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Characterizing The Controlled Release Of Glial Cell-Line Derived (gdnf) Neurotrophic Factor From Encapsulated Schwann Cells

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CHARACTERIZING THE CONTROLLED RELEASE OF GLIAL CELL-LINE DERIVED (GDNF) NEUROTROPHIC FACTOR FROM ENCAPSULATED SCHWANN CELLS

by

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ABBREVIATIONS

CNS   Central Nervous System

PNS   Peripheral Nervous System

NGF   Nerve Growth Factor

GDNF   Glial cell-line Derived Neurotrophic Factor

ECM   Extracellular Matrix

SCs   Schwann Cells

DRG   Dorsal Root Ganglion

mRNA   Messenger Ribonucleic Acid

DMEM   Dulbecco's modified Eagle's medium

DMSO   Dimethyl Sulfoxide

RH   Relative Humidity

MTT   Methylthiazolyldiphenyl Tetrazolium Bromide

MWCO   Molecular weight cut out

ANOVA Analysis of variance

Pon A   Ponasterone A

CAMs   Cell Adhesion Molecules

NC   Nerve Conduit

BM   Basement Membrane

N   Newton

Pa   Pascal

CHAPTER 1

INTRODUCTION

The fundamental cell of the brain is the neuron, which is very different from most other biological cells. Neurons are responsible for the reception, transmission and processing of stimuli by triggering certain cell activities, and releasing neurotransmitters and chemical messengers. Most neurons consist of three parts; the dendrites, which are specialized for receiving stimuli from the environment, sensory epithelial cells or other neurons; the cell body, which represents the trophic center for the whole nerve cell and is receptive to stimuli; and the axon, which is a single process, specialized for generating or conducting nerve impulses to other cells. Axons may also receive information from other neurons; this information mainly modifies the transmission of action potentials to other neurons. The distal portion of the axon is usually branched and terminates on the next cell in end bulbs, which interact with other neurons or non-nerve cells, forming synapses which transmit information to the next cell in the circuit [1] (Figure 1).
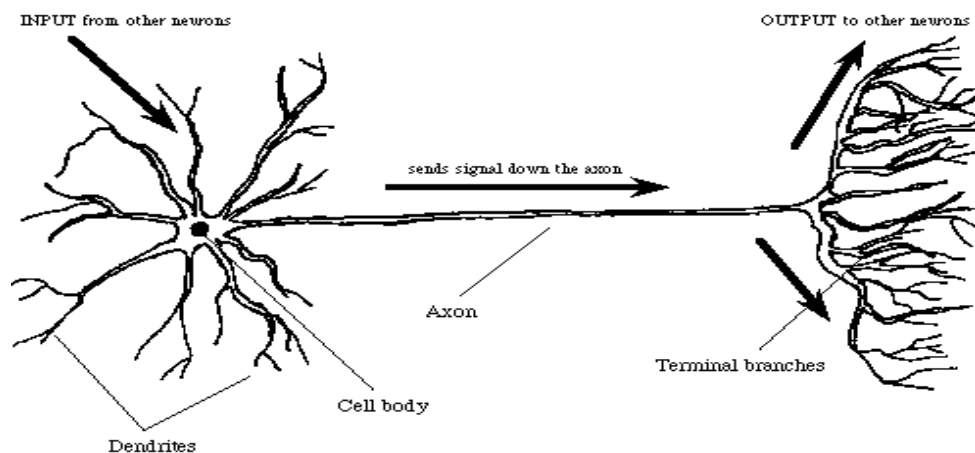
Figure 1. Diagram of a Neuron. (http://www.ccs.neu.edu) [2]

At the core of the human nervous system, is the central nervous system (CNS), which is composed of the brain and spinal cord. Using electrical signals that travel from the CNS through the peripheral nervous system (PNS), the brain controls effector cells, which carry out the physiological responses of the brain. The PNS resides or extends outside the CNS. The main function of the PNS is to connect the CNS to the limbs and organs. Unlike the central nervous system, the PNS is not protected by bone or by the blood-brain barrier, leaving it exposed to toxins and mechanical injuries. An important difference in the properties of the nerve fibers of the CNS as compared to those of the PNS is the network of nerve fibers that extends throughout the body. Most nerves have both sensory and motor fibers and are called mixed nerves; these nerves have both myelinated and unmyelinated axons. Myelin is an electrically insulating material that forms a layer, the myelin sheath, usually around only the axon of a neuron. It is essential for the proper functioning of the nervous system. Myelin is an outgrowth glial cell: Schwann cells supply the myelin for peripheral neurons, whereas oligodendrocytes supply it to those of the central nervous system. Schwann cells keep peripheral nerve fibers (both myelinated and unmyelinated) alive; and in myelinated axons, form the myelin sheath. The main purpose of a myelin sheath is an increase in the speed at which impulses propagate along the myelinated fiber. Along unmyelinated fibers, impulses move continuously as waves, but, in myelinated fibers, a nerve impulse is conducted rapidly in non gradual leaps.[2] The cell bodies and dendrites of peripheral nerves are located in the CNS or in peripheral ganglia. Their axons pass to the periphery and innervate body tissues including muscles and glands. These nerves establish communication between brain and spinal cord centers

and the sense organs and effectors (muscles, glands etc.). They possess afferent fibers which carry the information obtained from the interior of the body and the environment to the CNS and efferent fibers which carry impulses from the CNS to the effector organs commanded by these centers. Nerves possessing only sensory fibers are called sensory nerves; those composed only of fibers carrying impulses to the effectors are called motor nerves. Regeneration occurs because PNS cell bodies are sensitive to damage to their nerve processes, and they react by sending out a signal that passes along their axons to the periphery and innervate body tissues, which establishes communication between brain and spinal cord centers and the sense organs and effectors, which triggers the nerve fibers to regrow. [3] In PNS cells, an injury can stimulate the nerve cell to regrow to some capacity, but not normally back to the original state. PNS nerve regeneration makes it possible for severed limbs to be surgically reattached to the body and regain partial function.[4] When parts of the CNS are critically injured, the CNS cannot generate new neurons nor regenerate new axons of previously severed neurons. Severed CNS axons initially try to grow, but growth inhibitory molecules prevent axonal regeneration by inducing growth cone collapse while CNS cellular and matrix barriers block axon extension. [5]

GLIAL CELLS

The CNS and PNS have two distinct types of glial cells. Glial cells play an important role in the regulation of neuronal repair after injury; supporting neurons both physically and metabolically. [1] In the PNS, Schwann cells originate from the neural crest in a sequence of differentiating steps where neural crest cells generate Schwann cell

precursors, which in turn give rise to immature Schwann cells. The immature Schwann cells then develop into either of the two mature Schwann cell types; the 'myelinating' and the 'non-myelinating Schwann cell'. [6] Schwann cell precursors and to some extent immature Schwann cells depend on growth factors supplied by axons before the myelinating stage has been reached. [6] Without axonal support, Schwann cell precursors and premyelin immature Schwann cells are prone to die by apoptosis. [6]

During development, Schwann cells proliferate and myelinate the elongating neuronal axons. Once axons reach maturity, Schwann cells stop proliferating and become dormant.[6] It is recognized that Schwann cells play a vital role following nerve injury. Injured axons in the PNS receive structural guidance back to their targets by Schwann cells, which divide and line up into regeneration channels, that guides the redeveloping axon and produce extracellular proteins that direct axonal growth. In peripheral nerve damage, the Schwann cells in the distal end are known to secrete neurotrophic factors, which help in regeneration. In the absence of living Schwann cells, nerve regeneration is substantially limited.[7-8]

In the CNS glia cells suppress neuron repair and make up for the majority of cells here. Oligodendrocytes are a variety of neuroglia, whose main function is the insulation of the axons exclusively in the central nervous system of the higher vertebrates, oligodendrocytes are potentially what accounts for the discrepancy in the regenerative ability as compared to the PNS.[1] Astrocytes are glial cells in the brain and spinal cord and play a principal role in the repair and scarring process of the brain and spinal cord following traumatic injuries. Astrocytes enlarge and proliferate to form a scar and

produce inhibitory molecules that inhibit regrowth of a damaged or severed axon. Glia retain their ability to undergo cell division in adulthood, while most neurons cannot. However, there appears to be a general deficiency of the mature nervous system in replacing neurons after an insult or injury, such as a stroke or trauma, while very often there is a profound proliferation of glia near or at the site of damage. However, detailed studies found no evidence that 'mature' glia, such as astrocytes or oligodendrocytes, retain the ability of mitosis. Only the resident oligodendrocyte precursor cells seem to keep this ability after the nervous system matures.[9-10] On the other hand, there are a few regions in the mature nervous system, such as the dentate gyrus of the hippocampus and the subventricular zone, where generation of new neurons can be observed.[11-12]

NEUROTROPHIC GROWTH FACTORS

Neurotrophic growth factors support survival, differentiation and growth of neurons leading to improved nerve regeneration. Neurotrophic factors are secreted by target tissue and act to prevent neurons from initiating programmed cell death.[6] Migrating Schwann cells in developing or regenerating peripheral nerves are known to express dramatically increased levels of neurotrophic growth factors. [14] Glial cell line derived neurotrophic factor (GDNF) is a small naturally occurring protein found in the brain, which has been found to potently promote the survival of many types of neurons. The most prominent feature of GDNF is its ability to nourish and promote the growth, regeneration and protection of motor neurons. [14] GDNF promotes axonal initiation and elongation [15-18] in cultured neuronal cells and has been shown to counteract the effects chronic nerve injury in promoting axonal regeneration. [14, 19] Growth factor delivery from

polymeric microspheres have been considered for the delivery of neuronal growth factors in the context of both the implantation in the brain [20] and the combination with nerve conduit (NC) for peripheral nerve repair. [4] In gains to engineer a nerve guide which will improve functional recovery of damaged nerves, our research intends to incorporate Schwann cells which are modified to secrete glial cell line derived neurotrophic factor (GDNF).

