimental side muscle vs contralateral normal muscle weight) was calculated.

2.4.4 Footpad skin

In rats, the sciatic nerve supplies the skin on the sole and dorsum of the foot and toes except for a medial strip involving the first and second toes, which is supplied by the saphenous nerve subserving approximately 22% of the skin of the foot (Wall, 1979). In the 180 days PDS groups, a longitudinal strip of glabrous skin, 5-8 mm long, including some hypodermis and overlying the fourth metatarsal bone was harvested from the plantar surface of both hind paws (Stankovic et al., 1996). The skin was fixed by immersion in Zamboni’s solution as described in 2.4.1. The skin samples were blocked in OCT and orientated to allow cryostat sections to be cut perpendicular to the epidermal surface along the plane of the fourth metatarsus. Sections were processed for immunohistochemistry.

2.4.5 Dorsal root ganglia

Immersion fixation of central nervous system is inadequate due to slow penetration of the fixative into the tissue. To overcome this problem, fixation is carried out by trans-cardiac perfusion with a fixative.

In the rat, cardiac perfusion was carried out after anaesthetisation as described in 2.2.1, and after the nerve, muscle and footpad skin specimens were harvested. The animal was placed supine and the heart was exposed by bilateral subcostal incisions. Working
speedily, the left ventricle was incised and a blunt cannula introduced into the ascending aorta where it was clamped in position. The right atrium was opened to allow free drainage of blood and perfusate. The initial washout was carried out with PBS buffer until the perfusate draining from the right atrium was clear, ensuring that the animal was dead. Fixation was then achieved by perfusing with freshly prepared 4% paraformaldehyde in PBS confirmed by the liver becoming pale and hard and by the twitching of the limbs. The vertebral column was approached by a dorsal midline incision from the cervical region to the tail. The proximal stump of the sciatic nerve was identified and dissected up to its emergence from the vertebral foramina. The spinal cord was exposed by removing the posterior vertebral arches and incising the dura. The lower thoracic and lumbar regions of the spinal cord with the attached DRG and dorsal roots were carefully dissected (Fig.2.12) and removed en bloc, post-fixed in 4% paraformaldehyde for 6 hours at room temperature, followed by several washes and storage in PBS sucrose at 4°C. Paired L4 and L5 DRG were removed by dissection under an operating microscope and blocked in pairs in OCT using a small piece of rat liver to identify the DRG correspondent to the injured nerve. The tissue blocks were cut and the sections processed for immunohistochemical staining.
Fig. 2.11 Gastrocnemius muscle harvested bilaterally from
(a) PDS 'smooth' conduit gap repair group at 180 days (6 months) and
(b) PDS 'grooved' conduit group at 180 days.
Fig. 2.12  (a) Dissection in PDS nerve gap repair group to demonstrate the origin of the sciatic nerve in the rat from L4, L5 and L6 roots.
(b) Spinal cord with L3, L4 and L5 roots with the respective dorsal root ganglia.
2.5 MORPHOLOGICAL ASSESSMENT

2.5.1 Immunohistochemistry

2.5.1.1 The indirect avidin-biotin complex (ABC) method

Immunostaining of longitudinal nerve sections, dorsal root ganglia and skin was carried out according to the indirect avidin-biotin complex (ABC) peroxidase nickel enhancement procedure (Shu et al., 1988). Immunoreactive structures show as black permanent staining against a well contrasted pale background, which is optimal for image analysis (Terenghi and Polak, 1994). A known positive control was included in each batch of staining to ensure reliability of the technique (Emson, 1993).

Sections were cut on the cryostat, collected on slides coated with Vectabond (Vector Laboratories, Peterborough, UK) and air-dried for 4h at room temperature. The thickness of the sections was decided according to the type of tissue. Longitudinal nerve sections were cut at 15μm, skin at 20μm and DRG at 20μm. The slides were then wrapped in aluminium foil and stored at -20°C overnight. On the day of staining, the sections were thawed and allowed to air-dry for a minimum period of 4 hours. The contours of the sections were outlined with DAKO pen (DAKO A/S, Denmark) to limit the antibody solutions to the sections and prevent flooding of the slide. The sections were initially immersed in 0.2% phosphate-buffered Triton X for one hour. Any endogenous peroxidase was then inhibited by immersing the sections in 0.05% hydrogen peroxide (30% solution, BDH Laboratories, UK) in PBS for 20 min at room temperature. The sections were washed three times in plain PBS followed by incubation for 15 min at room temperature with normal serum (dilution 1:30 in PBS). This serum was derived from the same species as the second layer antibody and was used to block any possible background reaction, i.e. normal goat serum for polyclonal first layer antibodies (TAGO Inc., USA) and normal horse serum for monoclonal first layer
antibodies (ICN, UK). Following incubation, the normal serum was drained and the
appropriate first layer antibody was applied as approximately 100μl per section (cf.
2.5.1.2). The sections were incubated overnight at 4°C, following which they were first
washed by immersion in Tween/PBS (dilution 1:4000, Polyoxyethylensorbitan
monolaurate, Sigma, USA) for one min to remove unbound primary antibody, then
three times in plain PBS. The second layer biotinylated antibody (cf. 2.5.1.2) was
applied and incubated for an hour at room temperature in a humid chamber. A further
rinse in Tween/PBS for one min was followed by three more washes in plain PBS.
Peroxidase conjugated avidin-biotin (ABC, Vectabond, UK) was applied to the sections
and incubated for an hour at room temperature in a humid chamber. The sections were
finally washed in PBS before being equilibrated in 0.1M sodium acetate buffer for 10
min. The slides were then immersed in a developing solution containing 7.25g
ammonium nickel sulphate (Sigma, USA), 125mg 3,3 diaminobenzidine tetrachloride
dihydrate (Sigma, USA), and 600mg α (+) D glucose (Sigma, USA) dissolved in 300ml
of 0.1M sodium acetate buffer pH 6.0. The solution was activated by the addition of
6mg glucose oxidase (Sigma, USA) immediately prior to immersion of the slides for
stain development. The time required for development was determined by the degree of
staining which was monitored by examination under a light microscope of the known
control slide included in each batch, and as a norm spanned between 5 and 10 min
dependant on primary antibody used. After adequate development of immunostaining,
the reaction was arrested by immersion in 0.1M sodium acetate buffer pH 6.0. The
sections were then dehydrated through graded alcohols (industrial methylated spirit,
BDH Laboratories, UK), cleared in xylene (BDH Laboratories, UK) before being
mounted in DPX microscopy mountant (BDH Laboratories, UK).
2.5.1.2 Antibodies

A panel of antibody markers were used in this study to assess the volume and rate of axonal regeneration, the proliferation density of Schwann cells, the macrophage infiltration and the pattern of angiogenesis within the nerve grafts and conduits. Antibodies to neural peptides and proteins were also used to assess nerve terminal density in skin and neuronal cell bodies in the dorsal root ganglia. The antibodies reacted with known, specific antigens as described below and were applied at exact dilutions as summarised in Table 2.1:

Table 2.1

First layer antibodies and their specific dilutions used in indirect ABC immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5</td>
<td>Rabbit polyclonal</td>
<td>Affiniti</td>
<td>1:5000</td>
</tr>
<tr>
<td>CGRP</td>
<td>Rabbit polyclonal</td>
<td>Affiniti</td>
<td>1:8000</td>
</tr>
<tr>
<td>S-100</td>
<td>Rabbit polyclonal</td>
<td>Dako A/S</td>
<td>1:8000</td>
</tr>
<tr>
<td>ED-1</td>
<td>Mouse monoclonal</td>
<td>Serotec</td>
<td>1:600</td>
</tr>
<tr>
<td>VWf</td>
<td>Rabbit polyclonal</td>
<td>Dako A/S</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Protein gene product 9.5 (PGP) is a neuron-specific cytoplasmic protein (Jackson and Thompson, 1981). Antisera to this marker demonstrates all types of nerve fibres (Dalsgaard et al., 1989) and it is also considered to be the most suitable and practical
pan-neuronal marker for the demonstration of cutaneous nerves (Karanth et al., 1991; Kennedy and Wendelschafer-Crabb, 1993) allowing an accurate assessment of cutaneous reinnervation (Dalsgaard et al., 1989).

Calcitonin gene-related peptide (CGRP) is a 37 amino-acid peptide formed by tissue specific alternative splicing of the calcitonin gene product (Morris et al., 1984). It is found in small myelinated and unmyelinated Aδ and C sensory fibres (Kruger et al., 1989) and is generally the first neuropeptide to be detected in regenerating nerves (Zhao et al., 1993; Iwashita et al., 1994). It is anterogradely transported and accumulates in the distal ends of regenerating axons and is hence clearly visible in the regenerating front of injured nerves (Ekstrom and Tomlinson, 1989), as well as in neuronal cell bodies.

S-100 is a specific Schwann cell cytoplasmic antigen (Moore, 1965; Donato, 1986). The antiserum was used to identify the presence of Schwann cells within the graft or conduit, their progression and proliferation density.

ED-1 is a monoclonal marker specific for macrophages. The ED-1 antibody recognises a single chain glycoprotein expressed predominantly on the lysosomal membrane of tissue macrophages (Dijkstra et al., 1985; Damoiseaux et al., 1994). It was used to estimate the infiltration of the graft or conduit by macrophages, as an indication of the inflammatory response.

Von Willebrand Factor (vWf), previously known as factor VIII-related antigen is isolated from human plasma. The antibody reacts with vWf and positive staining is demonstrated in endothelial cells and megakaryocytes (Sehested and Hou-Jensen, 1981; Bukh et al., 1986). It was used to identify the presence of endothelial cells which in turn
demonstrated the pattern of angiogenesis and infiltration of the conduits by new capillaries.

The second layer biotinylated antibodies used were dependant on the species of origin of the first layer antibody, whether polyclonal or monoclonal, and are summarised in the following table (Table 2.2)

Table 2.2
Second layer antibodies and their specific dilutions used in indirect ABC immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin conjugated anti-rabbit</td>
<td>Goat polyclonal</td>
<td>Vector</td>
<td>1:100</td>
</tr>
<tr>
<td>Biotin conjugated anti-mouse</td>
<td>Horse monoclonal</td>
<td>Vector</td>
<td>1:100</td>
</tr>
</tbody>
</table>

2.5.2 Thionin blue and acridine orange staining

Semithin (1µm) transverse sections of the distal superficial radial nerve in the cat and the distal sciatic nerve in the rat were cut on a Reichert-Jung Ultracut E ultramicrotome, floated onto distilled water, collected on poly-L-lysine coated glass slides and dried on a hot plate. The sections were stained with thionin blue and counterstained with acridine orange to enhance the contrast of the myelin sheaths. Complete section adhesion to the slides was obtained by holding the slide in a yellow bunsen flame for a
few seconds prior to staining. The dried sections were stained with freshly-made filtered thionine (Thionine, Sigma, USA; 0.05g thionine in 22.5 ml absolute alcohol and 5ml 0.1M sodium hydroxide) for 45-60 seconds at 70°C on a hot plate. The slides were then washed in distilled water and stained on the hot plate at 70°C for 30 seconds with acridine orange (Acridine orange, BDH Laboratories, UK; 0.25g acridine orange added to 20ml distilled water and 5ml 0.1M sodium hydroxide). Following a further wash in distilled water, the sections were finally dried on a hot plate and mounted in DPX.

