Development of a nerve regeneration conduit

by

John Mahendra Kumar Daniel

A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Interdepartmental Program: Biomedical Engineering

Major: Biomedical Engineering

Iowa State University
Ames, Iowa
1988

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Until recently, a serious spinal cord injury was always considered to result in serious loss of function or death, but recent experiments show that nerve tissue can be transplanted or regenerated, breaks in axons can be jumped electrically and damaged nerve branches can be reconnected.

Several techniques have been tried to selectively control appropriate neural regeneration following nerve injury. One such technique is to use a cuff or sleeve to direct the regeneration, prevent self-reinnervation and permit substantial cross-reinnervation, particularly of motor axons. Interest continues in the pursuit of a suitable off-the-shelf prosthesis for supporting nerve regeneration and reconnection.

This work was undertaken to investigate the feasibility of developing a composite regeneration tube using current fabrication methods. Several methods were tried in an attempt to develop a simple, reproducible method of fabrication.

A nerve cuff was designed and fabricated by grafting 2-hydroxyethyl methacrylate (HEMA) onto a silicone rubber (Silastic^R) tube using a ^{60}Co radiation source. HEMA was chosen because the physical properties of hydrogels closely resemble those of living tissue, and the silicone rubber tube provides mechanical stability. A suitable hydrogel formulation was chosen to provide the required microstructure for incorporating a dye (Azo direct dye) in the hydrogel matrix. It is known that nerve

fiber growth follows a concentration gradient of a nerve growth factor or stimulant. Hence, a technique was developed to lay down a concentration gradient of the dye (which was used instead of the nerve growth stimulant) in the grafted hydrogel matrix along the axis of the tube. Also, a special setup was designed to position a glass mandrel coaxially in the lumen of the silicone rubber tube and hold it upright during irradiation. Visual and optical microscopic evaluations were done to determine the dye concentration in the grafted hydrogel.

The results of this study establish that a hydrogel having a controlled microstructure and a lengthwise distribution of a dye in its matrix can be grafted onto the inner wall of a silicone rubber tube. This investigation also indicates the feasibility of developing a nerve support tube or cuff by using the irradiation method of grafting hydrogels.

2. OBJECTIVES

The objective of this study is to design and develop a 2-hydroxyethyl methacrylate (HEMA) coated silicone rubber substrate nerve prosthesis which can be used to support an injured nerve and control the direction and rate of regeneration. The specific objectives include the following:

1. To choose a suitable formulation for the polymer,

2. To design the structure, determine the dimensions and develop a method to make several such conduits of varying cross section, and

3. To develop a method of impregnating the polymer, with a dye or fluorescent marker or an element that can be determined by X-ray microprobe analysis, in an increasing concentration along the axis of the conduit.

3. LITERATURE REVIEW

3.1. Nature of the Problem

3.1.1. The nervous system

The human nervous system consists of an extensive network of several billion neurons which are capable of receiving, storing and transmitting information. The neurons have long, thin extensions called axons. Neurons communicate with one another and with other nonneuronal cells through these axons. The axons are filled with cytoplasmic fluid which includes mitochondria, microtubules and neurofibrils. In addition to transmitting electrical signals, the axons also ferry nutrients and other vital substances to and from the cell body. Each neuron has only one axon, but each axon generally has several branches called collaterals.

The nervous system is separated into two major divisions based on structural locations: the central nervous system and the peripheral nervous system. The central nervous system includes the brain and the spinal cord. The peripheral nervous system which comes out of the spinal cord is composed of:

1. nerves which connect the distal body regions with the central nervous system.

2. ganglia (groups of nerve cell bodies) which are associated with the nerves.

The peripheral nerves are of 3 types: sensory neurons, which transmit nerve impulses from sensory receptor sites to the spinal cord; motor neurons, which innervate the skeletal muscles; and autonomic neurons, which innervate cardiac muscle, smooth muscle and glands.

Peripheral nerve injuries are usually caused by car or industrial accidents. But injured peripheral nerves can be made to function again because they are capable of growing after injury. The problem the growing neurons face is that they do not know which way to go when they grow back. Each peripheral nerve consists of several thousand individual nerve fibers and each of those fibers has its own protective sheath surrounding it. The sciatic nerve in the leg, which is the largest peripheral nerve, is made up of about 175,000 nerve fibers. After a nerve injury occurs, the growing nerve fibers must be able to cross a gap that usually contains blood and scar tissue for proper function to be restored.

Peripheral nerves have 3 supporting structures: the epineurium, the perineurium and the endoneurium. All three play an important role during the nerve repair. Each nerve fiber is individually wrapped by a thin connective-tissue sheath called the endoneurium. The nerve fibers are separated into bundles called fasiculi by another connective-tissue sheath called the perineurium. The epineurium surrounds several fasiculi, blood vessels and lymphatic vessels, all of which constitute a single nerve.

Each nerve fiber consists of one large axon (myelinated), or several small axons (unmyelinated). The axons are ensheathed by a chain of Schwann cells distributed at intervals along the length of the fiber. The axon relays messages and materi-

als between the neuron cell body and distal axon, and also between the neuron and Schwann cells (Daniller and Strauch, 1977). During development, some of the Schwann cells become associated with a single axon and by a process known as spiralization, surround the axon with compact layers of plasma membrane, or myelin. Each Schwann cell forms a myelin sheath for a regular repeating length of the axon that ends at the node of Ranvier. The integrity of the myelin sheath and node of Ranvier is essential for normal axonal impulse propagation (Spencer, 1974). Schwann cells associated with unmyelinated fibers enclose many small, naked axons within their cytoplasmic processes.

There is a thin extracellular coat called the basal lamina, which surrounds the outer surface of the Schwann cells of a myelinated or unmyelinated fiber. This forms a continuous tube that separates the nerve fiber from the surrounding endoneurial connective tissue.

3.1.2. Types of nerve injuries

The degree of damage to a nerve fiber increases in severity with the velocity, increasing temporal span, and force of the damaging agent (Daniller and Strauch, 1977). Myelinated nerve fibers are more susceptible to injury as compared to unmyelinated ones.

Selective damage to Schwann cells and the myelin sheath may occur during mild injury or indirect compressions. The damaged myelin is retracted (demyelination), and new myelin is produced around the denuded length of the axon (remyelination). Damage to the myelin sheath is also caused by a local chronic nerve constriction

which results in increased slowing of motor conduction. These types of injuries are classified as first degree nerve injuries.

Another example of this type of injury is termed axonal interruption (also called axonotmesis). In this type of injury, axonal continuity is lost, and the distal portion of the axon undergoes dissolution because of the separation of the axon from the supply of materials from the neuron cell body (Daniller and Strauch, 1977). The myelin collapses around the dying axon and the degenerating material is removed by phagocytosis. The entire process by which the fiber is removed from the endoneurial tube is termed Wallerian degeneration (Spencer, 1974). Motor, sensory or autonomic paralysis may be complete. The prognosis is less favorable if the supporting structures are also damaged along with the axons. Recovery may be incomplete and full function may not be restored. Regenerating axons become misrouted and fail to reach their target organs. This injury, known as neurotmesis, may result from endoneurial disruption, endo- and perineurial injury, or a complete severance of the nerve trunk (Spencer, 1974).

3.2. Present Treatment Methods

Current surgical techniques used in the repair of peripheral nerve injuries do not provide satisfactory results and complete restoration of function is difficult to achieve using these methods. End-to-end anastomosis using microsurgical principles and ground fasicular sutures, with or without a nerve graft bridging the nerve defects, is the most common procedure being followed by surgeons. (Grabb et al., 1970; Jabaley, 1981; Millesi, 1972, 1973). Suturing nerve injuries under tension is

also known to be unfavorable (Lundborg, 1975; Terzis and Williams, 1975).

There is a fundamental difference in the surgical techniques used in repairing an artery or bone and an injured nerve fiber. Tissue surgery is used to repair an injured artery or bone. But in the case of nerve injuries, only one cell is cut when a nerve fiber is cut. Hence, cellular surgical principles have to be applied to successfully repair cut nerves. There are several factors that have to be carefully considered when nerve injuries are surgically repaired. One, damage should not occur to the nerve fiber when the injured nerve ends are trimmed before reconnection. The nerve fiber can be frozen and then trimmed with a vibrating blade to avoid damage to the cell fibers. Two, to keep the interior of the nerve fiber from losing its chemical and ionic balance, the cut ends should be bathed in solutions that are identical to the axonal cytoplasm. Three, some kind of support structure has to be provided to hold the cut ends together. This support or guide is needed to direct the growing axons. They also serve as a reservoir to hold the secretions that ooze from the cut ends. These secretions, which have NGF in minute quantities, have a trophic effect on the growing axons.