Administration of GDNF is complicated by its poor penetration across the blood-brain barrier (BBB). [21] Since this factor does not cross the blood brain barrier very easily, it has to be administered locally rather than systemically. The problem with local administration of these factors would be that repeated invasive techniques have to be employed and this problem can be overcome by the local transplantation of cells that are genetically engineered to release specific nerve growth factors. To do so, a stable inducible system has been created to optimize neurotrophic factor release from a microencapsulated modified Schwann cell line. There are many parameters to be considered and discussed.

SCHWANN CELLS AND THEIR FUNCTION DURING NERVE REPAIR

Neurons are supported both structurally and functionally by glial cells, which are ten times more numerous than neurons in the nervous system. [22] Although these neural support cells do not participate directly in the transmission of electrical signals over long distances, they do communicate with neurons and with each other using electrical and chemical signals. Glial cells provide physical support for neurons since neural tissue has very little extracellular matrix (ECM). They also direct the growth of neurons during

repair and development. [22] During development, myelin forms when these glial cells wrap around an axon, squeezing out the glial cytoplasm so that each wrap becomes two membrane layers (Figure 2a). The difference is that oligodendrocytes wrap themselves around numerous axons at once, while a single Schwann cell makes up a single segment of an axon's myelin sheath (Figure 2b). Between the insulated areas, a tiny region of axon membrane remains in direct contact with the extracellular fluid. [22] These gaps are called the nodes of Ranvier and play an important role in the transmission of electrical signals along the axon. The PNS relies on myelin for insulation and as a method of decreasing membrane capacitance in the axon, allowing for salutatory conduction to occur and for an increase in impulse speed, without an increase in axonal diameter. Another type of glial cell is the nonmyelinating Schwann cell known as a satellite cell. These cells form supportive capsules around nerve cell bodies located in the ganglia, outside the CNS. They are also involved in the maintenance of axons and are crucial for neuronal survival.[23] Schwann Cells are very crucial for neuronal survival during the developmental stage, and in the case of damaged nerves, they play a very important role in successful regeneration and restoration of function.[24]

a)

b)
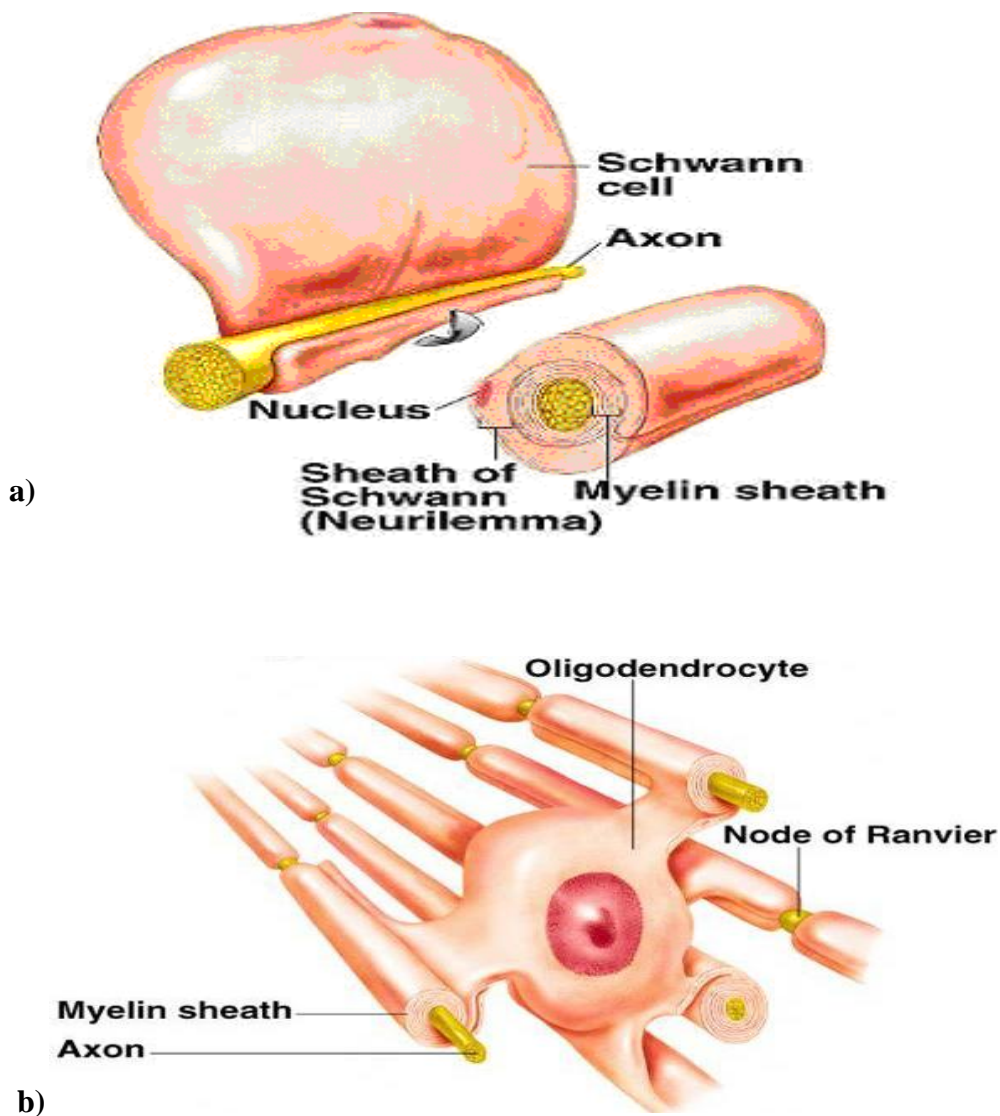
Figure 2.   a) Schwann cells are a variety of glial cell that mainly provide myelin insulation to axons in the peripheral nervous system (PNS) of jawed vertebrates. The vertebrate nervous system relies on this myelin sheath for insulation and as a method of decreasing membrane capacitance in the axon. Dysfunction of peripheral nerves results from damage to the neuron, to the Schwann cells, or to the myelin sheath. Schwann cells are the peripheral nervous system's analogues of the central nervous system oligodendrocytes.   b) Oligodendrocytes main function is the insulation of the axons exclusively in the central nervous system (CNS) of the higher vertebrates, a function performed by Schwann cells in the peripheral nervous system. (Source: McGraw Hill Companies Inc., 1999) [25]

NERVE INJURY AND REGENERATION

Schwann cells proliferate, migrate, and act as sources of neurotrophic support for nerve processes in PNS. They play a vital role in the development and regeneration of peripheral nerves through the excretion of ECM for axon migration and the production of bioactive factors that further improve nerve migration. [26-27] When the established neuron-Schwann cell relationship is disrupted by an injury of the nerve, a series of degenerative processes must take place, which result in the re-establishment of that relationship.[28] The success of regeneration depends largely on the severity of the initial injury and resultant degenerative changes. Pathological changes are mild or absent in first-degree injuries in which the mechanism is conduction block alone, and no true degeneration or regeneration occurs. In second-degree injuries (axonotmesis) there is little histological change at the injury site or proximal to it; however, distal to the injury site, a calcium-mediated process known as Wallerian (or anterograde) degeneration is known to occur [29] (Figure 3). Schwann cells play a key role in Wallerian degeneration. They initially become active within 24 hours of injury, exhibiting nuclear and cytoplasmic enlargement as well as an increased mitotic rate. [30] These cells divide rapidly to form dedifferentiated daughter cells that unregulated gene expression for a multitude of molecules to assist in the degeneration and repair process. Following nerve injury, the distal axons degenerate and the associated Schwann cells break down their myelin sheath. As myelin and axon disintegrate, the denervated Schwann cells and infiltrating macrophages remove axonal and myelin debris by phagocytosis. This is followed by Schwann cell proliferation, which begins 3 to 4 days after nerve injury[70]. Schwann cells line up in the basement membrane