2.6 QUANTIFICATION

Technical error and variation due to surgical procedure was reduced as all experiments were performed by myself and I had previous training and experience in microsurgical techniques. Thus it was ensured that intra-operative factors such as suturing, atraumatic handling, anastomotic tension and rotational alignment were performed in a reproducible manner. To minimise any variation in nerve regeneration attributable to difference in age, sex or genetic variability, all rats used were male eight-weeks old at implantation, from an inbred Lewis strain obtained from the same supplier.

Tissue analysis and all subsequent morphometric assessment were performed on coded sections without knowledge of the source. A computerised image analysis system (Seescan Analytical Services, Cambridge, UK) was used throughout the study in the morphometric quantification of immunostaining and myelinated axons. This system consists of an ORTHOLUX II microscope (Leitz Wetzlar, Germany) mounted with a CCD camera connected to the main computer terminal (Fig.2.13). This allows capture and digitisation of any single frame within a histological specimen. The light source illuminating the specimen under the microscope was maintained at a constant level by a
separate transformer which provides rectified and stabilised direct current to provide constant optimal tissue illumination. The amplitude and voltage were maintained at the same level while capturing images for a set of specimens in the same experimental series. The captured image was automatically edited by background subtraction, image enhancement and thresholding, this being within a narrow range for all measured sections.

Rate of regeneration

The rate of axonal and Schwann cell regeneration was calculated at x10 objective magnification by measuring the absolute distance of penetration in mm of the furthermost regenerating stained fibres or Schwann cells into the graft or conduit from the proximal anastomosis at each time-point. The site of proximal anastomosis in the conduit grafts was clearly identifiable and correlated well on the stained sections by its location 2mm distal to the proximal end of the section, by the constant presence of a short length of randomly orientated regenerating fibres and occasionally by the presence of suture holes. A correction factor of \( -2 \) was applied to compensate for the entubulation of the proximal stump 2mm into the proximal end of the conduit. Crossover of regenerating fibres across the 10mm nerve gap was confirmed by the presence of immunostained fibres in the distal nerve stump. In the autograft group, measurement of Schwann cells was not possible due to the inherent Schwann cell population in the grafted nerve.

Neuronal regeneration and Schwann cell proliferation density

The amount of axonal regeneration and the Schwann cell proliferation density in the graft or conduit was indicated by the staining per frame measured at a fixed point 2mm from the proximal anastomosis, using an eye-piece grid of 1mm length at x10 objective magnification. The staining per frame was measured at this point across the whole width of the graft or conduit at x25 objective magnification. The area of frame was kept
constant throughout the study. For all experimental groups (n=6), two randomly-chosen sections per specimen were used for image analysis.
To assess sensory re-innervation in footpad skin, 6 images of alternating frames of epidermis and sub-epidermis from two randomly chosen sections per specimen were captured. Using a similar quantification protocol, the area of immunoreactive epidermal and sub-epidermal nerve fibres per frame was quantified by assessing the percentage of red in frame by a process of background subtraction, image enhancement, thresholding (maintained within a narrow range for all sections), manual editing and quantification of the final edited image (Figs.2.14). The percentage of red in frame gave an indication of the amount cutaneous reinnervation following nerve repair.

**Macrophage infiltration**

The inflammatory reaction was measured by manual counting of macrophages across the whole width of the nerve at x20 objective magnification in two randomly chosen sections per nerve, stained with ED-1. In the PHB wraparound / primary epineural nerve repair experiments, the macrophages were counted in two adjacent fields on either side of the midpoint across the whole width of the nerve at x20 objective magnification. In the PHB and PDS nerve gap repair experiments, counts were carried out at the midpoint of the graft / conduit sample.

**Myelinated fibre counts**

From each nerve sample, four (cat superficial radial nerve model) or three (rat sciatic nerve model) non-adjacent fields at x40 objective magnification were captured from randomly chosen sections (Fig.2.15). The measured parameters for regenerating myelinated axons were fibre diameter, myelin thickness, g-ratio, shape factor and fibre count per frame, frame area being constant throughout the study for all specimens. The shape factor of a circle is 1, and measurements approximating this value indicate the regular shape of the axons. The percentage distribution of fibres according to their diameter was also charted for each group.
Fig. 2.13 Image analysis system consisting of a CCD camera attached to a light microscope. Images are captured and stored in an optical disc in the main computer.

Fig. 2.14 Quantification sequence to assess the re-innervation of skin.

(a) Thresholding.
Fig. 2.14  Quantification sequence of re-innervation of skin (contd)

(b) Editing – remove objects command

(c) quantification of final edited image.
Neuronal cell bodies

In the rat sciatic nerve model, immunostained cell bodies in DRG were captured at x25 objective magnification. The perimeter of each cell was delineated, and cell numbers and mean cell diameter were automatically calculated. From this data, the cell size distribution was assessed.

2.6 STATISTICAL ANALYSIS

2.6.1 Analysis techniques

Statistical analysis was carried out by Mrs Caroline Doré, Senior Statistician at the Royal Postgraduate Medical School, Hammersmith Hospital, London. STATA (STATA Corporation, Texas, USA) and SIGMASTAT (Jandel Scientific, Germany) software packages were used. The normality assumptions of all data were first checked using Shapiro-Francia's $W'$ test, and Bartlett's test was used to check the equal variance assumption.

The commonly utilised tests were $t$-test, one and two way analysis of variance (one-way and two-way ANOVA), all with or without Bonferroni adjustment. Statistical analysis to compare macrophage counts in the PHB wrap-around and epineural repair groups used the $t$-test, whereas a one-way ANOVA analysed myelinated fibres between PHB wrap-around, epineural repair and normal nerves.

In the rat sciatic nerve model, two-way ANOVA was used to detect significance in the rate and amount of regeneration and macrophage counts between PHB conduit and autograft groups at the early time-points of 7, 14 and 30 days. Schwann cell proliferation density between the same groups was compared with a one-way ANOVA.
Myelinated fibres at 120 days were analysed using the $t$-test. In Chapter 5 dealing with the effect of GGF delivery at the site of nerve injury, $t$-test was predominantly used to detect significance in the rate and amount of regeneration, Schwann cell density and macrophage counts between groups with and without GGF.

Difference between plain and grooved PDS conduits for rate and amount of regeneration, and Schwann cell proliferation density at 7, 14 and 30 days was detected by two-way ANOVA. One-way ANOVA was used in the analysis of data for macrophage and myelinated fibre counts. Data for central neuronal and peripheral target reinnervation was analysed using $t$-test.
Fig. 2.15  Myelinated fibres image analysis sequence comprising of (a) thresholding (b) zooming in to an area for manual editing as the programme is incapable of recognising these axons as separate entities.
Fig. 2.16  Myelinated fibres image analysis sequence (contd.)

(c) Separation of myelinated axons and

(d) quantification of the edited image.
CHAPTER III

PHB, A SYNTHETIC RESORBABLE WRAP-AROUND IMPLANT AS AN ALTERNATIVE NERVE REPAIR TECHNIQUE

3.1 Introduction

3.2 Aims

3.3 Materials and methods

3.4 Results
   3.4.1 Myelinated fibre analysis
   3.4.2 Macrophage counts

3.5 Discussion
3.1 INTRODUCTION

Current microsurgical techniques for peripheral nerve repair give functional results which are not always optimal (Terzis, 1990). The need to improve these results has led to the quest for a sutureless method of nerve repair which would minimally interfere with the internal environment of the injured nerve. The use of adhesives such as cyanoacrylate glue (Ferlic et al., 1965) and fibrin (Moy et al., 1988) have not improved results. Welding tissue with carbon dioxide lasers causes thermal damage to the nerve which exceeds any benefit gained by the absence of foreign material (Richmond, 1986). Nerve tubulization offers an alternative method of repair of severed nerves with maximal coaptation and minimal injury.

Tubulization has several advantages, as it protects regenerating fibres by reducing invasion and scarring of the nerve, and discourages the formation of neuromas. In a randomised clinical trial, Lundborg enclosed the ends of a transected nerve in a silicone tube, leaving a short gap in between to allow accumulation of neurotrophic factors inside the tube at the site of injury. As silicone is a nonabsorbable material, a second exploration of the repair site and removal of the tube was an ethically approved part of the study (Lundborg et al., 1997).

PHB (Astra Tech, Gothenberg, Sweden) is available in the form of sheets which have been used experimentally in cardiovascular surgery (Duvernoy et al., 1995; Malm et al., 1992, 1994). PHB is non-antigenic, biocompatible, easy to handle, has good tensile strength and is completely resorbed within 24-30 months by hydrolytic degradation (Gogolewski et al., 1993; Holmes, 1988; Malm et al., 1992, 1994). These qualities indicate that PHB may be ideally suited for use in nerve repair, particularly for tubulization in end-to-end primary repair.
3.2 AIM

The aim of this study was to test PHB sheets for coapting the severed ends of a nerve, providing an end-to-end primary repair without the need for epineural sutures. The extent of the axonal regeneration and the inflammatory response to PHB were assessed morphologically up to 12 months post-operatively, and compared to standard epineural nerve repair.

3.3 MATERIALS AND METHODS

Twenty adult female cats were divided into two groups (n=10). Under an operating microscope, the superficial radial nerve was exposed bilaterally on the mid-foreleg and transected. The divided nerve was repaired by two techniques (cf 2.1 and 2.2):

**PHB** A PHB sheet was wrapped around the nerve ends, leaving a 2-3mm gap between them, and sealed longitudinally with fibrin glue to form a tube which was secured by one 9(0) ethilon suture at each end away from the transected point (2.2.2).

**ER** Epineurial repair was carried out on the contralateral side in the same animal with 9-0 epineural sutures.

Ten animals from each group were sacrificed at either 6 or 12 months. The level of nerve regeneration was assessed in the nerve distal to the repair site by computerised quantification of myelinated axons (cf 2.5.2 and 2.6). The repair sites were assessed for an inflammatory response gauged by macrophage counts immunostained with a specific marker, ED-1 (cf 2.5.1 and 2.6).
3.4 RESULTS

3.4.1 Myelinated fibre analysis

On qualitative assessment, all specimens demonstrated a mixture of regenerating axons of varying diameters (Fig.3.1 and Fig. 3.2). Degenerating axons, present in minimal numbers, were not counted. Tables 3.1 and 3.2 summarise the values at 6 months, 12 months and in normal non-operated nerves respectively. Statistical analysis, using t-test, showed no significant difference for any measured value between the PHB and primary epineurial repair groups within each time-point. One way ANOVA comparing PHB wrap-around, epineurial repair and normal nerves at 12 months demonstrated a significant difference for all parameters between the PHB group and normal nerves, and similarly between epineurial repair and normal groups (p< 0.05).

Table 3.1. Myelinated fibre analysis: results at 6 months. All values are given as mean ± SEM. *g-ratio = axon diameter / fibre diameter.

<table>
<thead>
<tr>
<th></th>
<th>PHB</th>
<th>Epineurial Repair</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean axon count</td>
<td>971.7 ± 46.61</td>
<td>1029.3 ± 66.51</td>
</tr>
<tr>
<td>Mean fibre diameter (μm)</td>
<td>5.340 ± 0.020</td>
<td>5.306 ± 0.018</td>
</tr>
<tr>
<td>Mean myelin thickness (μm)</td>
<td>1.557 ± 0.004</td>
<td>1.516 ± 0.003</td>
</tr>
<tr>
<td>Mean g-ratio*</td>
<td>0.416 ± 0.002</td>
<td>0.428 ± 0.002</td>
</tr>
<tr>
<td>Mean shape factor</td>
<td>0.851 ± 0.0009</td>
<td>0.834 ± 0.0008</td>
</tr>
</tbody>
</table>
Table 3.2. Myelinated fibre analysis: results at 12 months and in normal non-operated superficial radial nerve. All values are given as mean ± SEM.