Experimentally, it has been shown that nerve defects can heal through bridging tubular implants of autogenic or synthetic origin (Lundborg and Hansson, 1980; Denny-Brown, 1946; Molander et al., 1982). Details of some of the tubular implants, the materials used to fabricate them and the type of nerves they were used on are listed in Table 3.1.

Table 3.1: Review of literature on tubular implants for bridging during nerve regeneration

Research Group	Animal and type of nerve	Type of cuff	Comments
Yannas, I.V. et al. (1985)	Rat Sciatic nerve (Gap of 15 mm)	Silicone tube packed with a protein collagen and a glycosaminoglycan (GAG) polysaccharide, chondroitin-6-sulfate. These materials were cross-linked to form a porous network that was degradable by enzymes at rates that could be controlled during preparation. The silicone tube was not biodegradable.	The regenerated nervous tissue was healthy, highly vascularized, and about 20 % of the axonal fibers were surrounded by a fatty myelin sheath produced by Schwann cells that migrated into the tube.
Madison, R. et al. (1985)	Rat Sciatic nerve (Gap of 5 mm)	Nerve guides were polymers of synthetic poly - D, L - lactates with 2% triethyl citrate added as plasticizer. These guides were lined with either a collagen matrix or a laminin-containing gel.	A laminin gel matrix lining the nerve guide significantly speeded up axonal regeneration *in vivo* in rat sciatic nerve.
Munz, M. et al. (1985)	Section of the optic nerve was replaced by a section of the sciatic nerve of rat.	No cuff was used	Severed axons of adult rat retinal neurons could regenerate through an entire 20-30 mm peripheral nervous system graft. Some restoration of function was observed.

Table 3.1: Continued

Research Group	Animal and type of nerve	Type of cuff	Comments
Chiu, D.T. et al. (1982)	A 1 cm segment of rat sciatic nerve was removed.	A segment of femoral vein was used to bridge the nerve gap	Histologic evaluation showed orderly growth of nerve fibers within the lumen of the vein grafts. They reached the distal stump within 2 months after repair. Nerve conduction studies showed restoration of conduction with muscle reinnervation.
Molander, H. et al. (1983)	Adult rabbit tibial nerve (Gap = 10 mm)	Polyglactin tube made of a polyglactin suture mesh with a pore size of 0.4 x 0.4 mm and a thread diameter of 140 ± 20 μm.	The polyglactin tube bridging a nerve defect gives regeneration as good as conventional nerve grafting. The tube influenced the direction taken by the axons and guided them into the distal segment.
Longo, F.M. et al. (1983)	Adult rat sciatic nerve (Gap = 10 mm)	Silicone chamber Inner diameter = 1.2 mm Outer diameter = 2.0 mm	The fluid that accumulates in the chamber has a trophic effect on nerve regeneration.

Table 3.1: Continued

Research Group	Animal and type of nerve	Type of cuff	Comments
Young, B.L. et al. (1984)	The nerve to the medial gastrocnemius muscle was crossed with that to the lateral gastrocnemius and soleus muscles in cats. Also the nerve to the medial gastrocnemius was crossed with that to the soleus muscle in cats.	Gore-tex^R (Expanded poly tetra fluoroethylene) Inner diameter = 1 mm Internodal distance = 30μm Length = 15 mm (was compressed to 5 mm for insertion)	Neural tissue invasion into the Gore-tex^R matrix does not occur even though other cell types invade and a good vascular supply is provided. Motor innervation was excellent but sensory innervation was depressed.
Midenberg, M.L. and Kirschenbaum, S.E. (1986)	39 year old Caucasian male sural nerve in the right leg	Size-4 Swanson-Ducker Silastic^R nerve cap	No chronic edema was seen. Silicone rubber nerve caps did not induce host tissue response; the exterior of the cap was found to be covered with a smooth capsule of mature connective tissue that prevented entrapment of the nerve in its cap in the surrounding tissue plane.

Table 3.1: Continued

Research Group	Animal and type of nerve	Type of cuff	Comments
Muller, H. et al. (1987)	Rat sciatic nerve (Gap = 10mm)	25 μliter silicone chamber (Inner diameter = 1.8 mm)	A mixture of laminin, testosterone, ganglioside, and catalase applied by multiple injections into the chamber was shown to advance substantially the progress of regeneration in 16 days, as compared to saline filled chambers. When sections containing axons 3 mm from the proximal stump were compared in experimental and control groups, computerized area measurements revealed an average 2-fold difference for the cross-sectional size of the whole regenerate, the endoneurium and the space occupied by blood vessels.

Table 3.1: Continued

Research Group	Animal and type of nerve	Type of cuff	Comments
Lundborg, G. et al. (1982)	Cross - anastomosed rat sciatic nerves (Gap = 6 mm and 10 mm). Two arrangements were examined after 1 month *in situ*: A proximal - distal (PD) system in which both proximal and distal stumps were introduced into the ends of the chamber, and a proximal - open (PO) system in which the distal stump was omitted.	Cylindrical silicone chamber. (Inner diameter = 1.2 mm, Outer diameter = 2.0 mm)	For the 6 mm gap, a regenerated nerve extended all the way through the chamber in both the PD and PO systems. For the 10 mm gap, a similar regrowth occurred in the PD chamber, whereas in the PO chamber proximal regrowth was partial or non-existent.

3.3. Nerve Growth Factor and Neuropeptides

3.3.1. General

Neuronal growth factors play a key role in the development of the nervous system and in neuronal repair mechanisms. When an injured axon starts to regenerate, there is an increased synthesis of proteins in the cell body and an enhanced transport of the newly synthesized materials to the growing axon tip. The extracellular matrix which consists of Schwann cells and fibroblasts also play an important role in regeneration. The denervated target organ and the Schwann cells that proliferate in the distal nerve segment start producing increased amounts of nerve growth factors. Their production is triggered by degeneration materials and modulated by contact with the new growing axons. Laminin, which is an extracellular matrix protein, stimulates neurite outgrowth *in vitro* and axonal regeneration *in vivo* (Dekker et al., 1987).

3.3.2. Nerve growth factor

Nerve Growth Factor (NGF) was discovered in 1953 by Levi-Montalcini. She observed that an implant of mouse sarcoma tissue caused a marked increase in the size of sympathetic and sensory ganglia in chicken embryos. Further experiments proved that this effect was due to the nerve growth factor. NGF was purified in 1960 by Stanley Cohen. It is a 13.25 kD, oligomeric protein complex, consisting of three subunits: alpha(2)-beta-gamma(2). The β-subunit (a dimer of two identical polypeptide chains of 118 residues, MW = 13,250, pI = 9.3) contains the biological activity (i.e., it affects the development of sympathetic and sensory neurons). The

polypeptide chain is stabilized by three covalent sulfur-sulfur bridges between units of the amino acid cysteine at different positions (15–80, 58–108, 68–110) along the chain. The α and γ subunits serve to activate, store and protect the NGF molecule.

The structure of the polypeptide chain of mouse NGF as described by Boyd et al. (1974) is:

NH_2-Ser-Ser-Thr-His-Pro-Val-Phe-His-Met-Gly-Glu-Phe-Ser-Val-Cys- Asp-Ser-Val-Ser-Val-Trp-Val-Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asn-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-Leu-Ala-Glu-Val-Asn-Ile-Asn-Asn-Ser-Val-Phe-Arg-Gln-Tyr-Phe-Phe-Glu-Thr-Lys-Cys-Arg-Ala-Ser-Asn-Pro-Val-Glu-Ser-Gly-Cys-Arg-Gly-Ile-Asp-Ser-Lys-His-Trp-Asn-Ser-Tyr-Cys-Thr-Thr-Thr-His-Thr-Phe-Val-Lys-Ala-Leu-Thr-Thr-Asp-Glu-Lys-Gln-Ala-Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asn-Thr-Ala-Cys-Val-Cys-Val-Leu-Ser-Arg-Lys-Ala-Thr-Arg-COOH.

The preceding abbreviations for the amino acids are explained in full in the appendix. The aggregate formed by the association of the β-subunit with the α and γ subunits is termed 7S NGF. The purified form of NGF obtained by dissociation of this aggregate is denoted as 2.5S NGF. Only the 2.5S NGF and the β-NGF component of the 7S NGF have biological activity.