tube and synthesize growth factors, including GDNF, which attract axonal sprouts formed at the terminal of the proximal segment of the severed axon. [31] Proliferating Schwann cells organize themselves as tube-like structures connecting the distal to the proximal end of the transected nerve. This allows the regenerating axons to be properly guided and seek out their original targets. In the absence of living Schwann cells, nerve regeneration is substantially limited. [7-8] Many experiments have revealed that axonal growth is significantly stimulated in the presence of Schwann cells, especially when the axonal growth occurs on the surface of Schwann cells. [27] Investigators have demonstrated that once the cultured Schwann cells survive *in vivo* and maintain normal functionality, host cells persistently elaborate neurotrophic factors, construct the neuronal cytoarchitecture, and successfully regenerate myelin axons. [26]
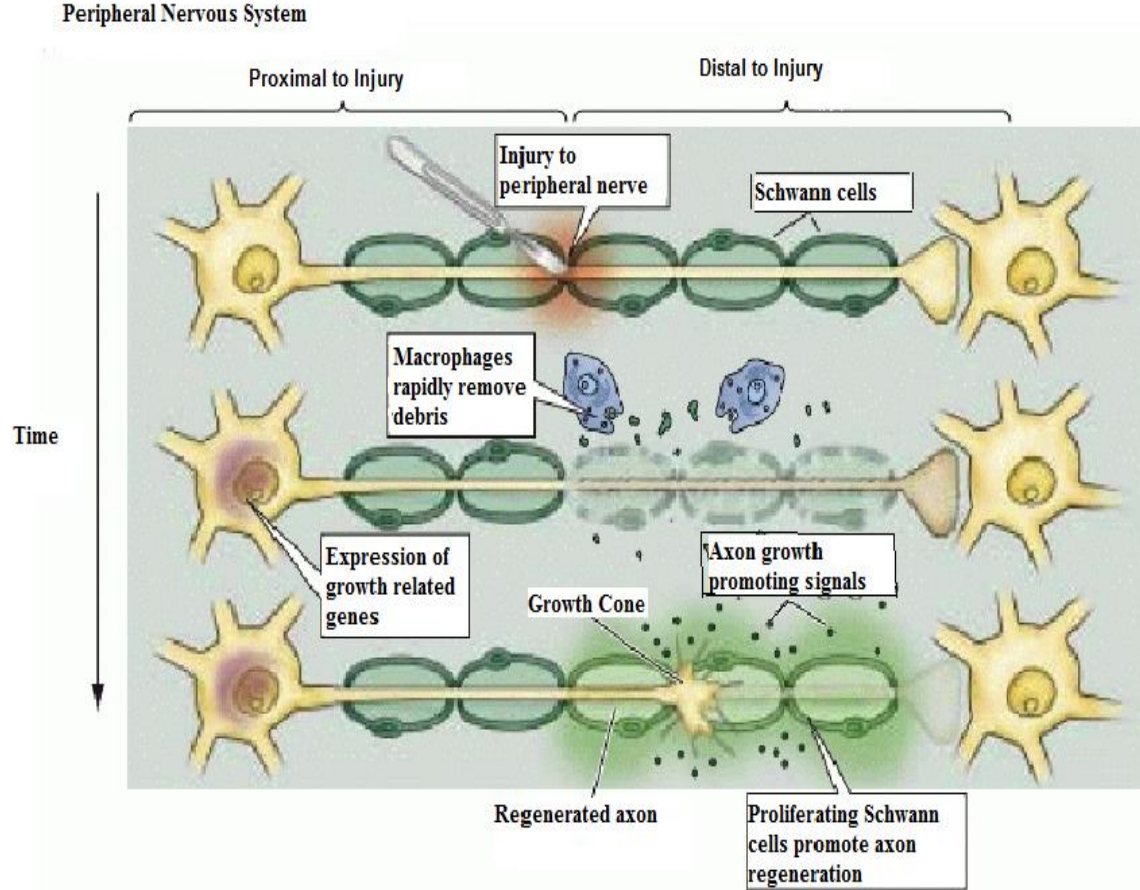
Figure 3. Illustrations showing the Wallerian degeneration of the peripheral nerve. Following nerve injury, the distal axons degenerate and the associated Schwann cells break down their myelin sheath. As myelin and axon disintegrate, the denervated Schwann cells and infiltrating macrophages remove axonal and myelin debris by phagocytosis. As the degradation of the distal nerve segment continues over time, connection with the target muscle is lost, leading to muscle atrophy and fibrosis. Once the degenerative events are complete, all that remains is a column of collapsed Schwann cells. Axon sprouts with a fingerlike growth cone advance using the Schwann cells as guides. After reinnervation, the newly connected axon matures and the pre-injury cytoarchitecture and function are restored.
(Source: http://www.medscape.com/viewarticle/480071_4) [32]

A key difference between the PNS and CNS is the capacity for peripheral nerves

to regenerate; CNS axons do not remarkably regenerate in their native environment.

Several glycoproteins in the native extracellular environment (myelin) of the CNS are

inhibitory for regeneration. [33-34] The physiological response to injury in the CNS is also different compared to that of the PNS. After injury in the CNS, macrophages infiltrate the site of injury much more slowly compared to macrophage infiltration in the PNS, delaying the removal of inhibitory myelin. This is largely a result of the blood-spine barrier, which limits macrophage entry into the nerve tissue to just the site of injury, where barrier integrity is weakened. In addition, cell adhesion molecules (CAM's) in the distal end of the injured spinal cord are not up-regulated greatly as they are in the PNS, limiting macrophage recruitment. Finally, astrocytes proliferate in a manner similar to that of Schwann cells in the PNS, producing glial scars that inhibit regeneration. [33]

GLIAL CELL-LINE DERIVED NEUROTROPHIC FACTOR (GDNF)

FOR NERVE REGENERATION

Glial cell line-derived neurotrophic factor (GDNF) is a naturally occurring growth factor that proved capable of protecting and promoting the survival of motor neurons in animal studies. [70] A growth factor is a growth inducing protein found in the human body. GDNF was first cloned and purified as a potent neurotrophic factor that enhances the survival of midbrain dopaminergic neurons and has since been shown to be a potent survival factor for spinal motorneurons, [35-36] and distinct sub populations of peripheral sensory, sympathetic and parasympathetic neurons. [37] The full-length GDNF consists of 211 amino acids and harbors both a signal sequence and a pro-region. GDNF is a growth factor which contains seven cysteine residues and forms three disulfide bonds resulting in a 'cysteine knot' quaternary structure. [35] The monomer of the three dimensional structure is characterized by two long fingers formed by pairs of anti-parallel β-strands connected

by a loop and a helical portion in the opposite site [38] (Figure 4). The structure-function analysis showed that the first 39 amino acids in the N terminus of GDNF are not required for its biological activities in motor neurons. The C-terminus is critical for the stability and biological activity of GDNF, the α-helix, finger 1 and finger 2 are involved in the binding of GDNF to its receptor. [38]
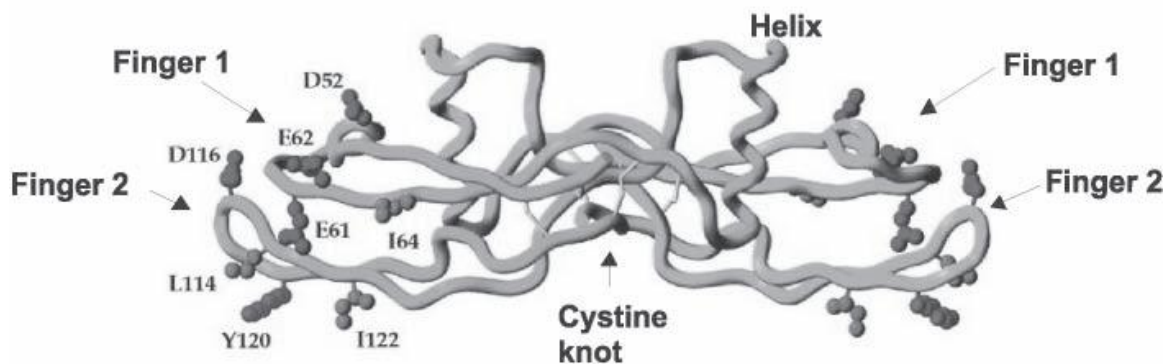
Figure 4. The 3D structure of GDNF. Indicated amino acids on the finger 1 and 2 are important for receptor binding. [26] Protein dimers of GDNF have a reported molecular weight of 33-45 kDa. [39]

