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Cell-based Therapeutics

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Nano- and Micro-Technology to Spatially and Temporally Control Proteins for Neural Regeneration

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1.1. INTRODUCTION

Nano- and micro-technologies in the field of neural tissue engineering have implications in the pursuit of spatial and temporal control of protein and sugar cues at the site of injury and in the control over cellular response to these cues to promote regeneration and healing. The nervous system consists of two main components that are relevant from a regeneration and tissue engineering perspective. These components are the central nervous system (CNS), consisting of the cells and processes contained within the spinal and cranial cavities, and the peripheral nervous system (PNS), comprising of the nervous system outside of the CNS. Although a third component, the autologous nervous system, exists and is important physiologically, this component will not be the focus of this chapter for the sake of relevance and brevity.

The cellular and molecular events that follow injury in the CNS and PNS are different. These differences have implications for the kinds of nano- and micro- scale control necessary to stimulate regeneration or healing after injury. The nerves in the PNS have the ability to spontaneously regenerate if the gap between the two nerve ends is less than 10 mm. However, in the CNS, nerves cannot regenerate after injury and permanent functional loss occurs.

1.1.1. Response after Injury in CNS and PNS

A possible reason for the CNS's relative inability to regenerate compared to the PNS is due to the body's wound healing response. After injury to the CNS, a glial scar forms and there is a migration of microglia/macrophages to the wound site to remove the debris produced [23]. At the injury site, there are cells that up-regulate or expose inhibitory molecules that make the environment non-permissive for nerve regeneration. For example, astrocytes up-regulate chondroitin sulfate proteoglycans (CSPGs) and oligodendrocyte debris, produced during injury, expose myelin associated glycoprotein (MAG) [43, 48], NOGO [50], and oligodendrocyte myelin glycoprotein (OMpg) [35]. Other inhibitory molecules are also present in the glial scar; however, the non-regenerative environment has been mainly attributed to CSPGs, MAG, NOGO, and OMpg.

In the PNS, a slightly different series of events occur after injury. Macrophages infiltrate the injury site within three days and deliver various growth factors, as well as encourage Schwann cells to start producing NGF [63]. Other events that occur during the 3rd and 4th days after injury involve mast cells, axonal sprouts, and fibroblasts [81]. Similar to the macrophages, the mast cells also release important growth factors and other molecules, such as cytokines and interleukins. The presence of these cells peaks around the 4th day post injury and declines back to normal levels around 4 weeks after injury [63]. Another event that occurs in the two nerve ends is angiogenesis. The combination of these events makes it conducive for regeneration to take place in the PNS. The delivery of various growth factors by the macrophages and the mast cells along with the angiogenesis create a microenvironment allowing axonal outgrowth to occur from the proximal nerve end to the distal end.

Macrophages have a similar role in both the CNS and the PNS as they enter the lesion site to remove debris. However, the required time for the macrophages to enter the glial scar is much longer in the CNS than in the PNS [2]. One cause is the macrophages have problems entering the injury site due to the blood-brain barrier [69]. Also, in the distal end of the severed nerve in the CNS, the cell adhesion molecules are not up-regulated like they are in the PNS [59]. Although there are morphological and physiological differences between the PNS and CNS, the basic approach to aid in regeneration in both of these systems is similar.

Currently the options for therapeutic application in the CNS after injury are limited. Anti-inflammatory agents, such as methylprednisolone, are administered after injury; however, this does not provide any type of regenerative stimuli. In the PNS, there are two types of clinical treatments that can be applied to reattach the severed nerve. The type of treatment depends upon the size of the gap between the nerve ends. If the gap is short, then the two ends can be sutured together, restoring the axonal connections. However, if the gap is longer, then typically autografts or allografts are used [59]. Although clinically autografts are the state-of-the-art to regenerate nerves in the PNS, they are not the ideal solution because the donor supply is limited and obtaining a nerve graft from the patient means that the donor site has been denervated. Therefore, there is a great need to find a tissue engineered therapeutic solution to regenerate axons in the CNS and the PNS.

1.1.2. Nano- and Micro-scale Strategies to Promote Axonal Outgrowth in the CNS and PNS

The current clinical challenges are in regenerating peripheral nerves across gaps greater than 20 mm and to obtain any regeneration with accompanying functional recovery

after injury in the CNS. As alluded to earlier, the crux of the approaches currently under development to promote regeneration in the CNS and PNS involve either understanding or manipulating of nano- and micro-scale events in a controlled spatio-temporal sequence.

To regenerate nerves across a PNS or CNS nerve gap, there are three basic areas that are being explored. These strategies are 1) to provide permissive bioactive substrates for axonal outgrowth; (2) to deliver trophic factors in order to stimulate growth; and (3) to alleviate signaling due to the inhibitory entities present in the extracellular environment to allow axons to regenerate between the proximal and distal ends. First, nerves are anchorage dependent and the design of a substrate or bridge between the severed ends is an opportunity to present the correct spatial and temporal cues to both guide and stimulate axonal growth across the nerve gap. Second, promotion and stimulation of axonal growth involves the application of specific factors known as neurotrophic factors in a spatially and temporally controlled manner *in vivo*. Third, any inhibitory cellular responses and/or any inhibitory cues that may be generated after injury must be modulated such that they do not interfere in any putative regenerative attempt after injury. This third aspect is especially critical in the CNS where an extremely inhibitory glial scar is generated at the distal nerve segment, often leading to regenerative failure in the CNS. There are a series of technologies and challenges that are encountered in achieving this goal and they are discussed below.