3.3.3. Sources of NGF

3.3.3.1. Submaxillary gland of the mouse The convoluted tubules (not the acinar part) of the male mouse submaxillary gland produces large amounts of NGF. This NGF is 10,000 times more active than that in mouse sarcoma and about

10 times more active than that in snake venom.

3.3.3.2. Mouse sarcoma NGF was first identified in mouse sarcoma – 180 tumor cells.

3.3.3.3. Snake venom NGF was isolated second in snake venom, which contains a high concentration of the enzyme phosphodiesterase. Crotalidae, Viperidae and Elapidae are 3 of the poisonous snake families from which NGF has been purified (Hogue-Angeletti and Bradshaw, 1978). The most extensively characterized NGF is the one isolated from cobra venom (Naja naja).

3.3.3.4. Prostate gland of guinea pig Harper et al. (1979) isolated NGF in the prostate gland of the guinea pig with levels comparable to those in the male mouse submaxillary gland.

3.3.3.5. Goldfish brain NGF has also been isolated in the brain of the goldfish by Benowitz and Greene (1979). Pheochromocytoma cells (PC12), which respond to NGF by producing new fibers were used by them in identifying NGF in goldfish brain.

3.3.4. Mechanism of action of NGF

The mechanism of action of NGF on neurons and other cell types is still unknown. Since NGF has multiple actions on most cell types, in any study the steps of different mechanisms are likely to be found intertwined. Pheochromocytoma cells and other cells are known to have membrane receptors on which NGF acts. Most

of the current research is focused on the effect of NGF on phospholipid metabolism as a possible transducing step in the neurite promoting effect of NGF (Dekker et al., 1987).

3.3.5. Chemotactic effect of NGF

Charlwood et al. (1972) used mouse sarcoma - 180 containing nerve growth factor to stimulate nerve fiber growth in the sensory ganglia of chick embryo, which was cultured in a liquid medium of rat tail collagen. They observed greater nerve fiber outgrowth from the side facing the source of NGF than from the opposite end. This effect was not seen when the ganglia were cultured in the absence of NGF. Since the concentration of NGF was greater on the side of the ganglion closer to the source, the fibers could have followed this concentration. However, when the experiment was done with 2 ganglia cultured in the presence of 1 source, they observed that fiber outgrowth was greater from the side of the more distant ganglion facing towards the source as compared to the outgrowth from the side of the nearer ganglion facing away from the source. From these experiments, they determined that the nerve fiber outgrowth follows the concentration gradient of NGF rather than its concentration. This effect is thus an example of chemotaxis. Charlwood et al. (1972) also suggested that other than influencing the growth of nerve fibers, NGF also may play a key role in directing the nerve fibers during development and regeneration.

In 1978, Letourneau used semisolid agar matrices containing concentration gradients of NGF to culture sensory neurons from dissociated dorsal root ganglia of

chick embryos. He observed a preferential orientation of more than half of the nerve fiber tips and a greater extension of fibers along NGF gradients. He also observed orientation of nerve fibers toward the NGF source in chambers with NGF sources ranging from 25 to 1000 ng/ml of β-NGF and from 400 to 5000 ng/ml of 7S-NGF, but saw no orientation when the concentration of the NGF source was 15000 ng/ml of β-NGF. Letourneau concluded that this orientation response apparently is not a concentration-dependent trophic response to NGF. His experimental evidence strengthens the belief that chemotaxis is an important regulatory factor in neuronal morphogenesis.

Carbonetto et al. (1982) have shown that cultured neurons become attached to the hydrogel substrates prepared from HEMA but grow few nerve fibers unless fibronectin, collagen, or β-NGF is incorporated into the hydrogel. They also have shown that there is a direct and selective interaction of the nerve fiber surface with defined molecules embedded in the gels.

3.3.6. Other neuronal growth factors

NGF was the only known growth factor for several years. In 1982, Barde et al. isolated a growth factor from pig brain. Ciliary Neuronotrophic Factor (CNTF) was purified in 1984 by Barbin et al. Even though the number of partially characterized factors are currently around twenty, it is probable that the actual number of growth factors will be smaller after final identification.

3.3.7. Commercially available NGF

Some of the nerve growth factors which are commercially available are

1. 2.5S-Nerve Growth Factor [1]

   From male mouse submaxillary gland.

   M.W. approx : 26,000

2. 7S-Nerve Growth Factor [1]

   From male mouse submaxillary gland.

   M.W. approx : 140,000

3. 2.5S-NGF [2]

   From mouse submaxillary gland.

   Endotoxin tested.

4. 7S-NGF [2]

   From mouse submaxillary gland.

   Lyophilized from phosphate buffer.

5. Nerve Growth Factor [2]

   From Vipera leletina venom.

   Lyophilized.

---

[1]ICN Biomedicals, Inc., Costa Mesa, California.

[2]Sigma Chemical Company, St. Louis, Missouri.

3.4. Properties of Materials

3.4.1. Silicone rubber

Silicone rubber (polydimethylsiloxane) is one of the best biocompatible polymers for implant applications. Medical-grade silicone rubber can be divided into the heat-vulcanizing variety and the room-temperature-vulcanizing variety (RTV) (Braley, 1970). The heat-vulcanizing variety of silicone rubber contains a finely divided silica filler with a particle size of about 120 to 300 Angstrom units. This filler adds strength to the silicone rubber and usually, the more filler used, the harder the rubber is. The RTV silicone rubber is divided into two component RTV and one component RTV. The two component RTV rubber (vulcanization requires a catalyst to be mixed into the base) contains diatomaceous earth particles for filler, ranging in size from 1 to 30 μm. The base is a fluid silicone polymer, mixed with filler and a cross-linking agent. The cross-linking of the silicone polymer units is initiated by the catalytic action of the organo-metallic compound, stannous octoate. These cross-linked polymers form the silicone rubber with filler dispersed in the matrix. In the case of the one component RTV, vulcanization is initiated by absorption of water vapor from the air.

3.4.2. Hydrogels

3.4.2.1. General Hydrogels are cross-linked polymeric networks capable of absorbing and holding large quantities of water without dissolution of the polymer network (Bruck, 1972, 1973). They were first used for biological applications in 1960 by Wichterle and Lim. The physical properties of hydrogels resemble those

of living tissue more than any other type of synthetic biomaterials (Ratner and Hoffman, 1976).

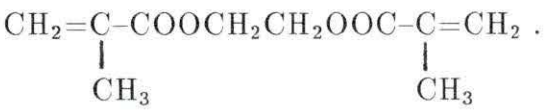
Hydrogels can be fabricated by different polymerization techniques or by conversion of existing polymers. 2-hydroxyethyl methacrylate (HEMA), cross-linked with ethylene glycol dimethacrylate, is one of the most popular types used for biological applications. The ease with which the combination of cross-linking and simultaneous solution polymerization occurs is an important advantage of the hydroxyethyl methacrylate esters. Details of the various techniques of synthesis and the resulting properties of hydrogels have been published by Refojo and Yasuda (1965), Wichterle (1971), and Ratner and Hoffman (1976).

There are several major advantages of hydrogels according to Ratner and Hoffman (1976). One, the expanded nature of the gel network facilitates the removal of unwanted initiator molecules, solvent molecules and other extraneous materials from the gel network before the hydrogel is used *in vivo*. Two, the physical irritation at tissue-polymer interfaces is minimized because of the soft and rubbery consistency of hydrogels. Three, the *in vivo* performance of hydrogels is greatly improved because the gel matrix allows small molecules and metabolites to diffuse across it (Levowitz et al., 1968). Four, hydrogels are chemically stable and will not easily cause dissolution of the polymer. Five, the low interfacial free energy and work of adhesion between the hydrogel surface and an aqueous surface (Andrade, 1973) reduces the tendency of proteins in body fluids to adsorb onto the gel surface (Hoffman, 1974). Six, the ease with which hydrogels can be made into a wide range of morphologies enables us to adjust the physical properties of hydrogels to suit

various applications.

3.4.2.2. Chemical properties and grafting methods Hydrogels are chemically stable due to the three-dimensional structure of the polymeric chains and the strong C-C bonds. The principal monomer used in the formation of hydrophilic gels is HEMA,

which is polymerized by conventional free radical methods in the presence of small amounts of cross-linking agents such as ethylene glycol dimethacrylate,



By varying the solvent content during polymerization, the amount of water taken up by the hydrogels made from the monomer and cross-linker described above can range from 35% to 90% of their weight. If polymerization is carried out in the absence of solvents for the polymer, a hard and brittle polymer is obtained. However, polymerization in a medium that contains a nonsolvent for the polymer results in precipitation of polymer and either an opaque gel or a microporous sponge with a range of pore sizes depending upon the polymerization conditions is obtained (Greer and Knoll, 1980).