Recent research shows that neurotrophic factors are present in the early development of the nervous system and are responsible for the initial growth and development of neurons in the PNS and CNS. They have been demonstrated to make damaged nerves regrow their processes *in vitro* and in animal models and insufficient expression of these proteins at the injury site *in vivo* has been shown to restrain the regeneration process. [40-43] An inherent advantage of Schwann cell transplantation over single or multiple protein delivery is the bio-responsiveness of Schwann cells to react to local environmental stimuli by secretion of a variety of appropriate growth factors. [44] In peripheral nerves,

adult Schwann cells express mRNA for GDNF and its co-receptor GFRα1 [45-47] and proven to be capable of presenting membrane bound and soluble forms of GDNF-GFRα1 complexes to adjacent cells. [35, 48] In mature healthy peripheral nerves, GDNF is suggested to be supplied continuously by Schwann cells at relatively low levels for maintenance of motor neuron survival and integrity. [49] However, synthesis and release of GDNF are drastically upregulated in response to nerve transection injury, specifically at the distal segment, implying that GDNF also has a role in peripheral nervous system repair aside from its anti-apoptotic and neurotrophic functions. [50] In response to nerve transection, the GDNF mRNA expression in Schwann cells of sciatic nerves and in dorsal root ganglion (DRG) rises dramatically, a finding that further implicates GDNF in peripheral-nerve regeneration. [48] Mammalian DRGs contain separate populations of neurons expressing receptors for different neurotrophic factors, essential for neuronal survival during development. [51] Expression of GDNF is six times higher than compared to an uninjured nerve, at one week post-trauma in a rat sciatic nerve model. [50] Afterwards, increase in the density of regenerating axons over time was coincident with the gradual decrease of GDNF from peak to baseline level suggesting that axonal contact to Schwann cells minimize the GDNF production. This aspect of phase and time-dependent release of GDNF may be diagnostic in the repair process and can potentially be considered in the design of a tissue-engineered system for peripheral nerve regeneration. Transplantation of naked cells, that have been genetically modified to secrete GDNF, also has the advantage of local delivery and de novo synthesis, of the active factor (e.g. GDNF) at the targeted treatment site. However, naked cells integrate

into the tissue where they have been transplanted making a termination of the treatment almost impossible. [52] Furthermore, transplanted naked cells may migrate once inside the brain and establish undesirable populations of GDNF secreting cells in the brain. Encapsulated cell bio-delivery combines the advantages of naked transplantations, avoiding the drawbacks, as the genome of the patient's cells is not affected and as the implanted devices can be retrieved if any untoward effect is observed. [28] An inherent advantage of Schwann cell transplantation over single or multiple protein delivery is the bio-responsiveness of Schwann cells to react to local environmental stimuli by secretion of a variety of appropriate growth factors. In contrast, man-made delivery systems are very limited with respect to bio-responsiveness, manageable number of growth factors, and fine-tuning of their release kinetics. [44]

The goals of the current study were to determine if a controlled GDNF secretion promotes further axonal regeneration and neuron viability. Encapsulated cell biodelivery combines the advantages of gene therapy while avoiding the drawbacks, as the genome of the patient's cells is not affected, and as the implanted devices can be retrieved if any unwanted effect is observed. [52] We are currently looking at ways to harness neurotrophic factors, such as GDNF, to induce the re-growth of damaged neurons.

ALGINATE AND MICROENCAPSULATION

Microencapsulation describes a technique of surrounding cells and tissue with a synthetic, possibly porous, matrix. The matrix is used to isolate the cells from the surrounding tissue, while still allowing chemical interaction. Encapsulation of cells has the potential to provide a protective barrier against immune cell interactions. Alginate is

a natural polymer recovered from seaweed (Figure 5). It is found in the intracellular matrix where it exists as a mixed salt of various cations, and the native alginate is mainly present as an insoluble Ca^{2+} crosslinked gel. [53]
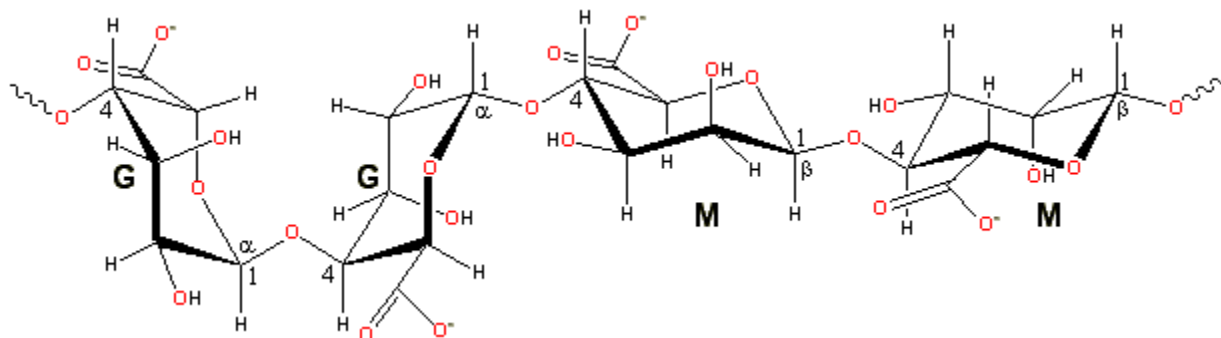
Figure 5: Chemical Structure of Alginate. Alginates are linear un-branched polymers containing β-(4)-linked D-mannuronic acid (M) and α-(1⟶4)-linked L-guluronic acid (G) residues. (http://www.lsbu.ac.uk/water/hyalg.html) [54]

Alginate has several unique properties that have enabled it to be used as a matrix for entrapment and/or delivery of a variety of cells. These properties include: (i) a relatively inert aqueous environment within the matrix; (ii) a mild room temperature encapsulation process free of organic solvents; (iii) a high gel porosity, which allows for high diffusion rates of micromolecules; (iv) the ability to control this porosity with simple coating procedures and (v) dissolution and biodegradation of the system under normal physiological conditions. [55] Alginate and other natural biomaterials have been used extensively for growth factor delivery since they have advantages over synthetic materials, such as similarity with natural ECM and their *in vivo* behavior, with respect to, binding and releasing growth factors. [56] Alginate is a common material used to encapsulate cells. The mechanical properties of alginate capsules depend on several

variables including alginate concentration, G arrangement and content, length of G blocks, molecular weight of alginate chains, homogeneity of the mix, and type and concentration of gelling cations. [57-58] One commonly used method for the encapsulation of cells is the alginate crosslinking method, which utilizes polyanionic alginate and polycationic polylysine polymers. Encapsulation by the alginate method typically occurs by the crosslinking of alginate via the Ca^{2+} ion and the interaction of polylysine with the alginate molecules. Such problems include the swelling of alginate microcapsules due to the presence of Ca^{2+} in the inner alginate core, insufficient mechanical strength of the alginate coating, and insufficient biocompatibility due to guluronic acid content in alginate/polylysine capsules. Cell culture media or the physiological environment surrounding microcapsules are usually rich in diverse ions, which can have an adverse effect on stability by destabilizing the polyelectrolyte interactions involved in microcapsule integrity. Insufficient biocompatibility can lead to fibrotic overgrowth of those capsules, a high rather than low guluronic acid content of the alginate has shown to be associated with a lower percentage of inadequate capsules, which produce smaller ranges of swelling and subsequent shrinkage during the encapsulation procedure in comparison to an increase in viscosity caused by applying a higher alginate concentration compensates for a low guluronic acid content. [71] Moreover, the process of alginate encapsulation is nonspecific and can result in the formation of microcapsules that do not contain the cells or cell groups intended to be encapsulated or that contain other non-target biological materials. Due to these problems, alternative methods for cell encapsulation have been investigated. An alternative to alginate crosslinking is alginate

gels, which are formed when multivalent cations (such as Ca^{2+}, Sr^{2+}, and Ba^{2+}) ionically displace sodium ions (Na^+) and associate with blocks of G monomers to form bridges connecting separate polymer chains. [59-60] Monovalent cations and Mg^{2+} ions do not induce gelation while Ba^{2+} and Sr^{2+} ions produce stronger alginate gels than Ca^{2+}. Ba^{2+} has a higher affinity to alginate crosslinking binding sites compared to divalent cations Ca^{2+} and Sr^{2+} suggesting better gel strength and minimal degradation (Figure 6). Consequently, droplets of cell suspension in unpolymerized liquid alginate held by surface tension form microspheres in air; then solidify via diffusion gelling when immersed in a solution containing free divalent cations. This process leads to encapsulation or entrapment of cells among linked alginate chains. The rate and extent of crosslinking are controlled by varying the alginate molecular weight, the alginate concentration, or the type and concentration of crosslinker, or by adding a chelating agent such as EDTA (ethylenediaminetetraacetic acid) or citrate. The relatively mild gelation process in alginate beading has enabled not only proteins, but also cells and DNA to be incorporated into alginate matrices with retention of full biological activity. [61] Furthermore, by selecting of the type of alginate and coating agent, the pore size, the degradation rate, and ultimately the release kinetics can be controlled. Gels of different morphologies can be prepared including large block matrices, large beads (1 mm in diameter) and microbeads (<0.1 mm in diameter). All these properties, in addition to the non-immunogenicity of alginate, have led to an increased use of this polymer as a protein delivery system. [61]
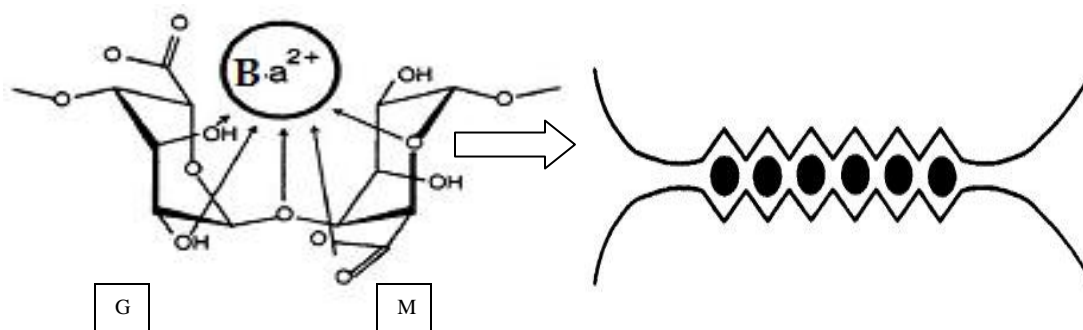
Figure 6. Ba^{2+} has a higher affinity to alginate crosslinking binding sites and is required to crosslink the polymer G and M chains together in order to make a gel. A higher affinity to the alginate crosslinking binding sites creates a mechanically stable gel with increased strength and minimal degradation.