P<0.05 for all measured parameters in Epineural repair vs Normal and PHB vs Normal, One way ANOVA all pairwise multiple comparison procedures (Tukey test).

<table>
<thead>
<tr>
<th></th>
<th>PHB</th>
<th>Epineural Repair</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean axon count</td>
<td>896 ± 62.62</td>
<td>942.7 ± 51.41</td>
<td>546.6 ± 26.31</td>
</tr>
<tr>
<td>Mean fibre diameter</td>
<td>6.276 ± 0.026</td>
<td>6.107 ± 0.024</td>
<td>9.238 ± 0.145</td>
</tr>
<tr>
<td>Mean myelin thickness</td>
<td>1.619 ± 0.004</td>
<td>1.557 ± 0.003</td>
<td>2.036 ± 0.026</td>
</tr>
<tr>
<td>Mean g-ratio</td>
<td>0.483 ± 0.001</td>
<td>0.490 ± 0.001</td>
<td>0.54 ± 0.006</td>
</tr>
<tr>
<td>Mean shape factor</td>
<td>0.839 ± 0.0009</td>
<td>0.847 ± 0.0008</td>
<td>0.836 ± 0.006</td>
</tr>
</tbody>
</table>

Comparison within the same experimental group through time (i.e. PHB or primary epineurial repair at 6 and 12 months) showed a significantly greater fibre diameter in both the primary repair group (Mann-Whitney rank sum test, p=0.003) and the PHB group (Mann-Whitney rank sum test, p=0.011) (Table 3.3). This increase in fibre diameter with time indicates a continuing maturation of the axon. This is more clearly shown in Figures 3.3 and 3.4, where the percentage distribution of myelinated axons has been plotted according to their size at both time-points and in normal non-operated nerves. At 6 months, the percentage pattern in PHB is similar to that of primary repair, both groups showing a unimodal distribution and a preponderance of smaller fibres with a major peak at 3-4 µm fibre diameter. At 12 months, the percentage pattern is again similar between the two groups. However, despite a persistent but smaller peak at 3-4µm, the fibre distribution is much wider, with a definite increase in larger diameter.
fibres (8-12 μm diameter). The normal uninjured nerves exhibited a bimodal distribution over a range of 2 to 15 μm with peaks at 5 and 11 μm. Both operated groups also showed a decrease in mean axon count at 12 months compared with 6 months, which may indicate a die-back of axonal sprouting with continuing maturation.

Table 3.3: Comparison of fibre diameter within each experimental group, PHB and epineurial repair: 6 months vs 12 months, P<0.05.

All values are given as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>PHB</th>
<th>Epineurial Repair</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean fibre diameter (μm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 months</td>
<td>5.340 ± 0.020</td>
<td>5.306 ± 0.018</td>
</tr>
<tr>
<td>12 months</td>
<td>6.276 ± 0.026</td>
<td>6.107 ± 0.024</td>
</tr>
<tr>
<td>P-value</td>
<td>0.011</td>
<td>0.003</td>
</tr>
</tbody>
</table>

3.4.2 Macrophage counts

On qualitative microscopic assessment, in the PHB group the polymer remnants appeared to be decreased at 12 months compared with 6 months with disruption in fibre continuity and increased fragmentation of the polymer (Fig. 3.5). Table 3.4 summarises the values of macrophage counts in the epineurial repair and PHB groups at 6 and 12 months. Although there was some difference between groups, this was not statistically significant difference (epineurial repair vs PHB at 6 months: P=0.536, at 12 months: P=0.798) indicating that the foreign body reaction to PHB is comparable to that of suture repair.
Fig. 3.1 Transverse sections of the distal nerve stained with thionin blue and acridine orange to enhance the myelin contrast at 12 months in:

(a) PHB wrap-around group (x 25 objective magnification)

(b) Epineurial repair group (x 25 objective magnification)
Fig. 3.2 Transverse sections of normal non-operated superficial radial nerve in the cat at
(a) x10 objective magnification to demonstrate the fascicular arrangement
(b) x40 objective magnification showing normal axons.
Fig. 3.3 Size distribution of myelinated fibres at 6 months. The percentage pattern in PHB is similar to that of epineural repair, both groups showing a preponderance of smaller fibres, with a major peak at 3-4 μm.
Fig. 3.4 Size distribution of myelinated fibres at 12 months (PHB and Epineurial repair) and in normal non-operated superficial radial nerves. The percentage pattern in PHB is similar to that of epineurial repair. The distribution of both groups is much wider with an increase in larger diameter fibres (8-12 μm). The distribution in normal nerve is bimodal with peaks at 5 and 11 μm.
Fig. 3.5 Longitudinal sections of the repair site in PHB group demonstrating
(a) fragmentation of the PHB polymer at x 10 objective magnification and
(b) immunostaining of macrophages with ED-1 antibody (x 40 magnification).
Table 3.4. Macrophage counts: Results of PHB vs epineurial repair at 6 and 12 months. Macrophages were counted over two adjacent fields on either side of the midpoint of the repair site across the whole width of the nerve at x20 magnification. All values are given as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>PHB</th>
<th>Epineurial Repair</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 months</td>
<td>100.83 ± 31.13</td>
<td>72.27 ± 21.19</td>
</tr>
<tr>
<td>12 months</td>
<td>92.68 ± 22.15</td>
<td>87.66 ± 19.30</td>
</tr>
</tbody>
</table>

3.5 DISCUSSION

The results of this study demonstrate that the axonal regeneration assessed by myelinated axon counts was similar in the two experimental groups and that PHB produced an inflammatory response gauged by the macrophage infiltration, similar to that found in primary epineurial repair with no evidence of scar tissue. The unequivocal conclusion is that PHB wrap-around is a good alternative to epineurial repair.

Sutures do not permit changes in the fascicular arrangements, which may be incorrectly made by a surgeon (Brunelli et al., 1994). Tubulization is a more ‘biological’ approach to nerve repair whereby the neural tissue is allowed to heal by its intrinsic capacity in a closed space with minimal surgical trauma. Encasing the ends of a transected nerve in a tube with a short gap between the nerve stumps allows accumulation of locally produced neurotrophic factors. In a series of 18 patients, comparing conventional microsurgical repair with silicone tubulization in median and ulnar nerve injuries, Lundborg et al. (1997) demonstrated no difference in the results between the two...
techniques. However, a second operation was necessary for removal of the silicone tube as it is non-absorbable and may cause local discomfort. Silicone entubulation can also lead to chronic nerve compression (Merle et al., 1989). As PHB is a bioabsorbable conduit, it would offer an alternative solution for the treatment of nerve injuries. Previous studies have shown that PHB is absorbed over 24 – 30 months (Malm et al., 1992, 1994). As expected, in this study the polymer underwent progressive degradation, qualitatively indicated at 6 and 12 months by disruption in the continuity of the fibres, fragmentation and a decrease in the polymer. The foreign body reaction to PHB as seen by the macrophage infiltration, was minimal and similar to the reaction to sutures used in epineurialrepair.

Morphometric analysis of the myelinated axons within the same experimental groups showed significant difference in the fibre diameter at 12 months in comparison to 6 months, with a progressive increase of fibre diameter indicating continuing axonal maturation. The normal non-operated nerves exhibited fibres which were significantly larger than at 12 months than in either of the two operated groups, implying that axonal maturation was still ongoing. However, in both PHB and epineurialrepair groups the axon counts at 6 months was greater than at 12 months. It is known that during regeneration after axotomy, there is sprouting of axon collaterals with subsequent pruning back, most likely as a result of successful connections with the target organs by surviving axons (Brushart,1993; Lundborg et al., 1994). The myelinated axon counts in normal uninjured nerves were significantly less than either the PHB or epineurialrepair groups at 12 months, indicating that further connections with target organs and axonal die-back continues to take place at 12 months. The size distribution pattern showed a consistent shift towards an increase in larger axons over time, with a decrease of smaller, possibly branching, fibres. A similar pattern was seen in other studies in which
a synthetic conduit was used to bridge a nerve gap (den Dunnen et al., 1931; Dellon et al., 1988).

There is a need to improve the functional results obtained from conventional microsurgical repair. Tubulization with a synthetic, bioabsorbable and relatively inert conduit such as PHB offers an alternative solution. It is easier and simpler to encase crushed, transected nerve stumps in a PHB tube allowing the nerve to heal by its intrinsic capacity rather than attempt to align the traumatised fascicles correctly by surgery with the risk of further damage. Although the technique of PHB tubulization may have some limitations at present, it has an attractive potential, especially in combination with exogenous neurotrophic factors which are known to improve nerve regeneration.
CHAPTER IV

PHB CONDUIT AS AN ALTERNATIVE TO NERVE AUTOGRAFT IN NERVE GAP REPAIR

4.1 Introduction

4.2 Aim

4.3 Materials and methods

4.4 Results
   4.4.1 Rate of axonal regeneration
   4.4.2 Amount of regeneration
   4.4.3 Macrophage infiltration
   4.4.4 Angiogenesis
   4.4.5 Myelinated fibre analysis

4.5 Discussion
4.1 INTRODUCTION

The use of nerve grafts to bridge irreducible nerve gaps results in sub-optimal functional results and donor site morbidity. This has led to a revival of interest in the search for a usable nerve conduit, which needs to have several properties. It should be inert, flexible, bioresorbable and inhibitory to pathological processes such as scarring and oedema, but beneficial to processes of healing and regeneration (Fields et al., 1989). Besides being bioabsorbable, the conduit should remain in situ without degradation beyond the period of time it takes the regenerating axons to cross the gap and penetrate the distal stump. Poly-3-hydroxybuytrate (PHB) possesses all these criteria, and the advantages of a PHB wrap-around in primary repair have been demonstrated (Hazari et al., 1999). In this study, the same material has been used as a conduit to bridge a 10mm gap in the rat sciatic nerve.

4.2 AIM

The aim of this study was to assess the level of regeneration in a PHB conduit bridging a nerve gap in comparison with the current surgical gold standard, a nerve autograft. Nerve regeneration was assessed in the short-term by quantifying the rate and amount of regeneration, and with myelinated fibre counts in the long-term.

4.3 MATERIALS AND METHODS

The rate and amount of regeneration was assessed by dividing 48 eight week-old inbred male Lewis rats into two groups. Under the operating microscope, a segment of the left sciatic nerve in the rat was excised and repaired using one of the two experimental techniques (cf 2.1.1 and 2.3):
A 5 mm nerve segment was resected to produce a 10 mm nerve gap which was then bridged using a 14 mm PHB conduit, entubulating 2 mm of the nerve stump at each end.

In the autograft group, a 10 mm nerve segment was resected, reversed and re-sutured in the gap with 10/0 ethilon sutures.

Six animals from each group were sacrificed at 7, 14, 30 and 120 days post-operatively. At the short-term time points, the repair sites (PHB conduit or nerve autograft) were processed (cf 2.4.1) and assessed morphologically for the rate and amount of regeneration, the Schwann cell proliferation density, the inflammatory response to PHB and the quality of angiogenesis (cf 2.6). Schwann cell proliferation density was measured only in the PHB group, as in the autograft group, measurement of S-100 was not possible due to the inherent Schwann cell population.