The biggest disadvantage of using hydrogels in biomedical applications is that the high water content of hydrogels makes them mechanically weak. This mechanical weakness can be circumvented by either radiation grafting the hydrogel onto

a substrate (Ratner and Hoffman, 1974) or impregnating a substance with the hydrogel (Predecki, 1974). By using these methods, the biocompatible properties of the hydrogel can be combined with the mechanical properties of the substrate.

The grafting technique has several advantages over other methods of incorporating HEMA onto a substrate polymer. First, successive graftings using different formulations can be used to fabricate complex surfaces. Second, variations in solvent and other grafting parameters can be utilized to make the hydrogel graft onto the surface, penetrate into the substrate, or disperse uniformly throughout a hydrophobic matrix. Third, there is no need for adding initiators to initiate polymerization, thereby eliminating one potential source of contamination in the final product.

The disadvantages of graft polymerization are polymer degradation, cross-linking and formation of unwanted chemical species. Using low dose levels can minimize the problems caused by degradation and cross-linking. Elimination of unwanted functional groups can be achieved by excluding oxygen and reactive solvents from the grafting environment.

3.4.2.3. Immobilization and entrapment of biologically active molecules on and within hydrogels Thre are several advantages in using hydrogels as a base material for "biologically active" biomaterials. One, the rate at which small molecules (drugs, enzymes) diffuse through the gel matrix can be controlled by co-polymerizing the hydrogel in varying ratios with other monomers. Two, the tendency to interact with the biologically active molecules is much less with hydrogels as compared to more hydrophobic materials. Three, they can be used to fabricate long-term treatment devices because of their ability of not causing any

reaction when left in contact with blood. Four, the large number of polar reactive sites on the hydrogels enables the biologically active molecules to be immobilized by simple chemical techniques (Ratner and Hoffman, 1976).

The biologically active molecules can be immobilized within the hydrogels either permanently or temporarily. Active biomolecules can be easily entrapped in the matrix of hydrogels which are formed by the solution polymerization method. In order to immobilize a biomolecule, the pore size or average interchain distance of the gel should be smaller than the size of the active molecule. Gutcho (as cited by Ratner and Hoffman, 1976) reports that a pore size of 35 Angstrom units or smaller should be suitable for retaining most entrapped enzymes. Controlled drug delivery devices can be fabricated if the hydrogel is designed to release the entrapped biomolecule at a preset rate. Different methods have also been developed for the covalent immobilization of active molecules to hydrogel surfaces.

3.4.3. Azo direct dyes

Azo direct dye consists of at least two ring structures connected by the azo chromaphore (-N=N-). The ring structure is made up of sulphonic acid solubilizing groups and hydroxyl groups.

The anionic charges of the hydroxyl groups on the dye molecules are repelled by similar charges on the hydroxyl groups of the hydrogel at neutral pH levels. Placing 20% (wt dye / wt hydrogel sample) of the dye and the hydrogel in a boiling salt bath (6 g NaCl in 200 ml H_2O) neutralizes the anionic charges and allows the dye to penetrate the hydrogel lattice. The dye molecules get trapped during cooling of

the lattice and are permanently secured to the gels by hydrogen bonds and Van der Waals forces (Eckstein and Pinchuk, 1982).

This dye technique is used to determine the presence and uniformity of the hydrogel graft when hydrogel is grafted onto polymeric substrates by radiation methods. Any dye that remains fixed to the sample surface indicates a grafted surface. According to the Beer-Lambert law, the depth of color of the dyed hydrogel indicates the relative thickness of the graft layer. In the case of a uniform hydrogel layer, the depth of color would represent a particular concentration of the dye.

4. MATERIALS AND METHODS

4.1. Fabrication Considerations

The development of a composite nerve regeneration tube with hydrogel impregnated into or bonded onto silicone rubber substrates requires approaching the problem from several viewpoints: fabrication considerations, chemical and microstructural properties considerations, design considerations, and biological variability considerations.

Based on previous nerve injury treatment methods, a tubular configuration was chosen. The HEMA which contained the Azo direct dye (Sirius Supra Yellow GD 167, Verona Dyestuff Div., Mobay Chemical Corp., NJ) was grafted onto the inner wall of the silicone rubber Silastic^R tube which provided the mechanical strength to the structure. The Azo direct dye was used to provide a visualization of a chemical gradient instead of nerve growth factor molecules.

The formulation for the HEMA to be used was dictated by the 'pore' size of the HEMA matrix because this is important in immobilizing the biologically active molecules. The composition chosen was (in volume percent): 20% HEMA (Polysciences Inc., Lot 2-2405), 39% methanol (100% pure), 2.0% EGDM (Monomer - Polymer and Dajac Labs Inc., PA, Lot 1-2-14), 39% distilled H_2O (50% methanol

/ 50% H_2O co-solvent). The microvoid size is $< 1\mu m$ for this formulation (Knoll, 1980).

The HEMA was radiation grafted (dose level = 0.25, 0.5 or 1.0 Mrad) onto the walls of 1.98 mm i.d. silicone rubber tubes (Dow Corning, Silastic^R Medical Grade Tubing, Lot HH055153, 1.98 mm inside diameter x 3.18 mm outside diameter). The silicone rubber tubes were placed in a specially designed glass support tube which held the silicone rubber tube upright during radiation. A glass capillary tube of 1.39 mm i.d. was placed coaxially within the silicone rubber tube to provide a hydrogel layer on the inner wall of the silicone rubber tube. By using various sizes of these capillary tubes, the lumen diameter can be easily altered to accommodate different nerve sizes.

4.2. Techniques

4.2.1. Grafting methods

Several methods have been developed for grafting hydrogels onto silicone rubber. In this study, several of these methods were tried in order to determine the technique best suited for this purpose.

The free radical initiated polymerization reaction method (Pinchuk and Eckstein, 1981), the interpenetrating polymer networks method (Predecki, 1974), the dip-coat method and the radiation grafting method developed by Ratner and Hoffman (1974) were applied to graft HEMA onto the walls of the silicone rubber samples.

Details of the different methods tried are listed in Table 4.1.

Table 4.1: Various techniques used in grafting HEMA onto silicone rubber

Author(s) and date	Technique used	Procedure	Comments
Pinchuk and Eckstein (1981)	Free radical initiated polymerization reaction	(i) Initiator solution [6 g $(NH_4)_2S_2O_8$ in 100 ml distilled water] (Fisher Sci. Co., Lot 743791) (ii) Co-initiator solution [12 g $Na_2S_2O_5$ in 100 ml of distilled water] (Fisher Sci. Co., Lot 745412) (iii) A mixture of monomer and cross-linker consisting of 50 ml HEMA (Polysciences Inc., Lot 64622) and 1 ml tetraethylene glycol dimeth-acrylate (TEGDMA) (Polysciences Inc., Lot 4-1686) and 17 ml of ethylene glycol (Polysciences Inc., Lot 24685). All three solutions were purged with N_2 gas separately for 30 minutes. 8 ml of HEMA-TEGDMA-ethylene glycol mixture was loaded in a 10 ml polyethylene syringe. 1 ml of initiator and 1 ml of co-initiator solutions were added separately to the mixture and agitated. This mixture was injected into the inside of previously washed and dried Silastic^R tubing, 1.98 mm i.d, and 100 mm long (Dow Corning, Lot HH055153), using a long stainless steel needle.	The HEMA - TEGDMA - ethylene glycol ratio (50:1:27) was different from the ratio used by Pinchuk and Eckstein (50:1:17). Reaction times were 100 – 120 minutes, whereas Pinchuk and Eckstein reported 15 minutes for polymerization.