THE OPTIMIZATION OF MICROCAPSULE PROPERTIES

Whole cell immobilization by entrapment is a widely used, simple technique, referred to as microencapsulation. Matrices of different polysaccharides, such as barium alginate, among others, appear to be a promising method for improving drug delivery.[72] Unlike most other polysaccharide gels, alginate gels can form and set at constant temperature, this unique property being particularly useful in applications involving fragile materials such as cells or tissue that have a low tolerance for high temperatures.[72] Alginate also has some useful pharmaceutical characteristics, especially the formation of a net-like lattice between cations and alginate in the gel which is responsible for the slow release of embedded drugs. This property can be useful for the development of alginate-based drug delivery systems.

In our research, alginates have been processed into gel beads by rapid crosslinking with barium ions (e.g. $BaCl_2$ solution) for immunoisolation. [34] This method involves diffusion of barium ions into alginate solution for crosslinking. [34] These beads

are ionically crosslinked and form with structural gradients. Therefore, there is a dependency of the gels' mechanical properties on the homogeneity of the gel. Consequently, droplets of cell suspension in unpolymerized liquid alginate held by surface tension form microspheres in air; then solidify via diffusion gelling when immersed in a solution containing free divalent cations. This process leads to encapsulation or entrapment of cells among linked alginate chains. The mesh-like crosslinked alginate nanometer-sized pores [62] are generally small enough for prevention of encapsulated cells to migrate and invade the host tissues and for protection against the aggressive cellular immune system; but large enough to allow transport of molecules required for cell survival and release of factors produced by cells.

Alginate is a polysaccharide copolymer made of guluronic (G) and mannuronic (M) acid groups. The stereochemistry of the G acid provides reactive carboxylic acid sites. The M acids are not reactive. The guluronic acid sites are active and can react with monovalent and divalent ions, such as sodium and calcium respectively. When reacted with sodium, the ion attaches to the guluronic acid block to form a stable and non-reactive alginate. But in the presence of divalent barium ions, the higher-free energy barium is ionically substituted for sodium at the carboxylic site. A second alginate strand can also connect at the divalent calcium ion, forming a link in which the Barium ion attaches two alginate strands together.[67] The result is a chain of barium linked alginate strands that form a solid gel matrix. The resulting alginate gel has non-adhesive, tissue-like mechanical properties and is over 95% water by volume. The bi-product of the alginate-barium chloride ionic reaction is saline (sodium and chlorine ions), which is

readily accepted by the human body. The concentration of G and M acids (the G/M ratio) contributes to varied structural and biocompatibility characteristics. Alginate's inert tissue-like properties maximize the effective therapy and minimize the potential for adhesion and tissue toxicity. [67]

The mechanical properties of alginate capsules depend on several variables including alginate concentration, G arrangement and content, length of G blocks, molecular weight of alginate chains, homogeneity of the mix, and type and concentration of gelling cations. [4] Barium ions (Ba2+) were chosen for alginate gelation since they were used for synthesis of mechanically stable microcapsules with negligible swelling. [73] Ba2+ has a higher affinity to alginate crosslinking binding sites compared to commonly employed divalent cations Ca2+ and Sr2+ [74], suggesting better gel strength and minimal degradation.

To preserve desired permeability of microencapsulated SC's it is necessary to focus on membrane molecular weight cut-off (MWCO) and cell viability. The homogeneity of microcapsule properties ensures structural integrity, uniform distribution of cells, and uniform porosity throughout the capsule. Controlling pore size is necessary to regulate exchange of nutrients and waste products for cells. Nutrients need to be able to diffuse through the capsules to reach the cells. Similarly, waste products need to diffuse out. However, pore sizes that are too large, will also provide entryway to immune cells that can harm allogenic cells and the developing tissue, as well as provide a means for the encapsulated cells to escape.

Cells immobilized in alginate gels are viable during long-term culture due to the mild environment of the gel network. For most uses, and in particular those involving immobilization of living cells, microcapsules are used because particles < 1 mm in diameter are less fragile than other sizes and have a high surface to volume ratio which allows good diffusion of oxygen an essential nutrients which may be limited in larger particles. At this microcapsule size, these substances do not reach the core and cause cell death because they are used to nourish peripheral cells. [72]

To determine the MWCO of our fabricated microcapsules, we performed a well known technique that used various sizes of, fluorescein isothiocyanate-labeled dextran (FITC-dextran). The major application of FITC-dextran is for characterizing the permeability of semi-permeable membranes either synthetic or natural (organ tissues). Using FITC-dextrans of various molecular weights allows for an estimation of material pore size. Other researchers have shown that secreted protein permeability of alginate microcapsules with pore sizes of 5 to 200 nm [49] ranges from 27 to 300 kDa. [63] The protein dimers of GDNF have a reported molecular weight of 33-45 kDa. [39]

The goal of this research is to characterize and optimize the microencapsulation of SC's for a controlled release of GDNF. In this study, we used a constructed stable cell line with controlled expression and secretion of GDNF with the ecdysone inducible mammalian expression system (Stratagene, La Jolla, CA). One of the advantages of this system when utilized *in vivo* is minimization of unwanted transcription of host genes since mammals lack ecdysone receptors. Thus, only exogenous engineered cells will be the induction target. Moreover, the inducer ponasterone A (ponA), an analog of

ecdysone, has no known detrimental effect on the host organism. PonA is a lipophilic compound that binds to nuclear receptors; hence it can easily penetrate peripheral tissues and cells to reach its target. We studied the effective release of GDNF from barium alginate solution loaded with the modified Schwann Cells (RT4-GDNF) via microencapsulation and assessed the viability of cells in long term microcapsules.

HYPOTHESIS AND SPECIFIC AIMS

In this study, we present the characterization of a microencapsulated modified Schwann cell line and its microcapsule structure, along with key physical characteristics including mechanical properties, permeability and durability. Our hypothesis is that optimizing the material properties of the microencapsulated system will enhance GDNF release along with Schwann cell survival and function within a semipermeable microcapsule. This research will provide knowledge that will be important for use of these constructs to further improve axonal regeneration. Thus, we will challenge our hypothesis through accomplishing the following three specific aims.

Specific Aim One – To microencapsulate modified Schwann cells and characterize their mechanical properties.

   a. Use alginate to encapsulate Schwann cells.

   b. Determine optimal crosslinking concentration and cell density for capsule.

   c. Compression properties.

   d. Permeability properties.

Specific Aim Two – To assess the viability of cells in long term microcapsules.

   a. Investigate the viability of encapsulated cells over 28 days.

      1. MTT assay to determine cellular proliferation.

      2. Confocal microscopy analysis.

Specific Aim Three – To determine their effective release of GDNF from the semi-permeable structure and examine the long term released kinetic of GDNF.

a. Optimize the concentration of the inducer which achieves GDNF synthesis

from encapsulated cells. Examine long term release of encapsulated cells.

CHAPTER 2

MATERIALS AND METHODS

SCHWANN CELL CULTURE AND SEEDING

The cells used in this study were Schwann cells, the principal neuroglial cells in the peripheral nervous system. The Schwann cell line (RT4-D6P2T) was previously modified utilizing an ecdysone-based stable transfection system to produce RT4-GDNF cells [4] The Schwann cell line from a rat source (RT4-D6P2T, ATCC, Manassas, VA) was cultured on a 75 cm^3 polystyrene flask in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 89%; fetal bovine serum, 10% and penicillin-streptomycin, 1%. Cells were maintained at 37°C, 5% Co_2 / 90% relative humidity and fed with fresh medium every two to three days. Cells were dissociated with 1X trypsin/EDTA solution (Invitrogen Corp., Carlsbad, CA), centrifuged and resuspended in medium to cell seeding.