1.1.2.1. Growth Permissive Substrates to Actively Support Growing Axons An important strategy used to promote axonal regeneration is to provide substrates for the outgrowth to occur. There are four types of substrates that have been investigated the most to provide an adequate scaffold; hydrogels, fibers, nerve guide conduits (NGCs), and transplanted cells. Proteins and oligopeptides are coupled onto the substrates to provide a more permissive surface that mimics the ECM for the axons to anchor up and extend through the nerve gap. Collagen and laminin (LN) are the more common proteins coupled to the substrates, as well as the oligopeptides, RGD, YIGSR, and IKVAV. The contribution of these proteins and oligopeptides will be discussed further later in this chapter. Transplanting cellular substrates is another approach to encourage axonal regeneration. Schwann cells, olfactory ensheathing glia (OEG), and astrocytes are examples of the cells that could be transplanted in the nerve gaps in the PNS and the CNS. These cells secrete proteins and growth factors that make the microenvironment less inhibitory for axonal outgrowth.

1.1.2.2. Stimulating Process Extension Using Trophic Factors Neurotrophic factors have an important role in the neural development and in adult life for axonal regeneration. Neurotrophins are a specific family of neurotrophic factors we are interested in to promote regeneration in the nervous system. The main focus in the PNS has been on two members of the neurotrohin family, nerve growth factor (NGF) and fibroblast growth factor (FGF). After injury, NGF is up-regulated. It was mentioned that Schwann cells and other cells that migrate to the injured area release trophic factors, one of them being NGF. FGF has also been shown to enhance axonal outgrowth [1] as well as angiogenesis [3]. In the CNS, brain-derived neurotrophic factor (BDNF) and neutrophin-3 (NT-3) have been investigated for their regenerative capabilities. Several studies, which will be discussed later, have shown that after administration of BDNF or NT-3, axonal regeneration was exhibited in spinal cord injured adult animals.

1.1.2.3. Alleviating Inhibitory Environment at the Site of Injury Neurites extend from the neuronal body and have a growth cone at the tip. The role of the growth cone is to read the environmental cues and decide which direction the neurite will grow towards. The growth cone extends filopodia and lamellipodia to read the cues. In the glial scar the inhibitory molecules prevent the extension of the filopodia and lamellipodia and induce growth cone collapse. Therefore, it is necessary to mask or remove these negative components from the microenvironment in order to support axonal outgrowth. Protein transduction is one of the methods utilized to promote and stimulate axonal regeneration. By intracellularly modulating levels of protein and other chemical concentrations, the growth cone can be manipulated to extend and grow into an inhibitory environment. Modulating Rho GTPases is one of the ways to overcome glial scar inhibition through protein transduction [21, 31, 45, 70]. Rho GTPases are involved in actin cytoskeleton dynamics, specifically promoting filopodial and lamellipodial extension [51]. By modulating Rho GTPases' levels in the neurons and elevating the concentration, the inhibitory effects of the glial scar will be masked and the growth cone will lead the neurite towards the distal nerve ending.

Other molecules that have been used to encourage neurite outgrowth in the face of inhibitory signals is cAMP and calcium in the CNS [41]. It has been shown that the modulation of cAMP, using the active and inactive analogs, can encourage neurite outgrowth through inhibitory substrates [5]. The activation of the signal transduction pathway by cAMP shows that axonal regeneration can be stimulated and promoted [54]. *In vivo* studies have also shown that increasing cAMP levels will promote axonal outgrowth [19].

The last method that will be reviewed is removing the inhibitory effects that do not allow regeneration to occur in the PNS and the CNS. An example of removing the inhibition is the enzymatic degradation of chondroitin sulfate proteoglycans (CSPGs) in the CNS. After injury, astrocytes and oligodendrocyte precursor cells release CSPGs into the glial scar matrix [46]. It has been demonstrated that both components, the protein core and the glycosaminoglycan side chain, contribute to the inhibitory nature of the macromolecule [28, 46]. Consequently, studies have shown that by treating the CSPGs with chondroitinase ABC, which cleaves the glycosaminoglycan (GAG) side chain into dissacharide units, axonal regeneration could occur and extend through the inhibitory glial scar region into the distal nerve end [12, 42, 82, 83].

In order to achieve axonal regeneration in the CNS and PNS it is important to construct a scaffold that allows axons to extend through; has the mechanical integrity to support cell migration, such as Schwann cells, into the scaffold; allows the delivery of growth factors, such as neurotrophins, that encourage axonal outgrowth; and integrate the scaffold and extracellular matrix (ECM) [22].

1.2. SPATIALLY CONTROLLING PROTEINS

In order for the three strategies to promote nerve regeneration, proteins must be controlled spatially and temporally in the CNS and PNS. Typically, biomaterial scaffolds are used for the spatial control of proteins in three-dimension (3D). An important factor when developing scaffolds is that it must mimic the ECM in order to encourage axons to grow through the glial scar. Therefore, in order to control the location of the proteins, 3D scaffolds,