Table 4.1: Continued

Author(s) and date	Technique used	Procedure	Comments
		In another set of experiments, 0.01 g of Azo direct dye (Sirius Supra Yellow GD, Verona Dyestuff Div., Mobay Chemical Corp., NJ, Lot 0121032) was also added to the HEMA-TEGDMA-ethylene glycol mixture.	
Predecki, P. (1974)	Diffusion bonding or surface impregnation of silicone rubber with HEMA. Interpenetrating Polymer Networks (IPN)	Previously cleaned Silastic^R tubes, 1.98 mm i.d, (Dow Corning, Lot HH055153) were preswelled in boiling xylene (Fisher Sci. Co., Lot 784866) for 10 minutes and transfered to a solution containing 20% HEMA (Polysciences Inc., Lot 2-2405) monomer, 75% xylene (Fisher Sci. Co., Lot 784866) and 5% ethanol (100% pure) by volume. The samples were held completely immersed in this solution at just below the boiling point (96-100°C) for 4 hours and 8 hours, respectively. The grafted samples were placed in an oven at 100°C for 48 hours to drive out the xylene, and then extracted in a 50% ethanol (100% alcohol) and 50% distilled water mixture. Finally, samples were washed in deionized water and dried in an oven at 100°C for 1 hour.	The procedure followed was the same as Predecki's, except that Predecki used 100% ethanol for extraction of grafted samples. Also the final wash with deionized water was not mentioned by Predecki before the drying.

Table 4.1: Continued

Author(s) and date	Technique used	Procedure	Comments
		In another experiment, 0.2 g of benzoyl peroxide initiator (Fisher Sci. Co., Lot 710664) was added slowly to the HEMA-xylene-ethanol mixture, and the IPN method was followed for grafting as explained above.	
Ryoo, J. (1986)	Solubilization of HEMA (Dip-coat method)	HEMA plugs were made up by the pressurized polymerization technique. The plugs were powdered using a ceramic crucible (Liquid N_2 was used to make the HEMA brittle). 1 g of powdered polymer was dissolved in 20 ml of N,N–Dimethyl Formamide (Fisher Sci. Co., Lot 856540) by heating the mixture at 50°C for 8 hours. Previously cleaned Silastic^R tubing (1.98 mm i.d., Dow Corning, Lot HH055153) was coated on the inside with this viscous mixture. Excess liquid was allowed to drip down and samples were allowed to dry in air at room temperature. This coat was repeated 5 times at $\frac{1}{2}$ hour intervals for a total of 6 coats.	Ryoo used this method to dissolve a methyl methacrylate-HEMA copolymer; however, the hydrogels solubilized similarly. Also, Ryoo used the dip-coat method for coating external surfaces of polymer tubes, whereas in the current work the coat was applied to the inner walls of silicone rubber tubes.

Table 4.1: Continued

Author(s) and date	Technique used	Procedure	Comments
		In another set of experiments, the free radical initiated technique was used to give a second coating of HEMA after the first coat was applied using the drip-dry method. In a separate set of experiments, Silastic^{R} sample tubes (1.98 mm i.d., Dow Corning, Lot HH055153) were first grafted using the IPN method and then were given a second graft using the free radical initiated polymerization method.	In Ryoo's work, no subsequent free radical initiated polymerization was used.
Ratner and Hoffman (1974)	^{60}Co irradiation while in contact with vapor or liquid solution of monomer.	Monomer solution was made up as follows: (in volume percent) 20% HEMA (Polysciences Inc., Lot 2-2405) 2% ethylene glycol dimethacrylate (EGDM) (Monomer-Polymer & Dajac Labs Inc., PA Lot 1-2-14) 39 % methanol (100% alcohol) 39 % distilled H_2O (50 % methanol / 50 % H_2O co-solvent)	Identical irradiation procedures to that of Ratner and Hoffman were used. The formulations chosen were based on achieving desired pore sizes for the polymer matrix.

Table 4.1: Continued

Author(s) and date	Technique used	Procedure	Comments
		The monomer solution was bubbled with N_2 gas for 45 minutes inside a N_2 purged glove bag. Previously cleaned Silastic^R tubes, 1.98 mm i.d. (Dow Corning, Lot HH055153) were filled with the monomer mixture. The ends of the tubes were sealed with plexiglass caps. The filled Silastic^R tubes were placed inside glass culture tubes. The culture tubes were filled with the monomer mixture and sealed. Radiation dose level = 0.25, 0.5 or 1.0 Mrad.	
		In separate experiments, 0.005 g, 0.01 g and 0.05 g of Azo direct dye were each added to 15 ml of monomer mixture, respectively. The silicone tubes were filled with the highest dye concentration at the bottom and lowest dye concentration at the top so as to form a gradual lengthwise concentration gradient of the dye.	The Azo direct dye was not used by Ratner and Hoffman. The dye was used in this study to help visualize a chemical gradient.

4.2.2. Sample preparation

Silicone rubber (SR) tubing (Dow Corning, Silastic^R Medical Grade Tubing, Lot HH 055153, 1.98 mm inside diameter x 3.18 mm outside diameter) was boiled in a 2% aqueous sodium bicarbonate (Fisher Scientific, Lot 855518) solution for 3 one-hour periods, using fresh solutions each time. This was followed by profuse rinsing with distilled water and 3 washings in distilled water for 30 minutes using an ultrasonic cleaner. Finally, samples were dried at 100°C for $2\frac{1}{2}$ hours.

4.2.3. Experimental set up and procedure for irradiation

A special device was designed to hold the Silastic^R samples in position during irradiation (Figure 4.1).

The Silastic^R sample (100 mm long) was fitted snugly inside a clean glass support tube (3 mm inside diameter x 5 mm outside diameter). A plexiglass cap was inserted into the bottom of the sample tube to press the expandable Silastic^R firmly against the inside of the glass support tube. Silastic^R Medical Adhesive Silicone Type A (Dow Corning, Lot H 102560) was used to seal the bottom cap. This was done 24 hours prior to filling the lumen with monomer mixture to allow the adhesive to polymerize and provide a good seal. A glass mandrel (capillary tube of 1.39 mm inside diameter with both ends sealed with Hemato-Seal^{TM} tube sealing compound, Fisher Scientific) was placed coaxially into the sample tube and was positioned by a slot drilled into the middle of the bottom plexiglass cap. This left a circular gap of 0.59 mm between the glass mandrel and the Silastic^R sample for grafting the hydrogel.