TESTING FOR BIOACTIVITY OF GDNF

Embryonic chick (day 5.5) ventral spinal cord motor neurons were isolated and cultured on plastic petri dishes coated with 5 μg/mL poly-D-lysine and 2 μg/mL laminin at an initial density of 2×104 cells/cm2 in L-15 medium with 2 mM GlutaMAX-I (Invitrogen), 1× N-2 Supplement (Invitrogen), 1× Antibiotic-Antimycotic solution, and 0.3% glucose as described by Loeb et al.[75] The cultured primary cells were divided into 4 treatment groups (each in triplicates) namely, induced (I), uninduced (U), positive control (+), and negative control (-). For group I, diluted conditioned culture medium from induced RT4-Gdnf cells containing 60 pg/Ml GDNF was added to the motor neurons.

Uninduced medium (diluted similarly as in group I) was added for the group U cells; while the + and - control groups were treated with 60 and 0 pg/mL of commercial rat GDNF (Sigma; product number G1401), respectively. Cells were incubated at 37°C/5% CO_2/90% RH (Relative Humidity) for two days and surviving motor neurons were quantified as those: with two or more neurites/neuron, with length of at least 1 neurite being 2× the diameter of the cell body, and without cytoplasmic vacuoles (Figure 7).
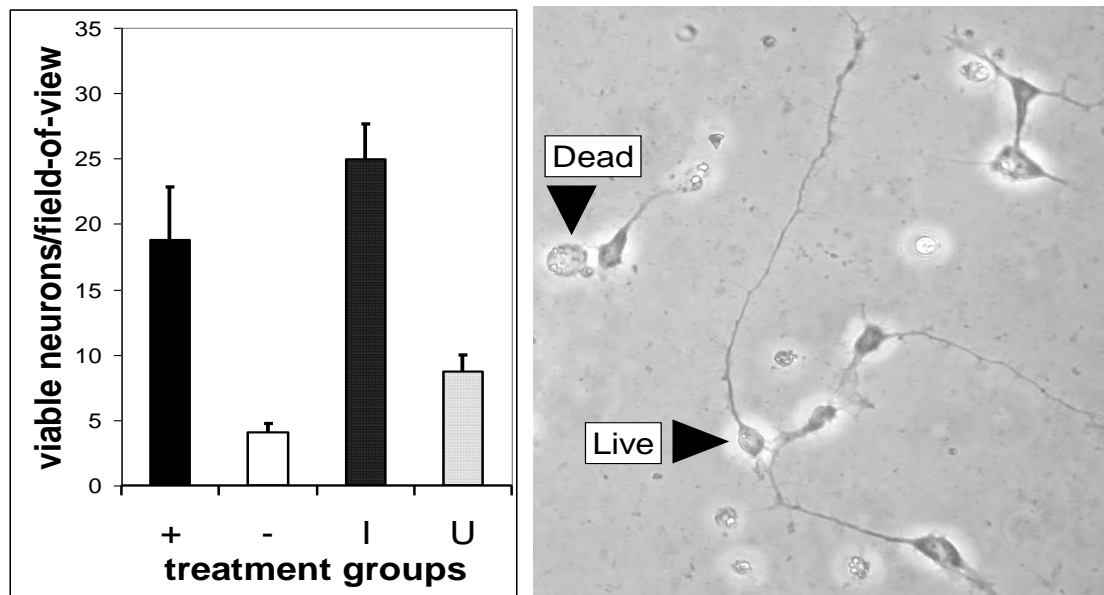
Figure 7. Cultured primary cells divided into 4 treatment groups namely, induced (I), uninduced (U), positive control (+), and negative control (-). Microscopic images of surviving and dead motor neurons.

ALGINATE PREPARATION AND CELL SEEDING USING 1% ALGINATE

Alginic acid sodium salt from brown algae (Fluka 71238, lot 1238898, filing code 50506157) powder with high M content was initially dissolved in wash buffer (13 mM HEPES and 0.84% NaCl, pH 7.4) to make a 1% working solution and then passed through a 0.22 μm filter for sterilization. RT4-GDNF cells growing as monolayer were trypsinized, counted, and centrifuged. The resulting cell pellet was gently mixed and

resuspended evenly in 1% alginate (while avoiding air bubbles). The cell-alginate suspension was loaded in a syringe connected to an R100-E syringe pump (Razel Scientific, St. Albans, VT), and subsequently ejected drop-wise (rate = 1 drop/s; height = 10 cm) through a 23G blunt-end needle into a gelling solution ($BaCl_2$ in wash buffer) to create microencapsulated cells (Table 1). Figure 8 is a display of the encapsulation process set-up. After 15 min of incubation at room temperature (RT) with shaking at 600 RPM, microcapsules were washed 5 times (3 min each) in 10 mL wash buffer to remove traces of free Ba^{2+} ions and finally transferred into a culture dish with RT4-GDNF culture medium for up to 28 days in vitro. Fresh culture medium was added two times/week.

Three cell groups (Low cell density: 20 cells/capsule; Medium cell density: 200 cells/capsule; and High cell density: 2000 cells/capsule) were formed to determine the effect on cell viability over time. In addition to the groups loaded with cells, cell-free capsules were formed and used as control groups. The cell-free capsules were made by dropping 1% alginate into a 50 mM $BaCl_2$ gelling bath.

Table 1. Microcapsule preparation and seeding: The viability of each group was tested over a 28 day test period.

Group			
Control	Low	Medium	High
[BaCl₂]			
50 mM	50 mM	50 mM	50 mM
Alginate Solution			
1%	1%	1%	1%
Cells/Capsule			
0	20	200	2,000
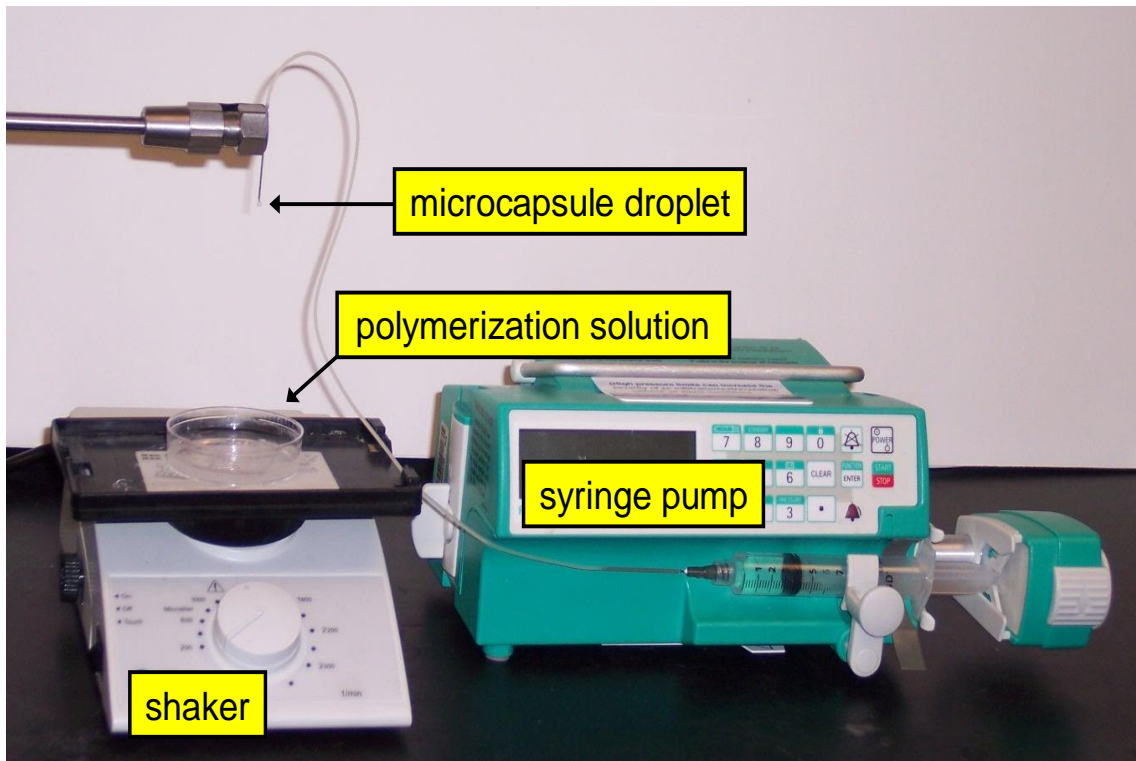
Figure 8. R100 E syringe pump system used for Microencapsulation. 10 ml syringe (23G 1½" needle). Height = 10 cm with 10 ml/hour flow rate; 1 drop/second. Average capsule d = 2mm.