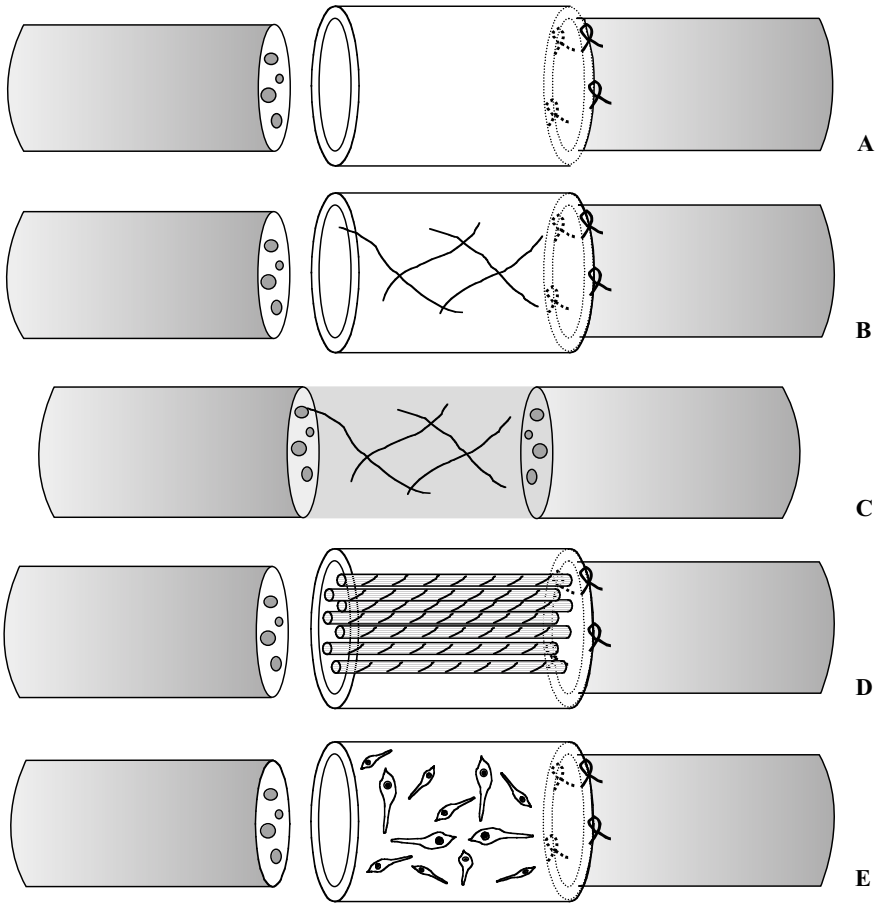

FIGURE 1.1. Spatial Control of Proteins. **A.** Nerve guide conduit (NGC) sutured to the proximal and distal ends of the nerve. **B.** Hydrogel filled NGC sutured to the nerve ends. **C.** Hydrogel scaffold injected between the nerve ends without the aid of the NGC. **D.** Fibers filled in the NGC to act as contact guidance cues for axonal regeneration. **E.** NGC containing transplanted cells, such as Schwann cells or olfactory ensheathing glia to aid in axonal regeneration.

such as hydrogels, fibers, NGCs, and cell transplantation can be utilized to promote axonal regeneration in the nervous system (Fig. 1.1).

1.2.1. Spatial Control: Permissive Bioactive Hydrogel Scaffolds for Enhanced Regeneration

The use of hydrogels provides a substrate for axonal outgrowth. Hydrogels are polymers that swell with the addition of water and are crosslinked. There are three main biomaterials belonging to the hydrogel family that have been used to provide a scaffold for axonal regeneration: (1) agarose, (2) alginate, and (3) collagen.

1.2.1.1. Agarose *as a Scaffolding Material* Agarose, which is a thermoreversible copolymer of 1,4-linked 3,6-anhydro-α-L-galactose and 1,3-linked β-galactose, is derived from red algae. Agarose is a beneficial biomaterial to use as a scaffold for a few reasons. The hydrogel is biocompatible as it causes no adverse reaction when implanted *in vivo*. Its porosity and mechanical properties can be manipulated and optimized to maximize axonal growth [47]. Most of all, agarose is beneficial because it can be used to control proteins spatially by binding proteins to the agarose and it can be used to support cell migration [6, 10].

Agarose gel can be used to encourage axonal outgrowth by covalently coupling growth promoting molecules to the agarose hydrogel, which would embody the characteristics of the ECM allowing axonal outgrowth into the glial scar and reconnect with the distal nerve. *In vitro* studies have shown that covalently coupling a growth promoting ECM molecule, such as LN, to the agarose gel encouraged neurite outgrowth compared to a scaffold that did not have any modifications [6, 80]. Along with coupling whole proteins, such as LN and collagen, oligopeptides can be bound to hydrogels as well. The oligopeptides of interest are the ones that influence cell-matrix interactions, such as RGD, which is responsible for the interaction between fibronectin and an integrin receptor, and YIGSR, which is a peptide on the β1 chain of laminin aiding in cell attachment [10].

The application of the engineered scaffold, which was a polysulfone tube containing LN-bound agarose and a slow release system of NGF, *in vivo* in the peripheral nervous system demonstrated that the regenerated myelinated axons were comparable to the regeneration found in autografts [79].

1.2.2. Spatial Control: Chemical vs. Photochemical Crosslinkers for Immobilization of Bioactive Agents

There are different types of crosslinkers that can be used to couple the proteins to the gel. There are thermochemical bifunctional crosslinkers, such as 1,1'-carbonyldiimidazole (CDI), which can couple the protein to the agarose. Another class of crosslinkers that can be used is photocrosslinkers. These photocrosslinkers are activated by shining UV light onto the agarose gel that contains the crosslinker [40]. Free radicals are created, which then can be bound to the protein of interest. Photocrosslinkers can be used to covalently bind macromolecules to the agarose hydrogel. Using UV light to produce free radicals is beneficial because laser beams can be used to create patterns in the hydrogel. One such application used UV laser beams to create channels through the agarose gel, encouraging the neurons to extend their neurites down the channel, providing directional cues for the neurites [40]. In a study, both CDI and a photocrosslinker, benzophenone, were used to couple YIGSR to agarose hydrogel. Results from both *in vivo* and *in vitro* experiments have shown that DRG neurite outgrowth was enhanced when cultured in 0.5% agarose. Additionally, *in vivo* the number of myelinated axons was higher in the agarose coupled to the YIGSR peptide than plain agarose [10]. This study also concluded that the effectiveness of the gel was not determined by the type of crosslinker used to couple the oligopeptide. Although there is not a functional difference between the types of crosslinkers, the advantage of using the photocrosslinker is the ability to pattern the hydrogel to favor the axonal growth in a specific direction.