In the long-term, at 120 days, the distal nerve was assessed for myelinated fibre counts (cf 2.5.2 and 2.6).

4.4 RESULTS
4.4.1 Rate of axonal regeneration

The rate of regeneration was measured as penetration of graft or conduit by the furthermost immunostained fibres from the proximal anastomosis. Table 4.1 outlines the distance of regeneration for PGP and CGRP immunostained regenerating axons. At 7 days, axons in the nerve graft had crossed 2/3 of the nerve graft and in the PHB conduit had penetrated 1.02 mm of the conduit (Fig.4.1). By 14 days, regenerating axons had reached the distal stump in the nerve grafts, whereas these were almost up to
the halfway mark in PHB conduits. At 30 days, regenerating fibres in all animals in both
groups had reached the distal stump. For PGP-immunostained axons, there was a
significant difference in the regenerative distance between the two groups along time at
7 and 14 days (two-way ANOVA, p <0.001). This pattern was reflected in CGRP-
immunostained fibres (Table 4.1).

4.4.2 Amount of regeneration
The values for percentage of immunostaining of PGP and CGRP at 7, 14 and 30 days
are given in Table 4.2. Comparison between the two groups at each of the time-points
was not statistically significant except for PGP at 30 days. There was also a linear trend
with time for both PGP (p=0.01) and CGRP (p=0.04) (Fig.4.2), indicating that though
axonal regeneration identified with the two markers is comparable between the graft
types and shows a progressive increase with time, the level of regeneration in PHB
conduit is slower, the difference being significant (p<0.05) by 30 days.

Schwann cell proliferation density measured in the PHB group showed an increase with
time, as shown in Table 4.2. Though there was a linear trend with time, the Schwann
cell density appeared to reach a plateau between 14 and 30 days (Fig.4.3). At this time,
the axons had reached the distal stump and the levelling effect may be a reflection of the
decreased impetus for Schwann cells to proliferate.
Fig. 4.1  Immunostaining of the regenerating front in a PHB conduit at 7 days with (a) PGP and (b) S-100 at x20 objective magnification.
Table 4.1  Maximum regeneration distance from the proximal anastomosis. Distances are given in millimetres as the mean ±SEM, (n=6). * p<0.001, AG vs PHB at 7-14 days. AG = nerve autograft; PHB = conduit.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PGP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>7.625±0.57*</td>
<td>&gt;10*</td>
<td>&gt;10</td>
</tr>
<tr>
<td>PHB</td>
<td>1.029±0.18</td>
<td>4.35±0.37</td>
<td>&gt;10</td>
</tr>
<tr>
<td><strong>CGRP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>8.15±0.28*</td>
<td>&gt;10*</td>
<td>&gt;10</td>
</tr>
<tr>
<td>PHB</td>
<td>0.791±0.17</td>
<td>3.566±0.49</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

Table 4.2  Percentage of immunostaining per frame 2 mm from the proximal anastomosis across the whole width of the nerve. All values are expressed as mean ± SEM. * p<0.001, *-test with Bonferroni correction, PGP at 30 days, AG vs PHB. AG = nerve autograft; PHB = conduit.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PGP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>3.318±0.81</td>
<td>5.842±0.81</td>
<td>7.125±0.89*</td>
</tr>
<tr>
<td>PHB</td>
<td>3.59±0.81</td>
<td>3.639±0.81</td>
<td>4.139±0.81</td>
</tr>
<tr>
<td><strong>CGRP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>2.724±0.61</td>
<td>3.196±0.61</td>
<td>5.002±0.67</td>
</tr>
<tr>
<td>PHB</td>
<td>2.867±0.61</td>
<td>2.884±0.61</td>
<td>3.277±0.61</td>
</tr>
<tr>
<td><strong>S-100</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHB</td>
<td>5.936±1.004</td>
<td>11.16±0.488</td>
<td>12.06±1.27</td>
</tr>
</tbody>
</table>
Fig. 4.2 Percentage of PGP-immunostaining showing the progression of axonal regeneration, AG vs PHB at 7, 14 and 30 days, with a significant linear trend. p<0.05, two-way ANOVA. All values are expressed as mean ± SEM.
Fig. 4.3 Schwann cell proliferation density in PHB conduit at 7, 14 and 30 days showing a significant linear trend. $p<0.001$, One-way ANOVA. Values have been expressed as mean ± SEM.
4.4.3 Macrophage infiltration

The inflammatory reaction to PHB was gauged by macrophage infiltration of the conduit. ED-1 immunostained macrophage counts for both groups are summarised in Table 4.3. Comparison between autograft and PHB groups showed no significant difference for macrophage counts at any of the time-points and demonstrates that the inflammatory response to PHB is not intense and was similar to that seen in nerve grafts. On the other hand, an interesting observation is that macrophage numbers appeared to increase from 7 to 14 days and then decreased to their lowest at 30 days. A two-way analysis of variance showed significantly higher numbers in both groups at 14 days compared to 30 days. This correlates well with an infiltration by macrophage scavengers at the injury site following transection, the activity increasing at 14 days and then tailing off as the regenerating axons reach the distal stump by 30 days.

Table 4.3 Macrophage counts. Results of AG and PHB at 7, 14 and 30 days.

Macrophages were counted one frame wide at the midpoint of the conduit /graft across the whole width of the nerve at x20 magnification. All values are given as mean ± SEM. Two-way ANOVA, p<0.05, both groups, 14 vs 30 days. AG = nerve autograft; PHB = conduit.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>15.83±1.82</td>
<td>20.08±1.53</td>
<td>12.5±1.6</td>
</tr>
<tr>
<td>PHB</td>
<td>16.08±2.14</td>
<td>18.66±2.7</td>
<td>11.91±1.2</td>
</tr>
</tbody>
</table>
4.4.4 Angiogenesis

A qualitative assessment of the vWf-immunostained sections showed good capillary penetration of the PHB conduit from either end along the proximal and distal nerve stumps and through the walls of the conduit (Fig. 4.4).

Fig. 4.4 Angiogenesis in PHB conduit at 7 days using the endothelial marker, vWf at x25 magnification, demonstrating new blood vessel formation through the walls of the conduit.
4.4.5 Myelinated fibre analysis

On qualitative assessment, there were regenerating axons of varying diameters. The measured parameters were axon counts, fibre diameter, myelin thickness, g-ratio and shape factor (Table 4.4). The shape factor of a circle is 1 and values close to one indicate the regular shape of axons. Axon counts were significantly greater in the nerve graft group compared to the PHB group. In the latter group, the fibres were also smaller in diameter with a significantly lower g-ratio (p<0.001, t-test with Bonferroni correction). However, the percentage distribution of myelinated fibres according to their size demonstrated that the pattern was similar in the two experimental groups (Fig. 4.5).

Table 4.4 Myelinated fibre analysis at 120 days. All values are given as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>PHB</th>
<th>Nerve Autograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon count</td>
<td>341 ± 82.43*</td>
<td>1105 ± 30.9*</td>
</tr>
<tr>
<td>Fibre diameter (µm)</td>
<td>3.931 ± 0.125*</td>
<td>4.67 ± 0.064*</td>
</tr>
<tr>
<td>Myelin thickness (µm)</td>
<td>1.058 ± 0.054</td>
<td>1.09 ± 0.013</td>
</tr>
<tr>
<td>g-ratio</td>
<td>0.441 ± 0.22*</td>
<td>0.5 ± 0.006*</td>
</tr>
<tr>
<td>Shape factor</td>
<td>0.859 ± 0.005</td>
<td>0.871 ± 0.01</td>
</tr>
</tbody>
</table>

* P<0.001, t-test with Bonferroni correction, PHB vs Autograft.

g-ratio = axon diameter / fibre diameter.
Fig. 4.5 Size distribution of myelinated fibres at 120 days. The percentage pattern pattern in PHB is similar to that in the nerve autograft group, both groups showing a preponderance of smaller fibres, with a peak at 3-4 μm.
4.5 DISCUSSION

The last 20 years have seen the development of many synthetic conduits and several excellent reviews are available summarising the strengths and failings of each of these (Fields et al., 1989; Seckel, 1990; Terris and Fee, 1993; Brunelli et al., 1994; Doolabh et al., 1996). Silicone has been the most widely used for experimental tubulization and has also been applied clinically (Lundborg et al., 1997). However, due to the lack of degradation of the silicone implants, it has been advocated that the next step in nerve gap repair is the use of a bioabsorbable synthetic conduit (Lundborg et al., 1997), which would elicit the most minimal of inflammatory reactions and would remain in situ long enough to support regeneration. Flexibility of the conduit is also necessary to enable continued protection of the regenerating nerve on initiation of mobilisation of the injured part.

PHB fulfils these criteria to a large extent. Besides being bioabsorbable, it elicits a low macrophage reaction comparable to that of a nerve graft. This was also observed in a previous study wherein a PHB wrap-around sheath to join the divided nerve was compared to primary epineural nerve repair (Hazari et al., 1999). In this study, macrophage numbers in both groups are similar with no significant difference, increasing from 7 to 14 days, this increase correlating well with the period of intense phagocytic activity during Wallerian degeneration (Perry and Brown, 1992). There is then a significant decrease by 30 days when the axons have reached the distal stump. Clinically, a low inflammatory response would be desirable to prevent adhesions to surrounding structures, in particular, to tendons in hand injuries.

The rate of regeneration measured by the regenerative distance into the conduit from the proximal repair site, though not fully equivalent to that of a nerve graft, still appears to
be comparable for both PGP- and CGRP-immunostained fibres. Regenerating axons
grow into the first part of the PHB conduit by 7 days, come up to the half-way mark by
14 days and reach the distal stump by 30 days. It is important to note that there was no
failure of regeneration in any of the implanted conduits. This time-scale is consistent
with observations made when other biodegradable conduits such as fibronectin mats
have been used (Whitworth et al., 1995). Despite good results obtained with fibronectin
mats, the clinical application of this type of conduit may be difficult as it undergoes
rapid reabsorption in 3 weeks and has inherent problems associated with pooled plasma
from which it is manufactured (Whitworth et al., 1995; Sterne et al., 1997). A longer re-
absorption time as with PHB, ensures that the regenerating and maturing nerve is able to
withstand the stress of mobilisation.

The volume of axonal regeneration quantified by the percentage of PGP and CGRP
immunostaining appears to be comparable between the two graft types. There is a
progressive increase in the amount of regeneration with time, although the difference
between nerve grafts and PHB becomes statistically significant by 30 days. The
regeneration pattern in the PHB conduit is reflected by the Schwann cell proliferation
density, which levels off between 14 and 30 days. This result correlates well with the
slowing down of the rate of Schwann cell proliferation demonstrated after the initial
burst during Wallerian degeneration, which is thought to be signalled by axonal contact
(Hall, 1986; DeVries, 1993).

Predictably, as only empty PHB conduits were used in the study, the number of
myelinating axons were less in the PHB group on comparison with the nerve graft
group. Though the fibre size was smaller in the PHB conduits, the size distribution
pattern was similar in the two groups. These results are in keeping with those obtained
using other conduits (Fields et al., 1989; den Dunnen et al., 1993; Whitworth et al., 1995). However, PHB has certain advantages over other conduits. It is bioabsorbable, has a low inflammatory response with good angiogenesis, does not compress the regenerating nerve and surgically, is an easy material to use.