EXAMINATION OF COMPRESSIVE PROPERTIES

Ten microspheres from each test group were uniaxially compressed using a BOSE Endura Tech 3200 Series compression machine with a load cell of 250 g and the cells were compressed at a rate of 0.01mm/second (Figure 9). All microspheres were wet with Phosphate Buffered Saline (PBS) buffer during testing procedures. Compressive forces were measured versus displacement. Ultimate compressive strength was identified as the data point where the capsule breaks. Data was graphed using Microsoft Excel.
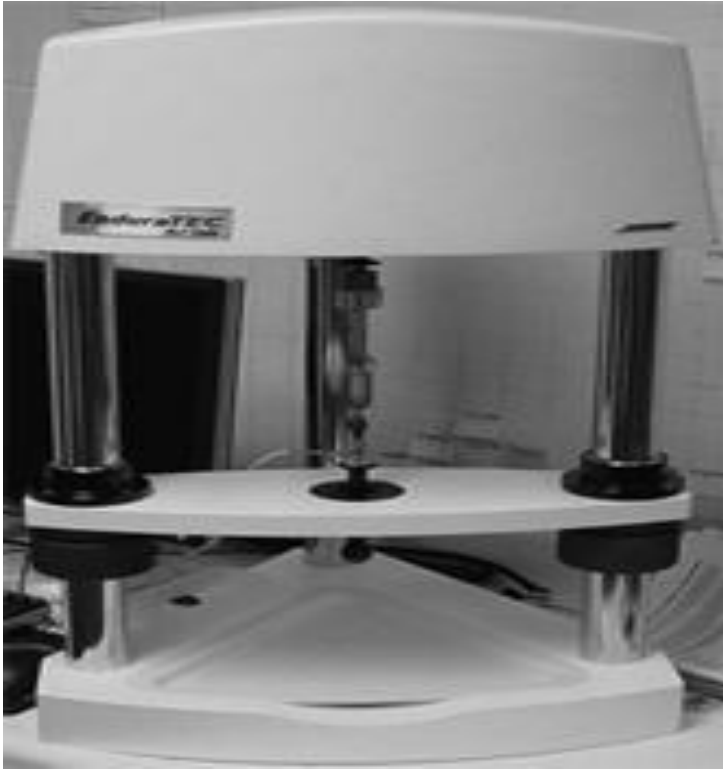
Figure 9. EnduraTEC ELF 3200 Series (BOSE). 250 g Load Cell; Compression Rate: 0.01mm/second.

EXAMINATION OF PERMEABILTIY PROPERTIES

Alginic acid sodium salt from brown algae (Fluka 71238, lot 1238898, filing code 50506157) powder with high 'M'(mannuronic acid) content was initially in (PBS) buffer to create a 1% working solution and then passed through a 0.22 μm filter for sterilization. RT4-GDNF cells growing as monolayer were trypsinized, counted, and centrifuged. The resulting cell pellet was gently mixed and resuspended evenly in 1% alginate (while avoiding air bubbles). RT4-GDNF cells were seeded at 200 cells/microcapsule. To examine permeability, the diffusion of fluorescein isothiocyanate-labeled dextran (FITC-dextran) with different molar masses (20, 40, and 70 kDa) were incorporated into the cell

suspension (2mg/ml). The test capsules were constructed according to Table 1. Using varying sizes of polymers such as dextran are ideal for permeability testing because they are uniform in chemical composition and shape, and form a random coil structure in solution. [64] FITC-dextran has been shown to be stable *in vivo* and have excellent biocompatibility. Excitation is best performed at 490nm and fluorescence measured at 520nm. The intensity is however, dependent on pH and measurements should be made under controlled conditions.

Most microcapsule permeability measurements which use dextran are made with a series of dextrans of narrow range of size, shape and mass characteristics. [64] Capsules will typically exclude a dextran with a molecular weight higher than the molecular weight cut out (MWCO) and allow the permeation of a dextran lower than the MWCO. These methods are used to determine the range of the MWCO. If a dextran molecule with a molecular weight close to the MWCO is used for permeability measurements, the fraction of the dextran which is less than the MWCO will permeate the capsule, and the fraction of the dextran which is greater than the MWCO will be excluded.

Table 2.   Permeability of the microcapsules: test capsule formation. Each capsule contained 200 cells per capsule, suspended in a 1% alginate solution.

Dextran-FITC (MW)		
20 kDa	40 kDa	70 kDa

Dextran-FITC		
0.2%	0.2%	0.2%

Alginic-Cell Solution		
10 ml	10 ml	10 ml

Group Label Name		
20-Dex	40-Dex	70-Dex

For test capsules, dextran-FITC (MW 20 kDa) was added to the alginic/cell solution to create a 0.2% FITC-dextran/alginic/cell solution (20-Dex); dextran-FITC (MW 40 kDa) was added to the alginic/cell solution to create a 0.4% FITC-dextran/alginic/cell solution (40-Dex); and dextran-FITC (MW 70 kDa) was added to the alginic/cell solution to create a 0.7% FITC-dextran/alginic/cell solution (70-Dex). The capsules were created using the alginate-dextran solution, 0.84% NaCl pH 7.4 and 13 mM HEPES. A cell solution without dextran-FITC (0-Dex) was used to produce a controlled group of capsules. The cell-alginate suspension was loaded in a syringe connected to an R100-E syringe pump (Razel Scientific, St. Albans, VT), and subsequently ejected drop-wise (rate = 1 drop/s; height = 10 cm) through a 22 G blunt-end needle into a gelling solution (BaCl2 in HEPES wash buffer) to create microencapsulated cells. The microcapsules were washed in their appropriate wash buffer made up of either, 0.2%, 0.4% or 0.7% FITC-dextran/PBS solutions to remove traces of

free Ba^2+ ions and finally transferred into a culture dish with RT4-Gdnf culture medium. The capsules were covered with aluminum foil to prevent light from reaching the capsules, which would affect the FITC molecules and stored at 37ºC with continuous shaking (250 RPM). The culture medium containing the diffused FITC-dextran solution was collected at specific temporal points: 3, 18, 24, and 48 hours. The solutions were collected in epindorf tubes and stored at 4ºC until spectroscopy analysis. Samples were scanned at 450 nm, 460 nm, and 490 nm. The ideal wavelength for analysis was determined to be 490 nm. Graphs were created using excel to represent the percent of diffusion over time.

QUANTIFICATION OF VIABLE ENCAPSULATED CELLS

Live/Dead Assay

Microcapsules were stained with LIVE/DEAD (Invitrogen) reagents and imaged under the Ultra VIEWERS dual-spinning disk Confocal system (PerkinElmer, Shelton, CT) at different time points (3, 7, 14, 21, and 28 days post-incubation). Green and red fluorescent images corresponding to live and dead cells, respectively, were captured approximately at the equatorial plane of the microcapsules using a 5× objective. Percent surviving cells were determined by measuring the area of green signals divided by the area covered by the total number of cells (green + red fluorescence) at a given field-of-view using Image J software (National Institutes of Health, Bethesda, MD).

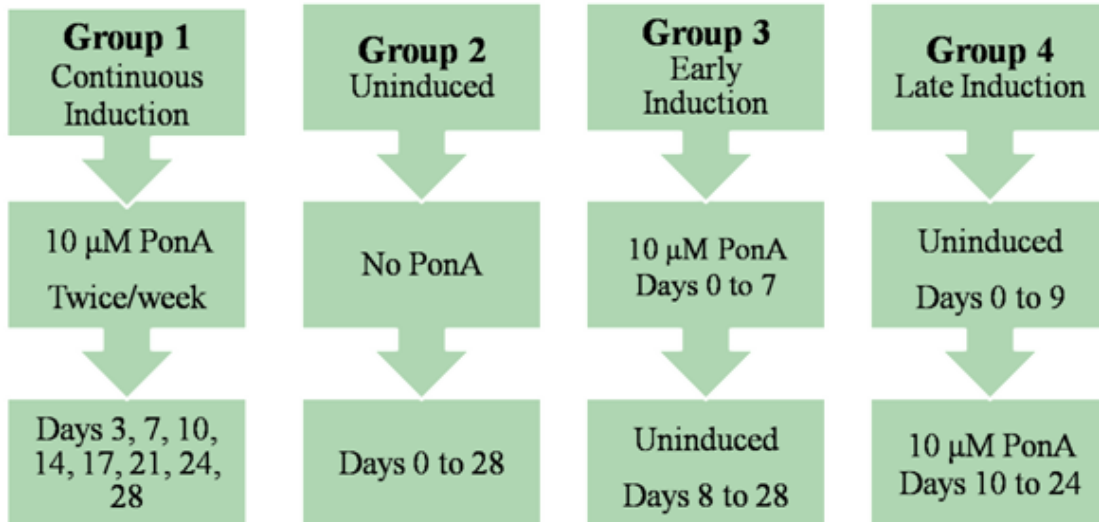### Methylthiazolyldiphenyl tetrazolium bromide (MTT) Assay

Methylthiazolyldiphenyl tetrazolium bromide (MTT) assay was performed to determine if encapsulated cells have the ability to proliferate or change their metabolic activity.

MTT reagent (0.5 mg/mL) was added to the culture medium then incubated for six hours in the dark at 37°C. Afterwards, culture media were replaced by dimethyl sulfoxide (DMSO) and the plate was incubated overnight with shaking to completely solubilize the formazan crystals within the alginate microcapsules. Absorbances were read at 570 and 650 nm in a microtiter plate reader. Optical densities (OD) of the samples were obtained by subtracting the 650-nm reference wavelength from the 570-nm absorbance reading. A standard curve was made to determine the number of cells based on OD values of serially-diluted encapsulated cells.