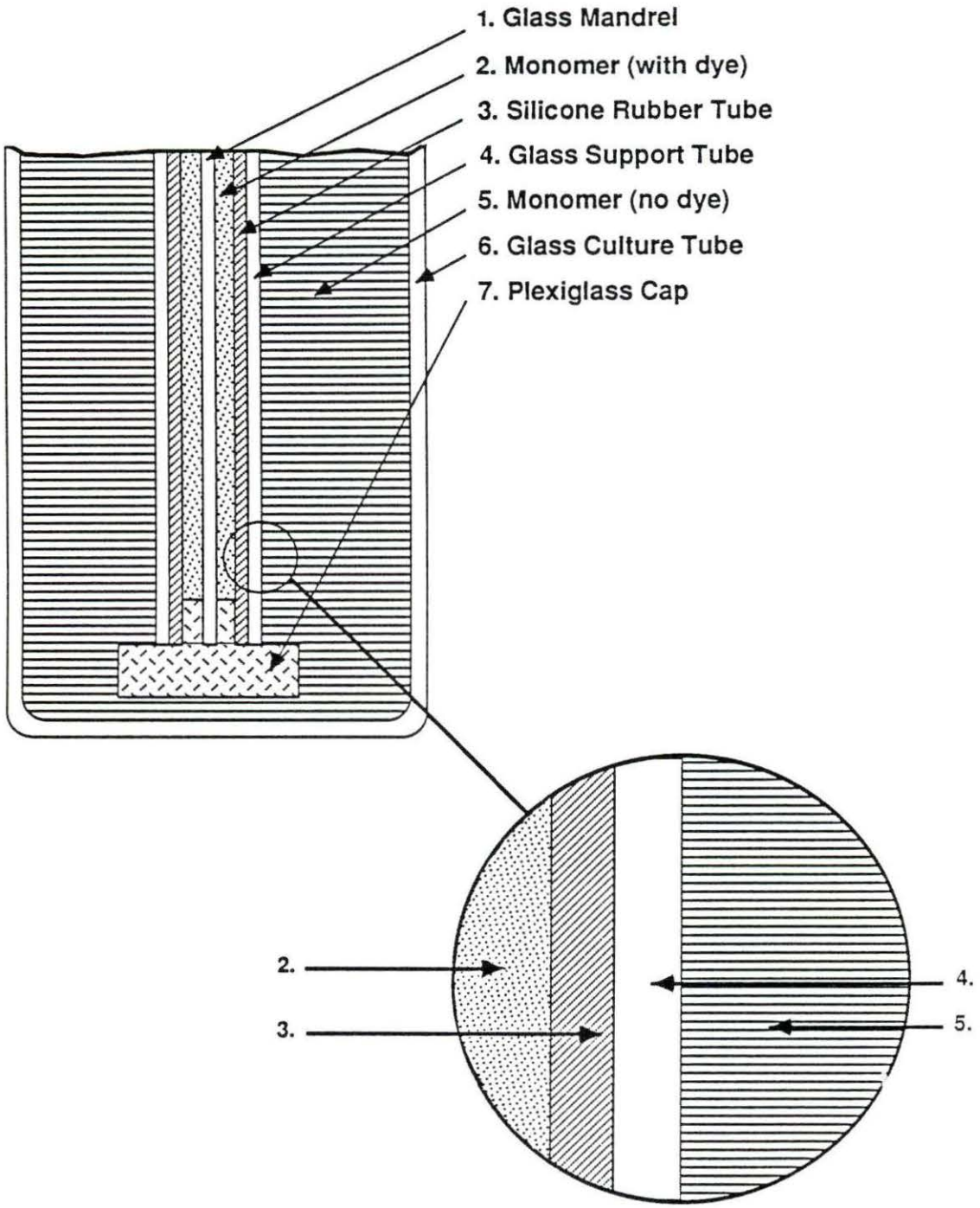


Figure 4.1: Schematic diagram of experimental set up used for ^{60}Co irradiation

A total of 4 formulations were prepared for radiation grafting (in volume %) :

1. A 100 ml mixture containing 20% HEMA, 1% EGDM, 39.5% methanol, and 39.5% distilled water. 0.01 g, 0.05 g and 0.1 g of Azo direct dye were each added to 15 ml of monomer mixture, respectively.

2. A 100 ml mixture containing 20% HEMA, 2% EGDM, 39% methanol, 39% distilled water. 0.01 g, 0.05 g and 0.1 g of Azo direct dye were each added to 15 ml of monomer mixture.

3. A 100 ml mixture containing 20% HEMA, 2% EGDM, 39% methanol, 39% distilled water. 0.005 g, 0.01 g and 0.05 g of Azo direct dye were each added to 15 ml of the mixture.

4. A 100 ml mixture containing 20% HEMA, 2% EGDM, 39% methanol and 39% distilled water. 0.1 g of Azo direct dye was added to 15 ml of the mixture.

Prior to irradiation, the space between the capillary tube and the inner walls of the sample tube was filled with the mixtures with different dye concentrations. Each of the three different dye solutions were loaded in 1 ml polyethylene syringes fitted with 23G hypodermic needles. The sample tube was flushed with a jet of N_2 to remove any O_2 present. The sample and support tube were held upright and 0.2 ml of each mixture was carefully injected into the tube. Any gas bubbles formed were carefully removed. The mixture with highest dye in it was at the bottom of the tube, and the mixture with the lowest dye in it was at the top of the tube. The top plexiglass cap was pressed onto the top of the support tube, and the assembly was carefully lowered into a Kimax^R culture tube (16 x 150 mm, Kimble, Division

of Owens-Illinois). The culture tube was filled with the monomer mixture that did not have any dye added to it and sealed. Teflon^R Thread Seal Tape was placed on the threads to make the cap airtight. The culture tube was placed horizontally in a N_2 filled desiccator and sealed. Movement of sample and handling was kept to a minimum before irradiation to prevent intermixing of various dye solutions. A series of radiation dose levels (0.25 Mrad, 0.5 Mrad and 1.0 Mrad) were used for the different formulations. The length of irradiation was 54 minutes, 110 minutes and 220 minutes for the three dose levels, respectively.

4.2.4. Characterization of grafted silicone rubber tubes

Visual and optical microscopic studies were done on all grafted samples. The samples were studied within a few hours after grafting while they were still wet. A section of each sample was also air dried for 24 hours and observations were made after the samples were dry. 1 mm thick cross sections of the samples were made using single edge industrial blades (Polysciences Inc., PA), and were viewed at 10x, 100x and 400x using an optical microscope (Balplan Microscope, Bausch and Lomb, Scientific Optical Products Division, Rochester, NY). Cross sections of the radiation samples were also observed at 30x using a stereo microscope (Nikon^R 98660, Japan). Longitudinal sections were also made using single edge blades, and samples were studied for dye distribution. The sections were mounted by laying the samples straight on a flat glass plate and taping the ends to the glass plate.

Weight measurements were made for the samples grafted by the IPN and dip-coat methods (Mettler H 31 AR weighing balance, Mettler Instruments Corp.,

Princeton, NJ). The initial weight of cleaned, dry samples and the final weight of dry grafted samples were obtained and the % weight change was calculated as follows :

% weight change = (final weight – initial weight) x 100 / initial weight.

5. RESULTS AND DISCUSSION

5.1. Results

5.1.1. Results from the different techniques used in grafting HEMA

The various methods which were tried in grafting HEMA onto silicone rubber tubes and the procedures followed are described in detail in Table 4.1. The results obtained are listed in Table 5.1.

5.1.2. Radiation initiated polymerization

Ratner and Hoffman (1974, 1975) and Knoll (1980) have reported the degree of graft and water imbibing properties of various radiation grafted hydrogel formulations on silicone rubber (nonporous) and polyethylene terephthalate (porous fabric), respectively. By comparison, this work reports that the same hydrogel formulation with varying amounts of Azo direct dye can be grafted on silicone rubber simultaneously using the radiation initiated polymerization method. Table 5.2 lists fabrication and graft data for a number of hydrogel formulations. The monomer-crosslinker-cosolvent compositions and dose levels had noticeable influences on the final hydrogel.

Table 5.1: Results obtained from the different methods used in grafting HEMA onto silicone rubber

Technique used	Results
Free radical initiated polymerization reaction	Even though Pinchuk et al. report that the reaction time was 15 minutes, in the current study the reaction time was seen to be longer (100–120 minutes) for polymerization. Complete polymerization could not be obtained using this method.
Interpenetrating Polymer Networks (IPN)	Significant grafting of the monomer onto the inner wall of the silicone rubber could not be achieved by the IPN method. Weight gain percentages were $< 1\%$.
Dip-coat method	Significant weight gains for silicone rubber tubes were not obtained for samples which received 6 coatings of the solubilized polymer.
Dip-coat method followed by the free radical initiated technique to give the second HEMA coat	Could not obtain significant grafting of HEMA onto the silicone rubber substrate.
The IPN method followed by the free radical initiated polymerization method	No significant grafting was achieved. The adhesion of HEMA onto the silicone rubber substrate was poor.
^{60}Co irradiation	Visual and microscopic observations indicated significant grafting of the HEMA. A layer 0.13 mm thick built upon the inner wall of the tube, and material penetrated 67 μm into the wall of the tube.
Radiation grafting with the various hydrogel-dye solutions. Formulation details are given in Table 5.2	A concentration gradient of the dye was evident in the grafted hydrogel matrix along the axis of tube.

Table 5.2: Fabrication data for radiation initiated polymerization of hydrogel on Silastic^R tubes

Composition (% by volume)					
		Solvent	Azo[a]	Dose	
HEMA	EGDM	Methanol/H_2O (100%)	Direct Dye (in grams)	Level (Mrad)	Observations
20	1.0	39.5/39.5	0.01	0.25	
20	1.0	39.5/39.5	0.05	0.25	No visible
20	1.0	39.5/39.5	0.1	0.25	reaction
20	2.0	39.0/39.0	0.01	0.25	
20	2.0	39.0/39.0	0.05	0.25	No visible
20	2.0	39.0/39.0	0.1	0.25	reaction
20	2.0	39.0/39.0		0.25	No visible reaction
20	2.0	39.0/39.0	0.1	0.25	No visible reaction
20	2.0	39.0/39.0	0.005	0.5	Firm gel stained
20	2.0	39.0/39.0	0.01	0.5	yellow; the dye
20	2.0	39.0/39.0	0.05	0.5	penetrated 5 μm into the wall of the tube.
20	2.0	39.0/39.0	0.005	0.25	Flaky loose
20	2.0	39.0/39.0	0.01	0.25	gel stained
20	2.0	39.0/39.0	0.05	0.25	yellow
20	2.0	39.0/39.0	0.005	1.0	Firm gel stained
20	2.0	39.0/39.0	0.01	1.0	yellow; a layer
20	2.0	39.0/39.0	0.05	1.0	0.13 mm thick built up on the inner wall of the tube, and material penetrated 67 μm into the wall of the tube.