In conclusion, this study demonstrates good axonal regeneration in PHB conduits with a low level of inflammatory infiltration. This is a good indication of the suitability of PHB as a resorbable synthetic conduit for nerve gap repair. We are aware that the rate and amount of regeneration in PHB conduits does not fully compare with that observed in nerve grafts, but this difference is probably due to the cellular elements inherently present in nerve grafts which aid regeneration. It is well known that de-differentiated Schwann cells synthesise neurotrophic factors (Zorick and Lemke, 1996) which are known to promote nerve regeneration when administered exogenously to an injured nerve (Whitworth et al., 1996; Sterne et al., 1997). It is tempting to speculate that the level of regeneration in a PHB conduit may be further improved by the addition of growth factors, bringing nearer the concept of a composite conduit to obtain optimal nerve regeneration.
CHAPTER V

GLIAL GROWTH FACTOR ENHANCES NERVE REGENERATION IN PHB CONDUIT

5.1 Introduction

5.2 Aim

5.3 Materials and methods

5.4 Results
   5.4.1 Rate of regeneration
   5.4.2 Amount of regeneration and Schwann cell proliferation density
   5.4.3 Macrophage infiltration
   5.4.4 Angiogenesis

5.5 Discussion
5.1 INTRODUCTION

Schwann cells share an intimate relationship with neurons and are essential for their regeneration (Hall, 1997). Glial growth factor (GGF) is a Schwann cell mitogen, first isolated from bovine pituitary (Raff et al., 1978). GGF is also trophic for Schwann cell precursors and at higher concentrations drives proliferation and maturation (Dong et al., 1995). At submaximal levels, GGF increases the motility of cultured Schwann cells and, applied focally, it causes directed migration in monolayer cultures, while at higher doses causes proliferation (Mahanthappa et al., 1996).

5.2 AIM

An increase in Schwann cell numbers would be beneficial to axonal regeneration. Therefore, it could be hypothesised that delivery of GGF in a synthetic conduit would indirectly enhance nerve regeneration. The aim of this study was to investigate morphologically the effect of GGF target administration on the level of nerve regeneration.

5.3 MATERIALS AND METHODS

Twenty-four 8 week-old inbred male Lewis rats were used in the study. Under the operating microscope, a 5 mm segment of the left sciatic nerve was resected to result in a nerve gap of 10 mm. The gap was bridged using a PHB conduit (cf 2.1 and 2.3). The animals were divided into two groups as follows:

<table>
<thead>
<tr>
<th>GGF</th>
<th>The PHB conduit was filled with GGF-augmented Matrigel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrigel</td>
<td>The PHB conduit was filled with plain Matrigel.</td>
</tr>
</tbody>
</table>
Silanised and pre-cooled apparatus was used in handling and delivering GGF-augmented Matrigel and Matrigel alone. Six animals from each group were sacrificed at 7 and 14 days post-operatively. The repair sites with a 2 mm length of proximal and distal nerve were harvested *en bloc* and assessed morphologically for the rate and amount of regeneration, the Schwann cell proliferation density, the inflammatory response and the pattern of angiogenesis.

5.4 RESULTS

5.4.1 Rate of regeneration

The penetration into the conduit of the furthermost immunostained regenerating axons was measured in mm from 2 mm within the proximal end of the PHB conduit (i.e. the end of the proximal nerve stump). The values are summarised in Table 5.1. At 7 days, PGP-immunostained axons had penetrated 2.25 mm in the GGF-augmented Matrigel filled conduits, reaching the half-way mark at 14 days. These were significantly further into the conduit than in the Matrigel only PHB conduits (p<0.05, *t*-test, GGF vs Matrigel at 7 days and at 14 days). A similar pattern was reflected in the CGRP-immunostained regenerating axons (Table 5.1).

5.4.2 Amount of regeneration and Schwann cell proliferation density

The amount of regeneration at 7 and 14 days for PGP and CGRP immunostained axons is given in Table 5.2. At 7 days, the difference between the two experimental groups was not significant. However, at 14 days, the amount of regeneration for PGP-immunostained axons was significantly higher in the GGF-augmented Matrigel group.
Table 5.1 Maximum regeneration distance in mm. All values are expressed as mean ± SEM. * p<0.05 (t-test) GGF vs Matrigel. GGF= GGF-augmented Matrigel, Matrigel= Matrigel only delivered in PHB conduits (n=6).

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td>GGF</td>
<td>2.25 ± 0.104*</td>
</tr>
<tr>
<td></td>
<td>Matrigel</td>
<td>1.75 ± 0.108</td>
</tr>
<tr>
<td>CGRP</td>
<td>GGF</td>
<td>2.03 ± 0.148*</td>
</tr>
<tr>
<td></td>
<td>Matrigel</td>
<td>1.60 ± 0.047</td>
</tr>
</tbody>
</table>

Table 5.2 Percentage immunostaining per frame across the whole width of the conduit, 2 mm distal to the proximal end of the PHB conduit. All values are expressed as mean ± SEM. *p< 0.05, t-test, GGF vs Matrigel. GGF= GGF-augmented Matrigel, Matrigel= Matrigel only delivered in PHB conduits.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td>GGF</td>
<td>5.58 ± 0.754</td>
</tr>
<tr>
<td></td>
<td>Matrigel</td>
<td>4.60 ± 0.346</td>
</tr>
<tr>
<td>CGRP</td>
<td>GGF</td>
<td>2.15 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>Matrigel</td>
<td>2.11 ± 0.22</td>
</tr>
<tr>
<td>S-100</td>
<td>GGF</td>
<td>11.29 ± 0.85*</td>
</tr>
<tr>
<td></td>
<td>Matrigel</td>
<td>5.85 ± 1.025</td>
</tr>
</tbody>
</table>
**Fig. 5.1** Percentage immunostaining per frame indicating the amount of axonal regeneration and Schwann cell proliferation density in PHB conduits with or without the addition of GGF at 7 and 14 days. *p<0.05, One way ANOVA (Tukey test) for S-100 at 7 days and PGP, S-100 at 14 days: GGF vs PHB and GGF vs Matrigel.
Schwann cell density was significantly greater at 7 days in the GGF-augmented Matrigel group compared to plain Matrigel due to the proliferative effect of GGF on Schwann cells (Table 5.2). At 14 days, the difference between the two groups was maintained, and this was reflected also by the increase in the amount of PGP-immunostained regenerating axons.

The difference between the two groups was also examined in relation to the results obtained with empty PHB tubes (cf chapter 4). The values for PGP, CGRP and S-100 immunostaining percentage at both time-points are shown in Figure 5.1. Differences between the groups were significant for S-100 immunostaining at both 7 and 14 days (p<0.001), with the GGF group showing higher values than that of the other two groups on both days. For PGP-immunostaining, the GGF group showed a greater amount of regeneration already at 7 days, although the difference became significant only at 14 days. At both time-points there was no difference for either PGP or S-100 immunostaining between the empty PHB and plain Matrigel groups, indicating that this substrate alone does not have any particular effect on nerve regeneration.

5.4.3 Macrophage counts

Macrophage counts are summarised in Table 5.3. The values for the two experimental groups were comparable reinforcing the point previously demonstrated in other chapters that PHB does not produce an intense inflammatory response.
Table 5.3 Macrophage counts at 7 and 14 days. All values are expressed as mean ± SEM. GGF=GGF-augmented Matrigel, Matrigel= Matrigel only group.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGF</td>
<td>11.91 ± 0.72</td>
<td>16.33 ± 1.95</td>
</tr>
<tr>
<td>Matrigel</td>
<td>12.41 ± 1.04</td>
<td>10.66 ± 1.07</td>
</tr>
</tbody>
</table>

5.4.4 Angiogenesis

Immunostaining for endothelial cells with ED-1 was carried out to determine the pattern of angiogenesis and blood supply to the regenerating nerve. Capillary infiltration appeared to be from the proximal and distal stumps into the conduit and through the walls of the PHB tube, in keeping with observations made previously (cf chapters 3 and 4).

5.5 DISCUSSION

The aim of the study was to determine whether the addition of glial growth factor in a PHB conduit would enhance the rate and amount of axonal regeneration. Indeed, the results have demonstrated that this growth factor indirectly enhances nerve regeneration by increasing the proliferation of Schwann cells.

Improvement in nerve regeneration has been seen in other nerve conduits with the addition of nerve growth factor (NGF), brain-derived growth factor (BDNF) and neurotrophin-3 (NT-3), and their effects on regeneration and reinnervation of motor and sensory target organs have been documented (Whitworth et al., 1996; Lewin et al.,
The main difference with the results outlined in this study is that these growth factors act directly on sensory or motor neuronal sub-populations (Lindsay, 1996).

Schwann cells are quiescent in adult nerves, but they retain the capacity to divide, de-differentiate and proliferate after nerve injury (Selzer et al., 1980). On transection of a nerve, factors promoting Schwann cell migration and proliferation, including neuronal-derived GGF, accumulate in the exudate at the injury site (Le Beau et al., 1988). Schwann cells, in turn, migrate out of transected distal nerve stumps (Martini et al., 1990) and produce a host of factors, which provide trophic support for the regenerating axons. In our experiments, this axonal-glial interdependence after nerve injury was already evident at 7 days by a GGF-induced increase in proliferation of Schwann cells. The effect of this upregulation of Schwann cell density in the GGF group was reflected by a significantly increased rate and amount of regeneration of PGP-immunostained fibres, which highlights the close interdependence between glial and neuronal cells in the regeneration process. It is known that GGF is synthesised in neuronal cells (Marchionni et al., 1994), and the effect of GGF is regulated through the expression of erbB receptors on the surface of Schwann cells. Rapid upregulation of the receptor in denervated nerve stumps has been correlated with the increase in GGF in response to axotomy (Carroll et al., 1997; Li et al., 1997). Conversely, in chronic denervation, the erbB receptors are down-regulated, with subsequent lack of response by Schwann cells to a delayed regeneration stimulus (Li et al., 1997).

The inflammatory response to PHB was not intense, in accordance with similar observations in other studies involving the use of PHB (cf chapters 3 and 4), indicating that GGF itself does not promote an inflammatory response. Similarly, infiltration of the
conduit by newly-formed blood vessels also appeared to follow the same pattern of angiogenesis seen previously (cf chapters 3 and 4).

In conclusion, GGF indirectly enhances nerve regeneration \textit{in vivo} when applied locally at the site of nerve injury. The results presented in this study outline the sequence of events that occur in the early phase of regeneration characterised by GGF-driven proliferation of Schwann cells, followed by an increase in Schwann cell-mediated axonal regeneration. It remains to be investigated whether this beneficial effect of GGF persists in the long-term and results in an improved level of nerve regeneration and re-innervation of target organs.
CHAPTER VI

PERIPHERAL NERVE REGENERATION IN POLYDIOXANONE CONDUITS

6.1 Introduction

6.2 Aim

6.3 Materials and methods

6.4 Results
   6.4.1 Rate of regeneration
   6.4.2 Amount of regeneration
   6.4.3 Schwann cell proliferation density
   6.4.4 Macrophage counts
   6.4.5 Angiogenesis
   6.4.6 Myelinated fibre analysis

6.5 Discussion
6.1 INTRODUCTION
In the previous chapters we have examined the suitability of PHB as a bioresorbable conduit material for nerve repair and grafting. Another material used for the preparation of nerve conduits is polydioxanone (PDS), which is widely used as a suture material in vascular, abdominal and plastic surgery (Berry et al., 1981; Chusak and Dibbell, 1983; Hoile, 1983; Myers et al., 1986). PDS was shaped as a tube conduit with either a smooth or grooved internal surface. Indeed, it is known that improvement of nerve regeneration occurs when there is longitudinal orientation of conduit material components, which produce micro-topographical control of Schwann cell migration (Curtis and Wilkinson, 1997). Etching of longitudinal grooves along the internal surface of PDS conduits provides this surface microgeometry and is likely to enhance regeneration.