1.2.2.1. Alginate as a Scaffolding Material Alginate is another scaffold material, similar to agarose, which can be utilized to control proteins spatially in order to influence axonal regeneration. Alginate can be found in brown seaweed and is a copolymer formed from α-L-glucoronic acid and β-D-mannuronic acid. The studies that have used alginate have been predominantly for axonal outgrowth in the PNS. Therefore, the use of alginate scaffold should support the migration of Schwann cells so that the cells can deliver LN, neurotrophic factors and cytokines. It was demonstrated that alginate sponge could support axonal regeneration from the proximal end to the distal end. The alginate allowed Schwann cell infiltration and enhanced the promotion of axonal growth through the nerve gap [26]. Growth promoting molecules can also be added to alginate gels similar to the agarose gels. Although soluble fibronectin added to the alginate gel did not exhibit any positive significance on axonal growth compared to empty poly-3-hydroxybutyrate conduits, the addition of soluble fibronectin in the alginate along with Schwann cells demonstrated a significant effect on axonal regeneration *in vivo* and shows promise as a possible method to attempt regeneration over long nerve gaps [47]. A study comparing the potency of implanted alginate with and without the use of a conduit demonstrated that in a 50 mm sciatic nerve gap made in cats, the alginate without the aid of a conduit was able to provide a microenvironment that allowed axonal outgrowth across the gap and the number of myelinated and unmyelinated axons that regenerated were the same in both conditions [62]. Regeneration in the PNS has mostly focused on using nerve conduits that are filled with a form of hydrogel. This study has suggested a possible alternative in case it is not possible to use the conduits. In addition, alignate sponge was investigated as a potential scaffold to promote regeneration in the CNS after the spinal cord was transected in rats. It was shown that regenerating axons infiltrated the alginate gel significantly higher compared to collagen gels [32]. It was also suggested that the formation of glial scar could be reduced by the alginate gel due to little infiltration of the connective tissue.

1.2.2.2. Collagen as a Growth Permissive Scaffold for Nerve Regeneration The third type of hydrogel applied as a scaffold for nerve regeneration is collagen, more specifically type I collagen. Collagen is found in the ECM and helps promote axonal outgrowth and cell adhesion. Comparisons among different types of gel matrices, collagen, methylcellulose, and Biomatrix, were conducted and showed that collagen along with methylcellulose had the best results in regenerating axons across a peripheral nerve gap [68]. It has been reported that filling tubes with ECM molecules, such as collagen, LN, and fibronectin, improves axonal regeneration. The affect of collagen and LN gels that are magnetically aligned improves the distance of axonal outgrowth compared to collagen added without any alterations [16, 66]. The collagen hydrogel has also been used in combination with GAG, and inserted into an NGC in the PNS. The combination of the collagen-GAG matrix exhibited a greater number of myelinated axons compared to unfilled conduits [17]. It is suggested that the collagen-GAG matrix elicited axonal outgrowth because it provided an adequate scaffold needed for attachment and cell migration. Collagen gels have also been inserted into lesions after dorsal transections in rat spinal cords. Although axonal regeneration did not occur through the entire lesion area, the collagen gel along with the neurotrophin encouraged outgrowth into the matrix [29]. The study also showed minimal glial scar formation, which would provide a more promoting microenvironment for axonal regeneration.

1.2.3. Other Hydrogel Scaffolds

There are other hydrogels that can be used as scaffolds besides the three main ones discussed above. Some of the other hydrogels are Matrigel, NeuroGel™, and Biomatrix. Matrigel is made out of a mixture of ECM proteins, such as LN and collagen. *In vivo* studies have shown that Matrigel alone is not adequate scaffold to promote axonal outgrowth [25, 65]. However, when the Matrigel is used in conjunction with Schwann Cells, axonal outgrowth is significantly noticeable. NeuroGel™ is a crosslinked copolymer hydrogel made of N-2-(hydroxypropyl) methacrylamide. When this hydrogel was inserted into the thoracic region of the spinal cord after a contusion injury, it was observed that the rats that had implanted NeuroGel™ in the lesioned cavity had an improved locomotion according to the BBB test and there was evidence of axonal fibers infiltrating the hydrogel, thereby crossing the tissue-implant interface [71]. NeuroGel™ also demonstrated the capability to hinder glial scar formation when it was implanted in the lesion of spinal cords in adult rats [72]. Biomatrix is a hydrogel, similar to Matrigel, made of ECM proteins, such as LN. However, Biomatrix does not appear to have as adequate regenerative capabilities as collagen and other hydrogels [68].

1.2.4. Spatial Control: Contact Guidance as a Strategy to Promote Regeneration

It was previously mentioned that besides the use of hydrogels as a scaffold, fibers could also be utilized to direct axonal growth from the proximal to distal ends of the nerve. This is another strategic technique to gain spatial control of proteins using a substrate. Tubes are inserted between the nerve gaps and then the nerve ends are sutured to the tubes with the fibers placed through the length of the tube (Fig. 1.1D). Due to the fibers being oriented longitudinally through the tube, it provides the orientation for the axons to grow from the proximal to distal end of the gap. Fibers are used to encourage the occurrence of two events in order to obtain successful myelinated axonal regeneration in the PNS. The first event is the formation of the fibrin matrix, which will have the same orientation as the filaments. The second event that needs to occur is the infiltration of Schwann cells. The goal is to have the Schwann cells adhere to the filaments and travel along the entire length of the filaments, which is the length of the nerve gap. This would then provide an environment which would encourage axonal outgrowth. Poly (L-Lactide) (PLLA) is another material that is commonly used to make filaments. In an *in vitro* study, it was demonstrated that if the PLLA was coated with LN, then the neurite outgrowth was significantly greater than neurite outgrowth on uncoated PLLA surface or the poly-L-lysine coated filaments [55]. Tubes are generally used to encapsulate the filaments and provide an environment for axonal growth along the filaments. However, in a study conducted in the PNS, collagen filaments were sutured to the proximal and distal ends of the nerve without the aid of tubes *in vivo*. The study showed that the number of myelinated axons that regenerated was greater to that found in the group that received the autograft, although it was not significantly greater [77]. This is the only study that did not use a conduit for the filaments or any neurotrophic factors, however, the regeneration was abundant and demonstrated that perhaps these two components are not completely necessary if the proper conditions are provided for axonal growth. Another variable that needs to be considered in the application of fibers is the number of fibers that should be inserted between the nerve ends. In studies conducted by Yoshii et al., collagen

filaments were sutured to the sciatic nerve ends without the aid of a tube, two thousand filaments were connected at the ends to keep them joined over a 20 mm and 30 mm gap [77, 78]. The myelinated axon regeneration was comparable to the results observed with autografts for the 20 mm gap [77]. However, in the case of the 30 mm gap, the axonal regeneration was significantly less. These studies suggest that a large number of filaments would aid in axonal outgrowth. However, in another study, which inserted PLLA filaments inside silicone tubes, demonstrated that a lower packing density of filaments elicited the greatest number of myelinated axons [49].