[a]A formulation is based on HEMA + EGDM + solvent = 100% by volume. The Azo dye is added to 15 ml of a formulation solution.

5.1.3. Optical microscopic results

Studies using compound and dissection microscopes were done on cross sections and longitudinal sections of the grafted samples as soon as possible after irradiation. Although the N-vinyl-2-pyrrolidone (NVP) graft penetrates the silicone rubber matrix, the HEMA graft forms a layer on the surface of the silicone rubber matrix (Horbett and Hoffman, 1975; Ratner and Hoffman, 1975; Ratner et al., 1975). Optical microscopic studies (at 10x) of the 20 % HEMA, 2% EGDM, 39% MeOH and 39% H_2O composition at 0.5 and 1.0 Mrad dose levels clearly showed the existence of a yellowish layer of HEMA on the inner walls of the tubes. The layers were approximately 50 μm thick. These layers were present even after extraction with a 50% ethanol and 50% H_2O mixture for 2 hours.

Longitudinal sections of a Silastic^R sample tube grafted with 3 different hydrogel-dye compositions are shown in Figure 5.1. The formulation chosen for this sample contained 20% HEMA, 2% EGDM, 39% MeOH and 39% distilled H_2O.

5.2. Discussion

The free radical initiated polymerization, the IPN, and the dip-coating methods of grafting HEMA onto silicone rubber tubes of 1.98 mm i.d. were not very successful. Most of the problems encountered with these methods were poor adhesion of the HEMA onto the silicone rubber, poor graft percentages and also very little control of the dye distribution in the HEMA matrix. Because of the sequential steps that have to be followed in applying these methods, more control over the dye distribution in the HEMA could not be achieved.

Figure 5.1: Longitudinal section of a 10 cm long radiation grafted Silastic^R tube (1.98 mm i.d. x 3.18 mm o.d.) showing the concentration gradient of the dye dispersed in the HEMA. Dose level used = 0.5 Mrad

The radiation initiated polymerization method worked very well for grafting the various HEMA-dye formulations onto silicone rubber. By using different dye amounts in the same hydrogel composition and by filling the lumen of the Silastic^R tubing with these different solutions, a concentration gradient of the dye was established along the axis of the tube. Diffusion of dye to areas of lower concentration is not a severe problem if the irradiation is done immediately after tubes are filled and if movement of samples is kept to a minimum. Diffusion can be kept to a minimum by filling the lower parts of the Silastic^R tube with the relatively high dye concentrations and the upper parts with relatively low dye concentrations. To reduce diffusion, it is also useful to lay the Silastic tubes horizontally after filling and maintain this position until irradiation begins. A little diffusion between the different liquid interfaces is advantageous because this will allow gradual changes of dye concentration rather than abrupt changes in concentration at the interfaces.

By varying the amount of HEMA-dye monomer mixture added to the interior of the Silastic^R tube, the distance over which the concentration gradient changes can be altered. By using different sized glass mandrels, the thickness of HEMA on the inner wall of a silicone rubber tube can be changed. Thus, different sizes of nerve cuffs can be fabricated by minor alterations in the fabrication method.

6. CONCLUSION

This study represents the initial steps of an attempt to utilize hydrogel formulations on a tubular substrate to produce a nerve cuff. Since nerve fibers follow a concentration gradient of the nerve growth stimulant, it is important to develop a method by which a chemical gradient can be established along the tubular substrate. The current work is successful in developing a technique to lay down a concentration gradient of a dye in the grafted hydrogel matrix along the axis of a silicone rubber tube. By replacing the dye with nerve growth stimulants, it is anticipated that hydrogel grafted silicone rubber tubes can be used as nerve cuffs. The ^{60}Co irradiation grafting method is satisfactory for fabricating hydrogel grafted tubes of varying lengths and diameters in which a dye is incorporated into the hydrogel matrix in increasing concentrations from one end of the tube to the other.

The chemotactic effect of NGF and other neuropeptides on nerve fiber regeneration has been emphasized by Charlwood et al. (1972) and Letourneau (1978). Based on these facts, emphasis was laid on the future development of a nerve cuff which will have biologically active molecules distributed along a lengthwise concentration gradient. This study shows that a substance (Azo direct dye in this case) can be distributed to form a concentration gradient in a HEMA matrix. This is grafted onto a tubular silicone rubber substrate by the radiation initiator method.

In these initial experiments, Azo direct dye was used instead of a chemotactic agent because it is easy to detect, procedures for use with hydrogels are known and it is less costly. The formulation containing 20% HEMA, 2% EGDM, 39% methanol and 39% H_2O (irradiation dose of 0.5 Mrad) was chosen based on 'pore' size requirements. The dye solutions containing 0.005 g, 0.01 g and 0.05 g of Azo dye each in 15 ml of the monomer mixtures worked best in visualizing the dye concentration gradients easily. The formulation chosen had a microvoid size of $< 1\mu$m, which was able to entrap the dye molecules (M.W = 624.56). Since the nerve growth factor molecules are larger (M.W = 13,250), this pore size should be able to also entrap the biologically active molecules. Also diffusion rates should be lower for the nerve growth factor molecules in the hydrogel matrix since they are larger. Hence, in future experiments with the growth factor, diffusion down concentration gradients should not be a critical factor.

Future research on this project might include substituting commercially available NGF in place of the dye, studying the effects of ^{60}Co radiation on the NGF (i.e., will radiation denature the protein), studying the interaction between growing neurons and the proteins trapped in the hydrogel matrix, and determining the adhesive requirements for attachment of neurons to the hydrogel substrate. Because of elevated temperatures reached during irradiation, the effects of increased temperature on the growth stimulants should be studied. Additional investigations of hydrogel formulations producing other characteristics (i.e., microporosity, chemical, mechanical strength) should be performed and results should be catalogued. These factors can be coupled to *in vivo* experiments, the results of which may demonstrate

the controlling factor(s) for nerve adhesion, fiber growth and direction of growth. All these factors have to be taken into consideration and all problems have to be overcome before a suitable off-the-shelf nerve cuff can be fully developed for *in vivo* applications.

7. BIBLIOGRAPHY

Andrade, J. D. 1973. Interfacial phenomena and biomaterials. *Med. Instrum.* 7:110–120.

Barbin, G., M. Manthorpe, and S.Varon. 1984. Purification of the chick eye ciliary neuronotrophic factor. *Journal of Neurochemistry* 43:1468–1478.

Barde, Y.A., D. Edgar, and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. *The EMBO Journal* 1:549–553.

Benowitz, L. I., and L. A. Greene. 1979. Nerve growth factor in the goldfish brain: biological assay studies using pheochromocytoma cells. *Brain Res.* 162:164–168.

Boyd, L. F., R. A. Bradshaw, W. A. Frazier, R. A. Hogue-Angeletti, I. Jeng, M. W. Pulliam, and A. Szutowicz. 1974. Minireview : Nerve growth factor. *Life Sciences* 15:1381–1391.

Braley, S. 1970. The chemistry and properties of the medical-grade silicones. *J. Macromol. Sci. Chem.* A4:529–544.

Bruck, S. D. 1972. Biomaterials in medical devices. *Trans. Am. Soc. Artificial Internal Organs* 18:1–9.

Bruck, S. D. 1973. Polymeric materials: Current status of biocompatibility. *Biomater. Med. Devices Artif. Organs* 1:79–98.

Carbonetto, S. T., M. M. Gruver, and D. C. Turner. 1982. Nerve fiber growth on defined hydrogel substrates. *Science* 216:897–899.

Charlwood, K. A., D. M. Lamont, and B. E. Banks. 1972. Apparent orienting effects produced by nerve growth factor. Pages 102–107. *In* E. Zaimis and J. Knight, eds. Nerve Growth Factor and its Antiserum. *The Athlone Press of the Univ. of London, London, England.*

Chiu, David T. W., Ivo Janecka, T. J. Krizek, M. Wolff, and R. E. Lovelace. 1982. Autogenous vein graft as a conduit for nerve regeneration. *Surgery* 226–233.

Daniller, A. I., and B. Strauch. 1977. *Symposium on Microsurgery. C.V. Mosby, St. Louis.*

Dekker, A., W. H. Gispen, and D. de Wied. 1987. Minireview: Axonal regeneration, growth factors and neuropeptides. *Life Sciences* 41:1667–1678.