6.2 AIM
The aim of this study was to determine the suitability of PDS as a conduit material and to assess the effect of surface microgeometry on the level of regeneration. Nerve regeneration was assessed in the short-term by quantifying the rate and amount of regeneration and by myelinated fibre analysis in the long-term.

6.3 MATERIALS AND METHODS
Forty-eight 8-week old inbred male Lewis rats were divided into two groups. Under the operating microscope, a 5 mm segment of the left sciatic nerve was resected. The nerve stumps retracted to produce a 10 mm gap, which was bridged using one of the two types of PDS conduits (cf. 2.1.1 and 2.3)
The internal surface of the PDS conduit was smooth.

The internal surface of the PDS conduits was etched with longitudinal grooves of 6-10 μm depth and 12.5 μm width (pitch = widthx2 = 25 μm).

Six animals from each group were sacrificed at 7, 14, 30 days and at 6 months. At the short-term time-points, the grafts were processed and assessed morphologically for the rate and amount of axonal regeneration, Schwann cell proliferation density, inflammatory response and the pattern of angiogenesis. In the 6 months group, myelinated fibres were analysed and counted. In addition, the central neuronal survival and target-organ re-innervation were assessed (cf chapter 7).

6.4 RESULTS

On macroscopic examination at harvesting of the repair sites, longitudinal fracturing of both types of conduit was found at 7 days (Fig.6.1). By 14 days, multiple fractures in the conduit material were seen. At 30 days, this had progressed to fragmentation and care had to be taken not to damage the fragile regenerating bridge. The fragments had to be removed piece-meal to facilitate harvesting of the specimen.

6.4.1 Rate of regeneration

The rate of axonal regeneration was measured as the penetration distance in mm of the furthermost immunostained fibres at the regenerating front from the proximal anastomosis (Fig.6.2). Table 6.1 summarises the results for PGP and CGRP-immunostained axons. At 7 days, in both groups the PGP-immunostained nerve fibres had penetrated less than a mm of conduit. By 14 days, the axons had penetrated up to a
third into the conduit and by 30 days had traversed the gap to the distal stump in both groups. There was no statistically significant difference between the values obtained for the two experimental groups at any of the time-points (t-test).

6.4.2 Amount of regeneration

Values of the percentage of immunostaining per frame for PGP and CGRP at 7, 14 and 30 days are given in Table 6.2. The amount of PGP and CGRP immunostaining in the two experimental groups was similar at each of the time-points. However, a significant linear trend through time was observed in the amount of regeneration of PGP-immunoreactive axons for both groups. The percentage immunostaining increased steadily from day 7 to day 30 (Two way ANOVA, p<0.05). The results indicate that the amount of regeneration was similar between the experimental groups but showed a progressive increase of axonal regeneration with time. For CGRP-immunostained axons there was also an increase with time, but this was moderate and did not reach statistical significance along time.

6.4.3 Schwann cell proliferation density

The increase in Schwann cell density through time was significant for both groups (two way ANOVA, p<0.05) (Table 6.2). However, comparison between plain and grooved conduits was not statistically significant, indicating that the presence of grooves did not significantly increase the density of proliferating Schwann cells.
Fig. 6.1 Intra-operative photograph at the time of harvesting of a PDS conduit 14 days post-operatively showing longitudinal fractures.

Fig. 6.2 Regenerating front of CGRP-immunostained fibres in PDS conduit at 7 days.
Table 6.1 Maximum regeneration distance from the proximal anastomosis. Distances are given in mm as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plain</td>
<td>0.30 ± 0.16</td>
<td>3.22 ± 0.29</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Grooved</td>
<td>0.47 ± 0.25</td>
<td>3.26 ± 0.29</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CGRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plain</td>
<td>0.33 ± 0.05</td>
<td>3.14 ± 0.41</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Grooved</td>
<td>0.40 ± 0.22</td>
<td>3.21 ± 0.22</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

Table 6.2 Percentage immunostaining per frame across the whole width of the nerve 2mm from the proximal anastomosis. All values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plain</td>
<td>1.863 ± 0.32</td>
<td>4.103 ± 0.42</td>
<td>5.19 ± 0.32</td>
</tr>
<tr>
<td>Grooved</td>
<td>2.327 ± 0.29</td>
<td>3.677 ± 0.32</td>
<td>5.87 ± 0.32</td>
</tr>
<tr>
<td>CGRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plain</td>
<td>1.214 ± 0.84</td>
<td>1.80 ± 0.31</td>
<td>1.93 ± 0.26</td>
</tr>
<tr>
<td>Grooved</td>
<td>1.176 ± 0.30</td>
<td>2.25 ± 0.17</td>
<td>2.81 ± 0.42</td>
</tr>
<tr>
<td>S-100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plain</td>
<td>2.462 ± 0.54</td>
<td>7.48 ± 0.65</td>
<td>7.91 ± 0.71</td>
</tr>
<tr>
<td>Grooved</td>
<td>2.642 ± 0.45</td>
<td>6.88 ± 0.24</td>
<td>9.03 ± 0.34</td>
</tr>
</tbody>
</table>
6.4.4 Macrophage counts

Macroscopically, a pseudo-capsule formation was seen enveloping the repair site (Fig.63). On microscopic examination, an intense polymorphonucleocytic (PMN) response was observed in the vicinity of the regenerating nerve in both experimental groups. The PMN infiltration appeared to be predominantly present around the nerve adjacent to the internal surface of the PDS conduit which had been removed prior to processing. Macrophages were also present in the same area in higher numbers than within the regenerating nerve.

The values for macrophage counts immunostained with ED-1 are summarised in Table 6.3. There was no significant difference between the two groups at each of the time-points and both groups demonstrated a decrease in the macrophage infiltration with time. However, the ‘grooved’ PDS conduits showed a more pronounced and statistically significant decrease through time (p<0.05).

Table 6.3 Macrophage counts. Results of ‘plain’ and ‘grooved’ PDS conduits at 7, 14 and 30 days. Macrophages were counted one frame wide at the midpoint of the conduit across the whole width of the nerve at x 20 magnification. All values are given as mean ± SEM. *p<0.005, one way ANOVA, for ‘grooved’ conduits (7days vs 14 days, 14 days vs 30 days and 7 days vs 30 days).

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain</td>
<td>49.25 ± 8.25</td>
<td>28.66 ± 2.4</td>
<td>24.83 ± 1.76</td>
</tr>
<tr>
<td>Grooved</td>
<td>56.75 ± 9.16*</td>
<td>29.33 ± 1.57*</td>
<td>22.5 ± 0.46*</td>
</tr>
</tbody>
</table>
Fig. 6.3 PDS conduit at 7 days showing the formation of a pseudo-capsule.

6.4.5 Angiogenesis

Qualitative assessment of angiogenesis assessed by vWF-immunostaining of endothelial cells showed newly-formed blood vessels penetrating into the graft from the proximal and distal nerve stumps and following the same direction as that of the regenerating axons. No penetration of blood vessels was seen from the micro-environment surrounding the conduits because of the impermeability of the material and despite fragmentation of the tube.

6.4.6 Myelinated fibre analysis

Long-term assessment of the level of regeneration was assessed by analysis of myelinated fibres. On qualitative assessment, regenerating axons of varying diameters
were seen. Quantification of the axons was also carried out and the measured parameters were axon counts, fibre diameter, myelin thickness, g-ratio and shape factor. The results are summarised in Table 6.4. There was no significant difference for any of the parameters between the two PDS groups. However, there was a significant difference for all parameters, except shape factor, between both types of PDS conduits and normal uninjured nerves (One way ANOVA, \( p < 0.05 \)). This is indicative of a continuing regenerating process in both PDS conduits, with more numerous axons, which are smaller in diameter and have an absolute thinner myelin coating. This is also demonstrated by the percentage distribution of myelinated fibres according to their size (Fig. 6.4). The graph shows a similar distribution pattern for both experimental groups with a unimodal distribution and a preponderance of smaller fibres. On the other hand, in normal nerves the distribution was bimodal with a greater number of large diameter fibres.

Table 6.4  Myelinated fibre analysis at 6 months. All values are expressed as mean ± SEM. * \( p < 0.05 \). One way ANOVA. Normal vs plain and grooved.

<table>
<thead>
<tr>
<th></th>
<th>Plain</th>
<th>Grooved</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon count</td>
<td>805 ± 52</td>
<td>920 ± 51</td>
<td>539 ± 29*</td>
</tr>
<tr>
<td>Fibre diameter (( \mu m ))</td>
<td>4.59 ± 0.116</td>
<td>4.55 ± 0.109</td>
<td>8.27 ± 0.212*</td>
</tr>
<tr>
<td>Myelin thickness (( \mu m ))</td>
<td>1.07 ± 0.025</td>
<td>1.18 ± 0.024</td>
<td>1.70 ± 0.059*</td>
</tr>
<tr>
<td>g-ratio</td>
<td>0.49 ± 0.02</td>
<td>0.44 ± 0.017</td>
<td>0.56 ± 0.009*</td>
</tr>
<tr>
<td>Shape Factor</td>
<td>0.89 ± 0.006</td>
<td>0.89 ± 0.006</td>
<td>0.88 ± 0.004</td>
</tr>
</tbody>
</table>
Fig. 6.4 Size distribution of myelinated fibres at 6 months. The percentage pattern in both types of PDS conduits is similar with a peak at 3μm, both groups showing a preponderance of smaller fibres. The distribution in normal nerves is bimodal with larger fibres and peaks at 3μm and 9μm.
6.5 DISCUSSION

This study demonstrates good axonal regeneration in PDS conduits. In both ‘grooved’ and ‘plain’ PDS conduits, PGP-immunostained fibres penetrated less than a mm into the conduit at 7 days, reached one-third of the way by 14 days and finally traversed the gap successfully at 30 days. In comparison to PHB tubes (cf chapter 4) and to other conduits (Whitworth et al., 1995), in both types of PDS tubes the rate of regeneration appears to be slow initially, but increases fast after 14 days with the regenerating fibres crossing the 10 mm nerve gap to reach the distal stump at 30 days.

The volume of axonal regeneration quantified by the percentage of PGP and CGRP-immunostaining was comparable between the two conduit types. There was a progressive increase in the amount of regeneration with time in both groups, but no statistically significant difference was observed between the experimental groups. This trend was reflected in the Schwann cell proliferation, which showed similar values in the ‘grooved’ and ‘plain’ PDS conduits. It is known that longitudinal orientation of Schwann cells occurs along unidirectional conduit material components (Curtis and Wilkinson, 1997) and therefore, longitudinal grooves along the internal surface of the PDS conduit would be expected to improve regeneration in comparison to a plain surface. The results obtained in this study do not match this expectation. A possible explanation lies in the biomaterial behaviour of PDS. Fractures in the material were seen to occur at 7 days, progressing to fragmentation by 30 days. This early degradation is likely to have interfered with the positive effect of the presence of grooves. Presence of sharp fragments is also known to elicit a more intense inflammatory response (Aebischer et al., 1990), explaining the intense polymorphonucleocytic reaction and the high macrophage counts seen in the vicinity of the conduit. In the clinical situation, this
would give rise to unacceptable adhesions between the injured nerves and surrounding tissue, in particular tendons, thereby hampering the mobility of the joint.