Although filaments are predominantly used in the PNS, studies have been performed where filaments were inserted in CNS to promote axonal outgrowth. Carbon filaments were implanted in the lesion of a fully transected rat spinal cord. The carbon filaments allowed a scaffold for axons to advance through the lesion [33]. This study was taken further, where 10,000 carbon filaments were cultured with fetal tissue and implanted into the spinal cord lesion. This condition exhibited an improvement in electrical conduction through the injured axons [39]. A study conducted by the same group who inserted 2000 filaments into a nerve gap in the PNS, utilized the collagen filaments to encourage axonal regeneration in the CNS after spinal cord injury (SCI) [78]. Four thousand collagen fibers were inserted between the two nerve ends parallel to the spinal cord. It was demonstrated that the collagen fibers provided an adequate scaffold to bridge the nerve ends and allow axons to extend across the gap.

It was previously mentioned that proteins and oligopeptides could be coupled to hydrogels. A similar method was used to couple peptides to fibers that could potentially be implanted as a scaffold in the CNS. Two laminin peptides, YIGSR and IKVAV, were coupled to poly(tetrafluoroethylene) (PTFE) fibers and DRGs were cultured to observe neurite extension [60]. The peptide surface modified fibers encouraged neurite outgrowth; however, the neurites could not extend along unmodified PTFE fibers. To have successful axonal regeneration using fibers as the scaffold, it is important to either use a biomaterial that encourages fibrin matrix formation and Schwann cell infiltration or to coat the fibers with a protein that does those things. Current research has demonstrated that fibers made out of collagen, coated with proteins, such as collagen or laminin, or oligopeptides have produced the most significant axonal regeneration. Controlling proteins spatially through fiber scaffolds allows a surface for axons to adhere, as well as orient the direction of growth.

1.2.5. Spatial Control: Nerve Guide Conduits Provide an Environment for Axonal Regeneration

The use of nerve guide conduits has greatly influenced axonal regeneration. They aid in providing a scaffold to promote axonal regeneration and have the potential to both spatially and temporally control the protein environment at the site of injury. Importantly, the conduit serves as a physical barrier to prevent proteins and other molecules from inhibiting axonal regeneration. When NGCs were first being used, it was believed that the best material for the tube was silicone due to its mechanical properties. However, silicone NGCs are non-absorbable, non-semipermeable and require a second surgery to remove the conduit, otherwise it could cause chronic tissue response, such as scar formation, as well as nerve compression [18]. Most NGCs in use today are semi-permeable and even biodegradable. However, as NGCs have been extensively reviewed elsewhere [8, 18, 30, 59], we choose

to concentrate this chapter on approaches where the NGCs are used as carriers for other bioactive agents to enhance their functionality.

1.2.6. Spatial Control: Cell-scaffold Constructs as a Way of Combining Permissive Substrates with Stimuli for Regeneration

Cell transplantation techniques are an elegant way to combine two promising strategies to elicit regeneration: permissive substrates and spatio-temporally controlled delivery of trophic factors at the site of injury. This strategy has been explored both in the CNS and the PNS and is described below. Typically, NGCs are used as carriers for the delivery of these cells to the site of injury in the PNS or the CNS (Fig. 1.1E).

Schwann cells and OEG are two cell types commonly used to promote regeneration in the CNS, while Schwann cells are typically the cells of choice in the PNS. These cells provide both trophic cues, as well as physical, contact guidance type cues in promoting regeneration as described below. The use of cells, such as these glia, utilizes the strategy that modulates intrinsic mechanisms to promote axonal outgrowth. The transplantation of Schwann cells and OEG allows for spatial control of growth factors and other proteins, which are secreted by the cells.

Schwann cells have been shown to enhance peripheral nerve regeneration. It was mentioned previously that infiltration by endogenous Schwann cells increased axonal regeneration [24]. Schwann cells were embedded in a scaffold, such as Matrigel, and transplanted into an NGC implanted between two nerve ends, myelinated and unmyelinated axons are regenerated [25]. It was believed that by implanting Schwann cells already present throughout the conduit, the pace of regeneration could be increased. Schwann cells align along the tube and arrange themselves so that they are end to end, which is called Bungner bands. It was demonstrated that syngeneic Schwann cells elicited a better axonal regeneration than heterologous Schwann cells, which elicited an immune response [25]. It was also shown that as the Schwann cell density increased in the NGC, the axonal regeneration improved along similar lines to nerve autografts. Schwann cells myelinate peripheral nerves and it has been established that transplantation of these cells encourages the outgrowth of myelinated and unmyelinated axons.

Schwann cells have also shown to promote regeneration in the CNS. In studies that transected rat spinal cords and then implanted grafts containing Schwann cells and Matrigel, it was demonstrated that the number of myelinated and unmyelinated axons was greater compared to grafts containing only Matrigel and the myelinated axons formed fascicles through the conduit [74, 75]. In another study that transplanted Schwann cells into the spinal cord, it was shown that Schwann cells that released increased amounts of NGF had significantly more axons growing into the graft compared to Schwann cells that were not modified to release increased amounts of NGF [67]. It was also demonstrated that these Schwann cells expressed the same phenotype and myelinated axons in the CNS as in the PNS. The combination of NGF and Schwann cells allows for the outgrowth of axons into the grafts due to the presence of NGF and then the Schwann cells provides direction for axonal growth due to the Bunger bands [67]. It was mentioned previously, cAMP has been investigated to promote axonal regeneration. In a study cAMP and Schwann cells were both inserted into the spinal cord to observe whether there was a synergistic effect [53]. The results demonstrated that by implanting Schwann cells and elevating cAMP, the

number of myelinated axons increased and functional recovery was observed compared to the transplantation of only Schwann cells.