Denny-Brown, D. 1946. Importance of neural fibroblasts in the regeneration of nerve. *Arch. Neurol. Psychol.* 55:171–215.

Eckstein, E. C., and L. Pinchuck. 1982. Dye technique for assessment of hydrogel grafts. *8th Annual Meeting of the Soc. for Biomaterials* 8:24.

Grabb, W. C., S. I. Bement, G. H. Koepke, and R. A. Green. 1970. Comparison of methods of peripheral nerve suturing in monkeys. *Plast. Reconstr. Surg.* 46:31–37.

Greer, R. T., and R. L. Knoll. 1980. Controlled microstructure of two stage polyhydroxyethyl methacrylate coatings on polyethylene terephthalate substrates for potential prosthetic applications. *Scanning Electron Microscopy* 3:277–290.

Harper, G. P., Y. A. Barde, G. Burnstock, J. R. Carstairs, M. E. Dennison, K. Su da, and C. A. Vernon. 1979. Guinea pig prostate is a rich source of nerve growth factor. *Nature (London)* 279:160–162.

Hoffman, A. S. 1974. Principles governing biomolecular interactions at foreign interfaces. *J. Biomed. Mater. Res.* 8:77–83.

Hogue-Angeletti, R. H., and R. A. Bradshaw. 1978. Nerve growth factor in snake venom. Pages 176–294. *In* C. Y. Lee, ed. Handbook of Experimental Pharmacology. Vol 52. *Springer, New York.*

Horbett, T. A. and A. S. Hoffman. 1975. Bovine plasma protein adsorption onto radiation-grafted hydrogels based on hydroxyethyl methacrylate and N-vinyl-

pyrrolidone. Pages 230-254. *In* R.F. Gould, ed. Advances in chemistry series. *Am. Chem. Soc., Washington, D.C.*

Jabaley, M. 1981. Current Concepts of Nerve Repair. *Clinics in Plast. Surg.* 8:33–44.

Knoll, R. L. 1980. Analysis of polyhydroxyethyl methacrylate coatings on polyethylene terephthalate fabric substrates for cardiovascular prosthetic applications. *Ph.D. Dissertation. Iowa State University, Ames, Iowa.*

Letourneau, P. C. 1978. Chemotactic response of nerve fiber elongation to nerve growth factor. *Developmental Biology* 66:183–196.

Levowitz, B., J. LaGuerre, W. Calem, F. Gould, J. Scherrer and H. Schoenfeld. 1968. Biologic compatibility and applications of Hydron. *Trans. Am. Soc. Artif. Int. Org.* 14:82–87.

Longo, Frank, M., M. Manthorpe, S. D. Skaper, G. Lundborg and S. Varon. 1983. Neuronotrophic activities accumulate *In Vivo* within silicone nerve regeneration chambers. *Brain Research* 261:109–117.

Lundborg, G. 1975. Structure and function of the intraneural microvessels as related to trauma, edema formation and nerve function. *J. Bone Jt. Surg.* 57A:938–947.

Lundborg, G., and H. A. Hansson. 1980. Nerve regeneration through preformed pseudosynovial tubes. *J. Hand Surg.* 5:35–38.

Lundborg, G., L.B. Dahlin, N. Danielsen, R.H. Gelberman, F.M. Longo, H.C. Powell, and S.Varon. 1982. Nerve regeneration in silicone chambers: Influence of gap length and of distal stump components. *Exp. Neurology* 76:361–375.

Madison, R., C.F. DaSilva, P. Dikkes, T. Chiu and R.L. Sidman. 1985. Increased rate of peripheral nerve regeneration using bioresorbable nerve guides and a laminin-containing gel. *Exp. Neurology* 88:767–772.

Midenberg, M. L., and S. E. Kirschenbaum. 1986. Utilization of silastic nerve caps for the treatment of amputation neuromas. *The Journal of Foot Surgery* 25:489–494.

Millesi, H. 1972. The interfascicular nerve grafting of the median and ulnar nerves.

J. Bone Jt. Surg. 54A:727–750.

Millesi, H. 1973. Microsurgery of peripheral nerves. *The Hand* 5:157–160.

Molander, H., Y. Olsson, O. Engkvist, S. Bowald, and I. Eriksson. 1982. Regeneration of peripheral nerve through a polyglactin tube. *Muscle and Nerve* 5:54-57.

Molander, H., O. Engkvist, J. Hagglund, Y. Olsson, and E. Torebjork. 1983. Nerve repair using a polyglactin tube and nerve graft: An experimental study in the rabbit. *Biomaterials* 4:276–280.

Muller, Harold., L. R. Williams, and S. Varon. 1987. Nerve regeneration chamber : evaluation of exogenous agents applied by multiple injections. *Brain Research* 413:320–326.

Munz, M., M. Rasminsky, A.J. Aguayo, M. V. Sanz and M.G. Devor. 1985. Functional activity of rat brainstem neurons regenerating axons along peripheral nerve grafts. *Brain Research* 340:115–125.

Pinchuk, L. and E. C. Eckstein. 1981. Pressurized polymerization for reaction casting of poly(2–hydroxyethyl methacrylate). *J. Biomed. Mater. Res.* 15:183–189.

Predecki, P. 1974. A method for Hydron impregnation of silicone rubber. *J. Biomed. Mater. Res.* 8:487–489.

Ratner, B. D., and A. S. Hoffman. 1974. The effect of cupric ion on the radiation grafting of N-vinyl-2-pyrrolidone and other hydrophilic monomers onto silicone rubber. *J. Applied Polymer Science* 18:3183–3204.

Ratner, B. D. and A. S. Hoffman. 1975. Radiation grafted hydrogels on silicone rubber as new biomaterials. Pages 159–171. *In* H.P. Gregor, ed. Biomedical applications of polymers. *Plenum Publication Corp., New York.*

Ratner, B. D., A. S. Hoffman, and J. D. Whiffen. 1975. Blood compatibility of radiation–grafted hydrogels. *Biomater. Med. Dev. Artif. Organs* 3(1): 115–120.

Ratner, B. D., and A. S. Hoffman. 1976. Synthetic hydrogels for biomedical applications. Pages 1–36. *In* J.D. Andrade, ed. Hydrogels for Medical and Related

Applications. *American Chemical Society, Washington, D.C.*

Refojo, M. F. and H. Yasuda. 1965. Hydrogels from 2–hydroxyethyl methacrylate and propylene glycol monomethacrylate. *J. Appl. Polymer Sci.* 9:2425–2435.

Ryoo, Je-Phil. 1986. In vitro studies of the release of tylosin tartrate from hydrogel ocular inserts. *M.S. Thesis. Iowa State University, Ames, Iowa.*

Spencer, P. S. 1974. The traumatic neuroma and proximal stump. *Bull. Hospital Joint Dis.* 35:85–100.

Terzis, Faibisoff and H. B. Williams. 1975. The nerve gap : Suture under tension vs graft. *Plast. Reconstr. Surg.* 56:166–170.

Wichterle, O. 1971. Hydrogels. Pages 273–291. *In* H.F. Mark and N.G. Gaylord, eds. Encyclopedia of polymer science and technology. Vol. 15. *Interscience of John Wiley, New York.*

Wichterle, O. and D. Lim. 1960. Hydrophilic gels in biological use. *Nature(London).* 185:117–118.

Yannas, I. V., D. P. Orgill, J. Silver, T. V. Norregaard, N. T. Zervas, and W. C. Schoene. 1985. Polymeric template facilities regeneration of sciatic nerve across 15 mm gap. *Am. Chem. Soc.* 190:35–36.

Young, Barbara L., P. Begovac, D. G. Stuart, and G. E. Goslow, Jr. 1984. An effective sleeving technique in nerve repair. *Journal of Neuroscience Methods* 10:51–58.

8. ACKNOWLEDGEMENTS

I wish to express my appreciation to my major professor, Dr. Raymond T. Greer, for giving me the opportunity to conduct this research, and for his support and guidance throughout this work. I would also like to thank Drs. Mary Helen Greer and F. Hembrough for readily agreeing to serve on my committee. Special thanks are extended to my friends for their suggestions and assistance.

Finally, I would like to thank my mother for the support, guidance and unending encouragement she has given me throughout my studies.

9. APPENDIX

Table 9.1: Abbreviations for amino acids

Amino Acid	Three-letter abbreviation
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic acid	Asp
Cysteine	Cys
Glutamine	Gln
Glutamic acid	Glu
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophan	Trp
Tyrosine	Tyr
Valine	Val