In the long-term, the myelinated fibre analysis profile seen with PDS conduits is similar to that observed using PHB (cf chapter 4). Axon counts were higher in the experimental groups in comparison with normal uninjured nerves due to the presence of axonal sprouting. Following nerve maturation and connection to target organs, the sprouts would be expected to die-back and the axon numbers to decrease with time (cf chapters 3 and 4). The fibres in the PDS groups were of smaller calibre with an absolute thinner myelin than in normal nerves, but their g-ratio indicates relatively thicker myelin coating. The shape factor in all groups, experimental and normal nerves, was close to 1 indicating that the axons were regular in shape. The size distribution pattern was similar to that seen with PHB (cf chapter 4), both PDS groups showing a preponderance of small diameter fibres. On the other hand, the percentage pattern of normal uninjured nerves in the rat sciatic nerve was bimodal, in keeping with the pattern seen previously in normal nerves.

The results of this study demonstrate that although PDS will act as a nerve conduit, problems of fragmentation resulting in an intense inflammatory reaction and potential damage to the fragile regenerating nerve pose the main drawbacks of this material over others. Despite these problems, axonal regrowth occurred, albeit slowly, and the regenerating fibres crossed the 10 mm nerve gap to reach the distal stump with a subsequent myelinated fibre analysis profile similar to that seen in other conduits (den Dunnen et al., 1993; Whitworth et al., 1997; Hazari et al., 1999). Further work on the biomaterial properties of PDS is necessary to resolve the problems seen in this study, prior to any applications in the clinical setting.
CHAPTER VII

CENTRAL NEURONAL SURVIVAL AND PERIPHERAL TARGET ORGAN REINNERVATION IN PDS CONDUITS

7.1 Introduction

7.2 Aim

7.3 Materials and methods

7.4 Results
7.4.1 Neuronal survival in dorsal root ganglia
7.4.2 Motor reinnervation
7.4.3 Sensory reinnervation

7.5 Discussion
7.1 INTRODUCTION

In Chapter 6, the level of axonal regeneration in PDS conduits was assessed up to 6 months. In this chapter, the central and peripheral effects at this long term time-point are assessed by quantifying the survival of neurons in the dorsal root ganglia and the reinnervation of motor and sensory target organs.

Following transection of a peripheral nerve, significant neuronal cell death occurs (Risling et al., 1983; Arvidsson et al., 1986), with a loss of 20-40% neurons in the dorsal root ganglia (DRG) despite microsurgical repair (Liss, 1994). The surviving neurons reorganise their metabolism by ceasing transmission and increasing synthesis of substances associated with axonal regrowth and cell survival (Aldskogius et al., 1992). The precise mechanisms involved in preferential survival of some neurons and cell death of others are not known. A comparison of the immunostained neuronal cell between the operated and uninjured non-operated DRG would reflect the central effect of nerve injury and repair, indicating the success of a particular method of operative intervention on the neuronal survival rate.

Transection of a nerve leads to denervation atrophy of its motor and sensory target organs. After denervation, skeletal muscles rapidly lose weight as the muscle fibres atrophy (Pellegrino and Franzini, 1963). With reinnervation, muscles gradually recover mass to a variable extent depending upon the degree of reinnervation (Bertelli and Mira, 1995), the mass correlating well with the maximum force of contraction (Gillespie et al., 1987). Individual fibres within a muscle differ in their contractile properties and metabolic needs, being classified as type I slow oxidative (SO) present in postural muscles such as the soleus, type IIa fast oxidative glycolytic (FOG) and type IIb fast glycolytic (FG) present in fast muscles such as the extensor digitorum longus (Burke et al., 1971; Peter et al., 1972). The gastrocnemius typifies a mixed muscle comprising of
both types of fibres in varying proportions. The muscle fibre phenotype is dependent on
the innervating nerve and changing the innervation leads to an alteration in the
phenotype of the muscle (Fex and Sonneson, 1970; Salmons and Sreter, 1975).
Following transection and reinnervation of the gastrocnemius muscle in the rat, its
normal mosaic muscle fibre distribution is replaced by clustering of reinnervated fibres
(Sterne et al., 1997b).

In sensory reinnervation, the quality of regeneration depends on the condition of the
denervated sensory target organ ranging from persistence of Merkel complexes until 35
days post-transection to the presence of Pacinian corpuscles up to a year (cf 1.2.3). A
correlation between histology and sensory recovery has been demonstrated after nerve
repair in humans with biopsies from fingertips (Dellon et al., 1983). The technique of
immunohistochemical staining has been used to demonstrate all nerve fibres in the skin
including those supplying the epidermis and their various sub-populations (Navarro et
al., 1997). Of the various antibody markers that are available, a pan-neuronal marker
protein gene product 9.5 (PGP 9.5) has been shown to demonstrate the overall
reinnervation because it reacts with all peripheral nerves (Karanth et al., 1991). In this
study, PGP 9.5 has been used to quantify the degree of reinnervation and, therefore,
draw an inference of the functional outcome.

7.2 AIM

The aim of this study was to determine whether the level of peripheral axonal
regeneration seen in the previous chapter translated into a proportionate survival of
dorsal root ganglia neurons centrally, and reinnervation of motor and sensory target
organs peripherally.
7.3 MATERIALS AND METHODS

Twelve 8-week old inbred male Lewis rats in the 6 months group were implanted with one of the two types of PDS conduits (cf 2.1.1, 2.3 and 6.2)

Plain The internal surface of the PDS conduit was smooth.
Grooved The internal surface of the conduit had longitudinal grooves.

The animals were sacrificed at 6 months. In addition to the grafted nerve (cf chapter 6), dorsal root ganglia were harvested and processed for PGP-immunostaining (Fig.7.1) (cf 2.4.5 and 2.6), while footpad skin and gastrocnemius muscle were assessed for reinnervation and muscle mass recovery respectively (cf 2.4.3, 2.4.4 and 2.6).

7.4 RESULTS

7.4.1 Neuronal survival in dorsal root ganglia

Values of neuronal cell counts and mean cell body diameter in L4/L5 DRG on the operated and contralateral non-operated sides are given in Table 7.1. The percentage of PGP-immunostained neuronal cells on the operated side in comparison to that in the uninjured non-operated side was 83% and 87% at L4 and L5 levels in plain PDS conduits whereas was reduced to 72% and 69% respectively in grooved PDS conduits. However, this difference in neuronal percentage between the two types of PDS conduits did not reach statistical significance (t-test), indicating that the choice of plain or
Fig. 7.1 PGP-immunostaining of L4 DRG neuronal cell bodies at 6 months following grafting of (a) grooved PDS conduit and (b) its corresponding non-operated contralateral side.
grooved conduits had no major effect on the loss of immunoreactive neurons. No significant difference was seen in the mean diameters of the PGP-immunoreactive neurons between the two experimental groups and their contralateral sides. To verify whether a particular sub-population of DRG neurons, classified according to their size, was particularly affected by the conduit grafts, the size distribution of neurons in all groups was plotted according to their diameter (Fig. 7.2). The percentage distribution pattern following plain and grooved PDS conduit grafting was similar to that of normal neurons, all groups showing a peak at 12-13 \( \mu \)m.

7.4.2 Motor reinnervation

The muscle mass of the gastrocnemius muscles on both operated and non-operated sides in each animal was weighed to allow an estimation of the muscle atrophy for each group (Table 7.2). On macroscopic examination, the muscles on the operated side appeared smaller than those on the unoperated control side. There was no difference in the weight of the contralateral normal muscles between the two groups. However, there was a significant difference between the muscle weight on the operated side in comparison to the contra-lateral normal side in both the experimental groups. The percentage of muscle atrophy was calculated according to the following formula:

\[
\text{% Muscle atrophy} = \left( \frac{\text{Weight of reinnervated muscle}}{\text{Weight of contralateral normal muscle}} \right) \times 100
\]

The difference of the percentage muscle atrophy between plain (52.27%) and grooved (58.15%) PDS conduits did not reach statistical significance. The results indicate that although there is no difference between the treatment types, the reinnervation of muscle is still poor at 6 months post-operatively.
**Table 7.1** Neuronal counts and mean diameters of PGP-immunoreactive neuronal cell bodies in L4 and L5 dorsal root ganglia in animals grafted with plain and grooved PDS conduits. All values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>DRG</th>
<th>Plain Operated</th>
<th>Plain Non-operated</th>
<th>Grooved Operated</th>
<th>Grooved Non-operated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>85.2 ± 13.09</td>
<td>102.8 ± 11.18</td>
<td>127.8 ± 15.05</td>
<td>174.6 ± 27.48</td>
</tr>
<tr>
<td>L5</td>
<td>64.4 ± 9.44</td>
<td>73.6 ± 5.46</td>
<td>73.8 ± 9.6</td>
<td>105 ± 13.66</td>
</tr>
<tr>
<td></td>
<td>Diameter in µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>13.24 ± 0.38</td>
<td>14.47 ± 0.31</td>
<td>14.83 ± 0.29</td>
<td>14.69 ± 0.52</td>
</tr>
<tr>
<td>L5</td>
<td>13.14 ± 0.30</td>
<td>13.56 ± 0.38</td>
<td>13.78 ± 0.40</td>
<td>14.12 ± 0.64</td>
</tr>
</tbody>
</table>

**Table 7.2** Gastrocnemius muscle mass weight at 6 months post-operatively for plain and grooved PDS groups. All values are expressed in mg as mean ± SEM. * p< 0.05, t-test. Non-operated vs operated side.

<table>
<thead>
<tr>
<th></th>
<th>Operated side</th>
<th>Non-operated side</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain</td>
<td>124.2 ± 9.95</td>
<td>237.6 ± 11.76*</td>
</tr>
<tr>
<td>Grooved</td>
<td>126 ± 10.51</td>
<td>216.66 ± 10.12*</td>
</tr>
</tbody>
</table>
Fig. 7.2 Size distribution of neurons in the L4 DRGs at 6 months in plain and grooved PDS conduits and corresponding contralateral non-operated side. The distribution pattern in the experimental groups is similar to that in uninjured DRG, all groups showing a peak at 13\(\mu\)m diameter.
7.4.3 Sensory reinnervation

On macroscopic examination, clawing due to loss of intrinsic muscle innervation and muscle wasting was present on the operated side, with the plantar skin being thinner and drier compared to the non-operated side. No signs of footpad ulceration or self-mutilation were present.

Qualitative microscopic examination of normal skin revealed presence of PGP-immunoreactive fibres in the deep dermis and sub-epidermis. Nerve terminals arising from the sub-epidermal plexus traversed the epidermal papillae to reach the uppermost layers of the epidermis as free nerve endings (Fig.7.3). In the skin on the operated side reinnervation had taken place in both PDS groups, but the nerve density was less in comparison to that in normal unoperated footpad skin.

Results of the quantification of cutaneous PGP-immunoreactive fibres in footpad skin are given in Table 7.3. There was significant difference between the density of innervation of the skin on the operated and normal sides (p<0.05), with a reduction in cutaneous innervation of 47% for plain PDS and 50% for grooved PDS conduits. However, comparison between skin reinnervation density between the two conduit types was not significant, indicating that the use of either type of conduit did not affect the reinnervation of sensory target organs.