Unlike Schwann cells, which can be transplanted in both the PNS and CNS, OEG is primarily transplanted in the CNS to promote axonal regeneration. OEG ensheath olfactory axons and shield the axons from inhibitory molecules exposed in the environment, thus allowing the axons to regenerate throughout adult life [58]. OEG demonstrates a promising method to ensheath the axons in other areas of the CNS that are injured and aid in regeneration. The olfactory bulb is the main supplier for OEG and one of the main benefits of using this source for OEG is because the glia can migrate into other regions of the CNS and integrate with other CNS glia [58].

Comparisons have been made between Schwann cells and OEG for their effectiveness in promoting axonal regeneration in the CNS. In a study that was comparing the response of astrocytes and CSPG expression after OEG or Schwann cell transplantation in the CNS, it was demonstrated that OEG elicited less of an astrocytic response and lower expression of CSPG compared to Schwann cells [37]. Although OEG do not induce as severe a response as Schwann cells do, Schwann cells have shown more promising results in improving locomotor performance compared to OEG after adult rats have suffered from contused thoracic SCI [64].

It was mentioned earlier that astrocytes can also be used as a substrate for axonal outgrowth. These studies were performed *in vitro*. It was demonstrated that uniformly orienting the astrocytes and organizing the ECM and cell adhesion molecules in order to culture neurons on the astrocytes lead to the enhancement of neurites extending in a direction parallel to the astrocytes [9]. The use of glial cells, such as astrocytes, as a substrate can be combined with a biomatrix to enhance neurite extension in a specific direction [20]. Glial cells were cultured on the biodegradable poly(D,L)-lactide matrices to orient the cells in a specific direction. Although this substrate did not enhance either the number of extended neurites or the length of the neurites, the cultured cortical neurons extended neurites along the orientation of the glial cells/biomatrix substrate.

1.3. TEMPORALLY CONTROLLING THE RELEASE OF PROTEINS

As important as it is to control the proteins spatially, it is equally imperative to control the amount of protein delivered over a period of time. Regeneration over long nerve gaps requires several months. Therefore, for axonal outgrowth to occur during this time period, the microenvironment must be actively supportive over this time scale. If proteins, such as Rho GTPases and neurotrophic factors, are only administered as a single dose at the time of implantation of the scaffold, then some of the protein will be taken up intracellularly, diffuse into the surrounding tissue, and degrade. Then there will not be a therapeutic level of protein to promote axonal outgrowth over the time necessary to have complete regeneration. For example, it was concluded that after local administration of NGF into the brain, the half-life of NGF was 30 minutes [36]. Once the effective concentration for the proteins is known, then it can be delivered and sustained. Sustaining the presence of proteins at the effective concentration can be achieved through a controlled slow release delivery system. There are currently four main techniques that are being investigated for controlling protein concentration at the site of injury over time: (1) osmotic pumps, (2) embedded

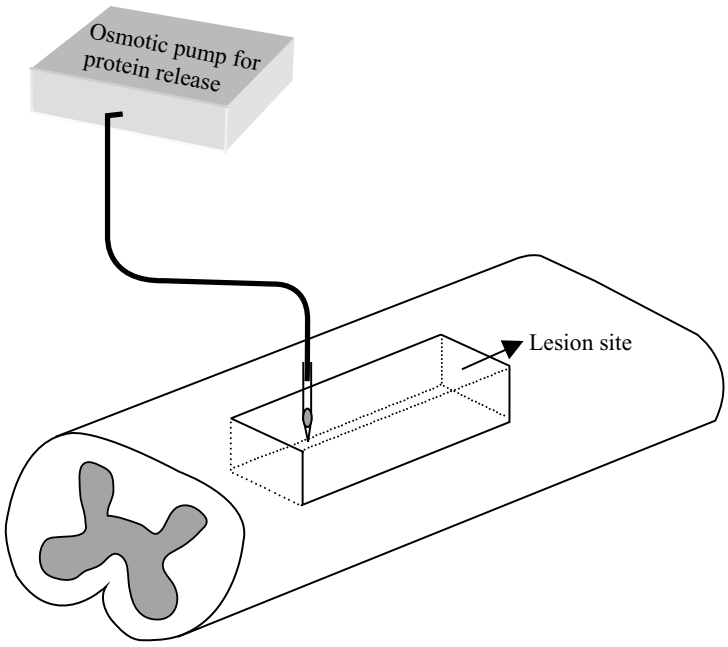

FIGURE 1.2. Osmotic Pumps for Temporal Control of Proteins. An external osmotic pump provides a reservoir of protein that is delivered via a catheter implanted near or at the lesion area.

microspheres, (3) microtubules and (4) enzyme dependent demand-driven trophic factor release.

1.3.1. Temporal Control: Osmotic Pumps Release Protein to Encourage Axonal Outgrowth

Osmotic pumps can be used to deliver proteins, such as neurotrophic factors, to promote axonal regeneration. Osmotic pumps are mostly utilized to deliver the proteins in the CNS. There are two parts to this delivery system, one component is the infusion pump that is usually implanted under the skin on the back of the animal, and other component is the catheter that is inserted in the lesion of the nerve (Fig. 1.2).