7.5 DISCUSSION

The results of this study show that neuronal cell survival at 6 months following tubulization with PDS conduits was shown to be between 69-87%, a loss of 15-30% neurons compared to normal values. The difference between the two experimental groups did not reach significance and is likely to be due to early fragmentation of the
Table 7.3 Cutaneous reinnervation measured as PGP-immunoreactive fibres / \( \mu \text{m}^2 \) following grafting of plain and grooved PDS conduits at 6 months post-operatively. All values are expressed as mean ± SEM. *p<0.05, t-test. Non-operated vs operated side.

<table>
<thead>
<tr>
<th></th>
<th>Operated side</th>
<th>Non-operated side</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain</td>
<td>500.75 ± 157.43</td>
<td>1058.86 ± 119.98*</td>
</tr>
<tr>
<td>Grooved</td>
<td>642.42 ± 56.96</td>
<td>1274.32 ± 116.68*</td>
</tr>
</tbody>
</table>

Fig. 7.3 Cutaneous innervation in normal footpad skin revealed with PGP immunostaining. Note that the nerve fibres arising from the sub-epidermal plexus reach the upper layers of the epidermis as free nerve endings.
conduits negating any beneficial effects of the presence of grooves. The percentages of cell loss observed in this study are in keeping with reports from other studies on rat dorsal root ganglia following transection of the sciatic nerve (Arvidsson et al., 1986). Results from studies in other animal species using conventional histology have shown considerable variation, with report of negligible neuronal death (Carlson et al., 1979) or substantial cell loss (Risling et al., 1983). This may be probably due the different methodological approaches used. Indeed, using retrograde tracing of dorsal root ganglia with horseradish peroxidase (HRP), following nerve injury, more consistent results have been reported, with neuronal cell losses varying between 20-30% (Liss et al., 1994) and 50% (Peyronnard et al., 1986; 1988). Though the exact cause of selective cell death is still unclear, these results have been reversed by application of nerve growth factor (Rich et al., 1987).

Percentage atrophy of muscle mass after denervation and tubulization was 52% and 58% of normal after grafting of plain and grooved PDS conduits, the difference not being significant between the two groups. Similar results of muscle atrophy have been reported in the rat, with a rapid loss of weight following nerve transection, this being slowly reversed to 70% of normal by 120 days and then remaining steady up to 8 months post-operatively (Sunderland, 1978; Gillespie et al., 1987; Bertelli and Mira, 1995; Sterne et al., 1997b). Cutaneous sensory reinnervation was 47% and 50% of normal for plain and grooved PDS conduits respectively. As in motor reinnervation, these differences were not statistically significant, indicating that the type of conduit used in repair did not influence the cutaneous reinnervation. Again our results were consistent with previous results, as up to 60% cutaneous reinnervation values have been reported following rat sciatic nerve transection and primary repair or grafting (Whitworth et al., 1995; McAllister et al., 1997).

Hence, it would be appear that the use of PDS conduits is not detrimental to sensory and motor target organ reinnervation. However, a longer time course than was possible in this experiment would be necessary to fully evaluate a complete functional recovery. In
conclusion, the results of this chapter and of Chapter 6 demonstrate that PDS may represent a potentially interesting material for use in nerve repair. The present results would also indicate that the presence of grooves in the conduits did not influence the outcome of nerve regeneration, neuronal survival and target organ reinnervation. However, because of the problem arising from the brittleness of the material, it cannot be completely excluded that surface microgeometry might be beneficial to improved nerve regeneration.
The aim of this study was to investigate morphologically two bioresorbable conduits, PHB and PDS, for peripheral nerve gap repair. It was also of interest to determine whether the addition of a growth factor, GGF, would improve the overall nerve regeneration. The results set out in the preceding chapters have shown that both PHB and PDS may prove to be suitable materials as nerve conduits, and that the addition of GGF to the microenvironment may have an indirect enhancing effect on nerve regeneration. The results also allow a comparison between the two types of material tested. The main criteria for a clinically suitable conduit for nerve repair are that it should be bioabsorbable, elicit the most minimal of inflammatory reactions and should remain in situ long enough to support regeneration. Flexibility of the conduit is a desirable requirement, particularly when the conduit is used in the vicinity of joints, such as the wrist, to enable gliding of surrounding structures like tendons and other nerves.

To a large extent PHB fulfilled these criteria. It was bioabsorbable, underwent progressive degradation with fragmentation of the polymer fibres and a decrease in its mass at the repair site. Regeneration in the conduits with penetration of the distal stump by the regrowing fibres took place by 30 days, a period when support from the conduit material was essential. A longer reabsorption time, as shown by PHB, ensured that the nerve would be able to withstand mobilisation both during its regenerative phase and whilst undergoing maturation. PHB did not provoke intense inflammation as shown by low macrophage counts up to 12 months (cf. 3.4.1, 4.4.3, 5.4.3). This is obviously an advantage, as it is unlikely PHB would give rise to adhesion formation with surrounding structures in a clinical application. The rate of regeneration in PHB conduits was consistent with previous observations following the use of other biodegradable conduits such as fibronectin mats (Whitworth et al., 1995; Sterne et al., 1997a and b), with axons
bridging the nerve gap by 30 days (cf 4.4.1), and the regeneration showing a progressive increase with time (cf 4.4.2). Furthermore, the results of this study have shown that PHB could be used as a wrap-around for direct repair (cf chapter 3), avoiding the use for epineural sutures and facilitating the overall regeneration process. This application, in particular, is likely to progress to clinical trials in the very near future.

PDS also proved to be suitable but with some limitation. Regeneration rate progressed similarly to that in PHB conduits, with the regenerating axons bridging the nerve gap by 30 days (cf 6.4.1). The volume of regeneration also showed a progressive increase with time (cf 6.4.2). However, comparison between grooved and plain PDS tubes did not show any major difference. This conclusion was contrary to that expected, as it is known that longitudinal orientation of Schwann cells occurs along unidirectional conduit material components (Curtis and Wilkinson, 1997); hence it was thought that the internal microgeometry would indirectly enhance regeneration in grooved PDS tubes. A possible explanation may be found in the bio-behaviour of PDS. Early degradation of the material was seen to occur from 7 days, which had progressed to fragmentation by 30 days. This is a time when the regenerating nerve is at its most susceptible, and it is likely that the grooves had been disrupted and disintegrated, and therefore failed to have any effect. It is also possible that the volume of Schwann cells orientating themselves longitudinally along the conduit grooves was small compared to the mass of glial cell progressing within the core of the regenerating front, therefore failing to make an impact on the overall rate and amount of regeneration. PDS also elicited an intense inflammatory reaction with accumulation of large numbers of polymorphonucleocytes and high macrophage counts in the vicinity of the conduit material (cf 6.4.4). This would be unfavourable in the clinical situation as it would be likely to give rise to unacceptable levels of adhesions with surrounding structures, thus
hampering movement. Nevertheless, the results in the long-term were similar to that seen in PHB, with comparable distribution pattern and profile of myelinated fibres (cf 6.4.6). Also, neuronal survival in the dorsal root ganglia was good (cf 7.4.1), and peripheral target organ reinnervation, as seen with skin reinnervation and muscle mass recovery was comparable to that by other investigators (cf 7.4.2 and 7.4.3). The initial work carried out with GGF by its local delivery at the site of nerve injury demonstrated enhanced nerve regeneration. However, due to time constraints, the long term effects of GGF could not be studied. Further work is necessary to validate its usefulness in nerve regeneration.

To summarise, PHB has certain advantages over PDS. Though both are bioabsorbable, PHB degrades at a slower rate than PDS, protecting the fragile regenerating nerve during regeneration and maturation. PHB has a low inflammatory response with good angiogenesis, whereas PDS elicited an intense inflammatory reaction. As PDS is impermeable, penetration of the regenerating nerve by new blood vessels was only from the ends of the conduit (cf 6.4.5). On the other hand with PHB, neo-vascularisation occurred through the walls of the conduit. Surgically, PHB is an easy material to handle and use, while problems with the rigidity and brittleness of PDS made it difficult to implant. In conclusion, PHB is potentially the more promising of the two materials tested, although further testing will be needed before progressing to clinical trials. In particular, the tests should examine the nerve regeneration behaviour in the longer repair gaps (>3-4 cm) and in larger calibre nerves. This could be done in other animal species, like rabbit and sheep, and would approximate better to the clinical situation found in man.
Though the production of an 'artificial nerve' is still far from being a reality, the work set out in the preceding pages serves to illustrate that the concept of an off-the-shelf composite conduit is a real possibility for the future. This would obviate the need for nerve grafting and its associated morbidity, resulting in better patient outcome.
BIBLIOGRAPHY


Verga, G. (1918) Results of operations on peripheral nerves. *Inter-Allied Conference Aftercare Disabled Men* 2:441-443.


APPENDICES

APPENDIX I – SOLUTIONS

Phosphate buffered saline (PBS)

To make 10 litres

Add the following to 5 litres of distilled water

NaCl 87.9g
KH$_2$PO$_4$ 2.72g
Na$_2$HPO$_4$(H$_2$O)$_2$ 3.9g
or Na$_2$HPO$_4$ 11.35g

Stir and leave for a few hours, then add another 5 litres of distilled water. Mix and allow to stand for at least 8 hours. Check pH and adjust to pH 7.3.

Sucrose-PBS

15% sucrose + 0.1% sodium azide in PBS

For 1 litre

Sucrose 150g
Sodium azide 1g

Dissolve in 700ml PBS and when dissolved make up to 1 litre.

Triton X-PBS

0.2% Triton X-100

Mix 2ml with 1 litre PBS.
**Antibody Diluent**

0.03% Triton X-100  
0.1% bovine serum albumin (BSA)  
0.1% sodium azide  

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

Dissolve in 100ml of PBS.

**Zamboni’s solution**

2% paraformaldehyde  85ml  
Saturated picric acid  15ml  

Dissolve 20g paraformaldehyde in 800ml 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.

**4% Paraformaldehyde**

Dissolve 40g paraformaldehyde in 800ml PBS at 60°C max and stir until solution is clear. If necessary, add few drops of 10N NaOH to clear the solution. Adjust volume to 1 litre and allow to cool before use. Store at 4°C and use within 24 hours. Alternatively,
divide into aliquots and keep frozen at -20°C until required. Defrost at room temperature or by immersing in beaker of hot water. Once defrosted, do not re-freeze.

4% Glutaraldehyde

Dissolve 40g glutaraldehyde in 800ml PBS and stir until solution is clear. Adjust volume to 1 litre and store frozen until required.

0.1 Sodium Acetate

Dissolve 82.03g sodium acetate in 700ml of distilled water. Make up solution to 1 litre and adjust pH to 6 using NaOH or acetic acid.

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.
APPENDIX II – PREPARATION OF GLASS SLIDES

Vectabond coated slides

The slides were treated to enhance adhesion of sections and prevent loss of tissue during the indirect ABC nickel enhancement procedure.

1. Clean slides in dilute chromic acid and rinse thoroughly in water.
2. Immerse slides in acetone for 5 min, then remove and drain well.
3. Prepare Vectabond™ reagent treatment solution by mixing 7ml of reagent in 350ml acetone.
4. Remove slides, drain well and minimise excess reagent by dipping in distilled water.
5. Dry slides at 37°C.
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PRESENTATIONS AND PUBLICATIONS

Publications


Presentations