1.3.1.1. Temporal Control: Using Osmotic Pumps to Stimulate Process Extension by Sustained, Local Trophic Factor Delivery Several studies have investigated the benefits of continuous infusion of the neurotrophic factors BDNF and NT-3 after SCI. Typically, after SCI, methylprednisolone (MP) is administered to the patient. It has been demonstrated that the levels of BDNF and NT-3 decrease after the administration of MP. In a study, after treatment of MP, it was concluded that if BDNF was continuously delivered, then the rats locomotor function improved [34]. In a study that delivered both BDNF and NT-3 over a short time period (2 weeks) and a longer time period (8 weeks), it was shown that only the rats treated with BDNF and NT-3 over the 8 week time period allowed for the survival of the rubrospinal neurons [52]. However, rubrospinal axonal regeneration was not observed.

In another study that delivered either NT-3 or BDNF for 4 weeks into the spinal cord after it was crushed, the rats treated with BDNF did not exhibit any axonal regeneration. However, fiber sprouting was observed into and through the lesion in the rats that had NT-3 administered to the spinal cord lesion [11]. In a study that infused only BDNF for two weeks into the rat motor cortex after SCI, sprouting of corticospinal fibers was observed; however, axonal regeneration did not occur into the peripheral nerve transplant that was placed in the lesion [27]. The constant release of neurotrophic factors using the osmotic pump appears to exhibit therapeutic results. The site of administration seems to affect the response of axonal regeneration and fiber sprouting. The only disadvantage of utilizing the osmotic pump is the different locations of its components.

1.3.1.2. Temporal Control: Alleviation of Inhibitory Environments by Using Osmotic Pumps It was mentioned above that osmotic pumps can be used to deliver neurotrophic factors to the CNS to modulate intra-neuronal mechanisms. Osmotic pumps have also been utilized to infuse IN-1 antibody that neutralizes NOGO-A, an isoform of NOGO that is one of the main inhibitory molecules located in the glial scar [13]. It was observed that after 2 weeks of IN-1 delivery, regenerating fibers were observed through the lesion in the thoracic region into the lumbar region of the spinal cord. Therefore, the use of osmotic pumps can also be used to deliver proteins that can neutralize the inhibitory environment of the glial scar.

Other than the use of osmotic pumps to deliver proteins, Gelfoam, an insoluble gelatin sponge, was used to deliver chondroitinase ABC into the spinal cord lesion. The animals treated with chondroitinase ABC filled Gelfoam displayed axonal regeneration of the Clarke's neurons through the lesion area and it was exhibited that CSPG was digested by the chondroitinase ABC [76].

1.3.2. Temporal Control: Slow Release of Trophic Factors Using Microspheres

Microspheres, used in drug delivery applications, are being investigated to deliver protein to the PNS and CNS in order to encourage axonal outgrowth (Fig. 1.3). Microspheres have an advantage over osmotic pumps because a single administration is needed to release the protein over time. The size of the microspheres depends upon the application. The size of the microparticles in the studies that use microspheres to promote axonal outgrowth is around 12-16 μm. The materials that are used to make the microsphere are typically biodegradable polymers. The use of copolymers and altering the ratio of the polymers can affect the biodegradation profiles because the polymeric characteristics, such as glass transition temperature and hydrophilicities, change [61]. The polymeric materials mostly used for the microspheres are poly(lactic acid) (PLA), the copolymer poly(lactic-co-glycolic acid) (PLGA) and polyphosoesters. When investigating a specific polymer or another biomaterial, it is important to make sure that when the material degrades it does not denature the protein due to the possible immunogeneic response it can cause, thus altering the release profile and bioactivity [61].

1.3.2.1. Temporal Control: Use of Microspheres to Stimulate Process Extension in the PNS and CNS Most of the research, currently, focuses on delivering NGF loaded microspheres to regenerate nerves in the PNS. In a study performed by Xu et al., NGF

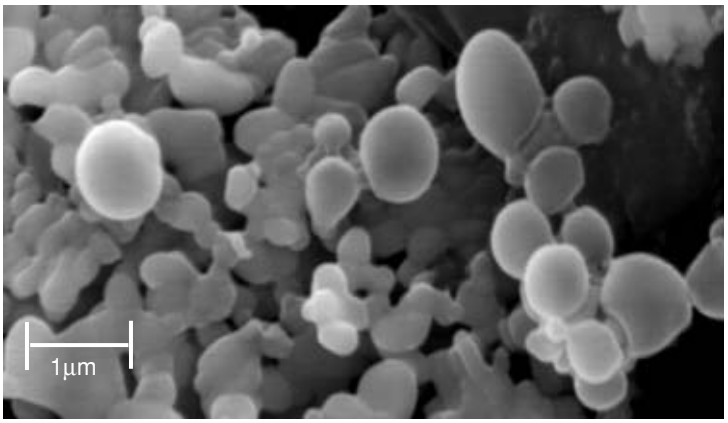

FIGURE 1.3. SEM Image of Microspheres. Microspheres can be used to encapsulate protein that will be slowly released as the microsphere degrades. Scale bar = 1μm. Figure courtesy of YT Kim and RV Bellamkonda, Department of Biomedical Engineering, Georgia Institute of Technology.

was loaded into poly(phosphoester) (PPE) microspheres. First, in *in vitro* studies, it was determined that the microspheres released bioactive NGF up to 10 weeks. The NGF loaded PPE microspheres in a saline solution were loaded into PPE NGCs. When these constructs were implanted into rat sciatic nerves, it was observed that treatment with NGF loaded microspheres in the NGC had a cable that bridged the entire 10 mm gap between the nerve ends. Also, compared to the controls, there were more myelinated axons, higher fiber density, and thicker myelin sheath [73].

In the CNS, one of the first studies conducted using microspheres to deliver protein to the CNS was by Camarata et al. In order to combat neurodegenerative disease, they inserted microspheres loaded with NGF that could be released *in vivo* for 4 to 5 weeks [14]. In another *in vitro* study, the number of days NGF was released was increased to 91 days. Various ratio of PLGA were tested to determine the release characteristics, as well as poly(ε-caparolactone) (PCL) [15]. The surface morphology of the microspheres that are loaded versus unloaded ones is different. The surface of protein loaded microspheres is rougher, whereas the unloaded microspheres have a smoother surface. The smaller the microsphere, the greater the surface area, thus increasing the degradation rate of the microsphere and release of the protein.

1.3.3. Temporal Control: Lipid Microtubules for Sustained Release of Stimulatory Trophic Factors

Another method to slowly release protein in the CNS and PNS is the use of lipid microtubules, also referred to as microcylinders (Fig. 1.4). These microtubules are hollow cylinders with a diameter of 0.5 μm [44]. The length of the microtubules varies based on the time period in which the protein, DNA, or other desired molecule needs to be released. The molecule is released at the ends of the microtubules, which is the reason why the length of the microcylinders controls the release profile of the protein. In a study previously mentioned, to aid axonal regeneration in the PNS, a two-step slow release system was developed. The first step was NGF loaded microtubules, which had a length of 40 μm, and

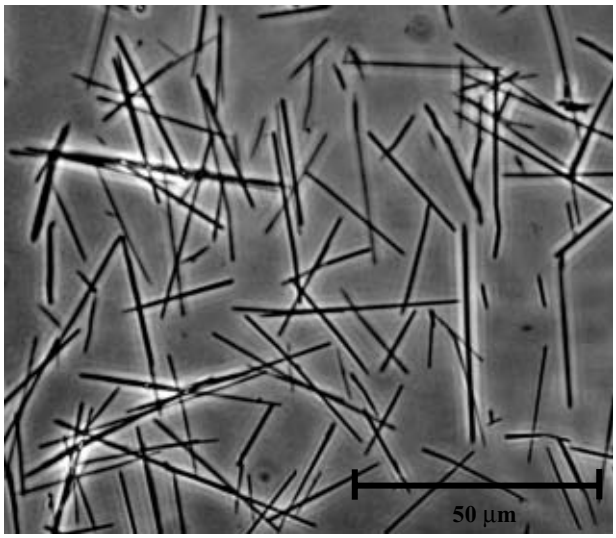

FIGURE 1.4. Micrograph Image of Lipid Microtubules. The image depicts the lipid microtubules being on average 40 μm in length. The microtubules release the protein from the ends. Scale bar = 50 μm.

the second step was the loaded microtubules embedded in agarose hydrogel [79]. The two step release system was thus, first the diffusion of the NGF from the microtubules into the agarose and then the release of the NGF from the agarose into the gap between the two nerve ends. This slow release system allows the NGF to last longer in the nerve gap and prevents degradation or dilution by macrophages and other fluids. Two months post-implantation, a cable formed, the number of myelinated axons was statistically similar to the autograft condition, and the density of myelinated axons was similar to that of the autograft and a normal sciatic nerve.

1.3.4. Temporal Control: Demand Driven Release of Trophic Factors

Another form of controlled release of a protein is the fibrin matrix, which was initially developed for wound healing. Cells that migrate to the area degrade the matrix through proteolysis, thereby releasing the contained protein (Fig. 1.5) [57]. A fibrin matrix covalently coupled to heparin that interacted with neurotrophins, NGF, BDNF, and NT-3 was developed. It was demonstrated *in vitro* that the neurite outgrowth was enhanced when the neurotrophins were released using this delivery system compared when soluble neurotrophins were added to the fibrin matrix [56]. When the heparin immobilized fibrin matrix was implanted in a nerve gap in the PNS, fiber sprouting was observed through the conduit to the distal end [38].

1.4. CONCLUSION

The advancement in CNS and PNS regeneration has been due to the utilization of nano- and micro-technologies. Most of the technology that has been developed has been geared

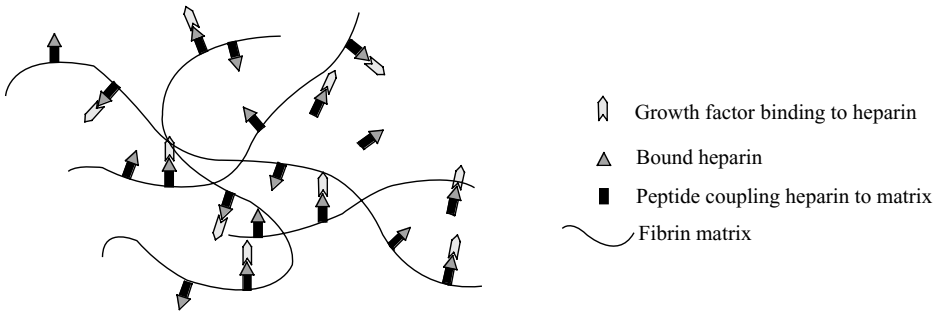

FIGURE 1.5. Schematic of Fibrin Matrix Releasing Protein. The peptides bind the heparin to the fibrin matrix. The growth factor or protein is then able to bind the heparin, thus attaching the growth factor to the matrix. The cells migrating to the area will then degrade the matrix releasing the growth factor. Figure adapted from Ref. [57].

towards controlling proteins spatially and temporally. There are three main strategies used to elicit axonal outgrowth after injury, which allows spatial and temporal control of proteins. The three strategies mentioned are to 1) provide permissive bioactive substrates for the axonal outgrowth; (2) use trophic factors to stimulate growth; and (3) alleviate signaling due to the inhibitory entities present in the extracellular environment to allow axons to regenerate between the proximal and distal ends.

This chapter briefly describes studies that have incorporated various nano- and micro-technologies using biomaterials based design. While, for analytical convenience we divide this chapter into sections with various strategies, it is becoming evident that a coordinated, multiple component strategy may be required for successful regeneration. For example, one approach is to design a substrate that is coupled to proteins, contains either Schwann cells or OEG, and has a delivery vehicle slowly releasing proteins. The key combination remains elusive and is the focus of active, ongoing investigation.

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