INDUCTION OF GDNF SECRETION

Microcapsules were separated into four groups of triplicates. Thirty capsules/group/replicate were placed into the wells of a 12-well tissue culture plate with 800 μL culture medium/well. Group 1 (G1) capsules were subjected to continuous induction in which 10 μM of ponA inducer was added two times/week throughout the 28-day incubation; while group 2 (G2) capsules were left uninduced. For the capsules in group 3 (G3) (early induction), the inducer was added only at day 0 (start of culture) until day 7, and subsequently left uninduced. In contrast, group 4 (G4) capsules (late induction) were only induced from day ten until day 24 (Table 3). As a non-encapsulated control (Mono), 6×10^5 RT4-GDNF cells growing on monolayer were seeded/well, subdivided into 4 groups, and treated with ponA inducer in a similar fashion as the encapsulated cells. Conditioned culture media were collected in both encapsulated and monolayer cells at days 3, 7, 10, 14, 17, 21, 24, and 28 post-incubation. Secreted GDNF levels were quantified via GDNF ImmunoAssay (Promega) ELISA kit.

Table 3. Induction of test groups with ponA.

Group 1 Continuous Induction	Group 2 Uninduced	Group 3 Early Induction	Group 4 Late Induction
10 µM PonA Twice/week	No PonA	10 µM PonA Days 0 to 7	Uninduced Days 0 to 9
Days 3, 7, 10, 14, 17, 21, 24, 28	Days 0 to 28	Uninduced Days 8 to 28	10 µM PonA Days 10 to 24

The inducible Mammalian Expression System is designed to provide the tightest control of expression available in mammalian cells. The system is based on a synthetic ecdysone-inducible receptor and a synthetic receptor recognition element that modulates expression our gene of interest GDNF. After addition of the inducer, ponasterone A, a conformational change of the receptor subunits removes transcription repressors and recruits transcriptional machinery to activate transcription.

CHAPTER 3

RESULTS

MICROENCAPSULATED RT4-GDNF IN BARIUM ALGINATE CAN SURVIVE LONG-TERM IN VITRO

Varying the initial number of seeded cells per capsule in 50 mM $BaCl_2$ concentration in the gelling buffer did not affect the ability of encapsulated cells to survive in a static culture condition at 3, 7, 14, 21, and 28 days. Figure 11, is an image of an alginic microcapsule from each of the three seeded test groups.
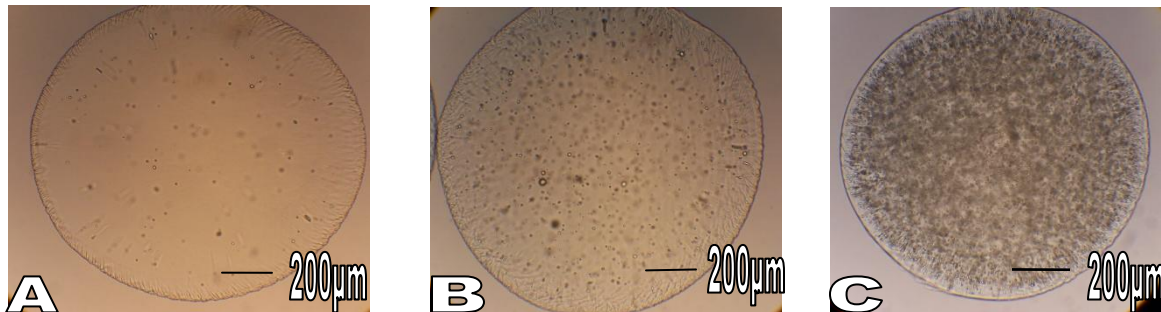
Figure 10 A) Low cell density: 20 cells/capsule B) Medium cell density: 200 cells/capsule and C) High cell density: 2000 cells/capsule.

The stained microcapsules were imaged under the Ultra VIEWERS dual-spinning disk Confocal system (PerkinElmer, Shelton, CT) and captured approximately at the equatorial plane of the microcapsules using a 5× objective (Figure 12). Cell viability was consistently high between 88 to 100% (Figure 13) for all capsule parameters. Results imply that regardless of the amount of loaded cells and barium ions used for crosslinking, RT4-GDNF cells can be maintained long-term within barium alginate microspheres

without suffering significant cell death as long as the medium is replenished every three

or four days.

Figure 11. Confocal microscopy analysis of the stained microcapsules from Low, Medium, and High cell/capsule test groups: approximately at the equatorial plane of the microcapsules using a 5× objective.
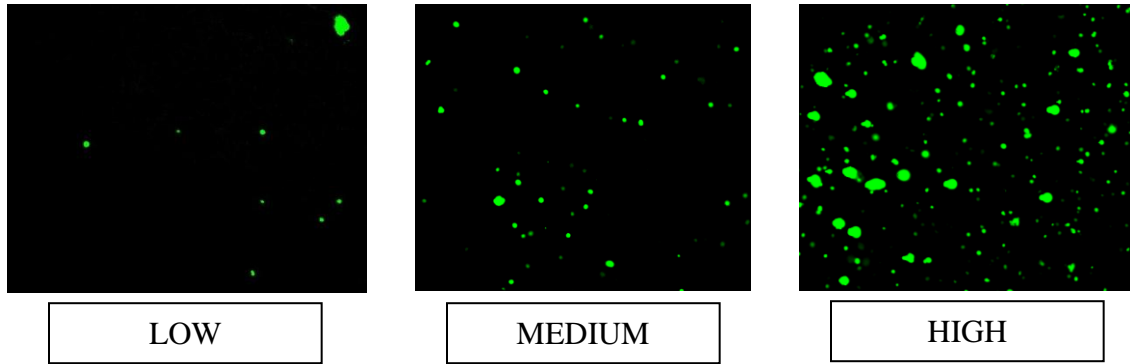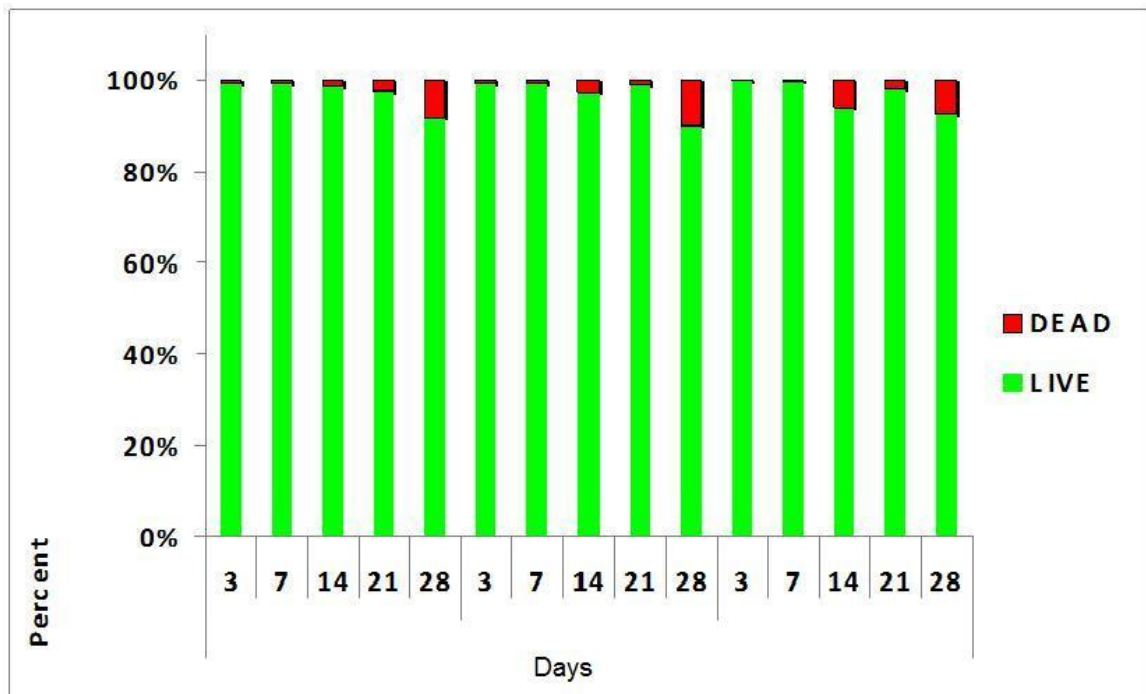

Figure 12. Green and red fluorescent images corresponding to live and dead cells from Low, Medium, and High cells/capsule test groups. Cell viability was consistently high between 88 to 100%.

A contour plot of data from capsules with high cell count displayed interpolated pattern indicating that the 50 mM $BaCl_2$ gelling buffer supported the highest cell viability compared to other concentrations (Figure 14 A-B); thus these parameters were employed in the future testing of GDNF induction, permeability, and compression tests.
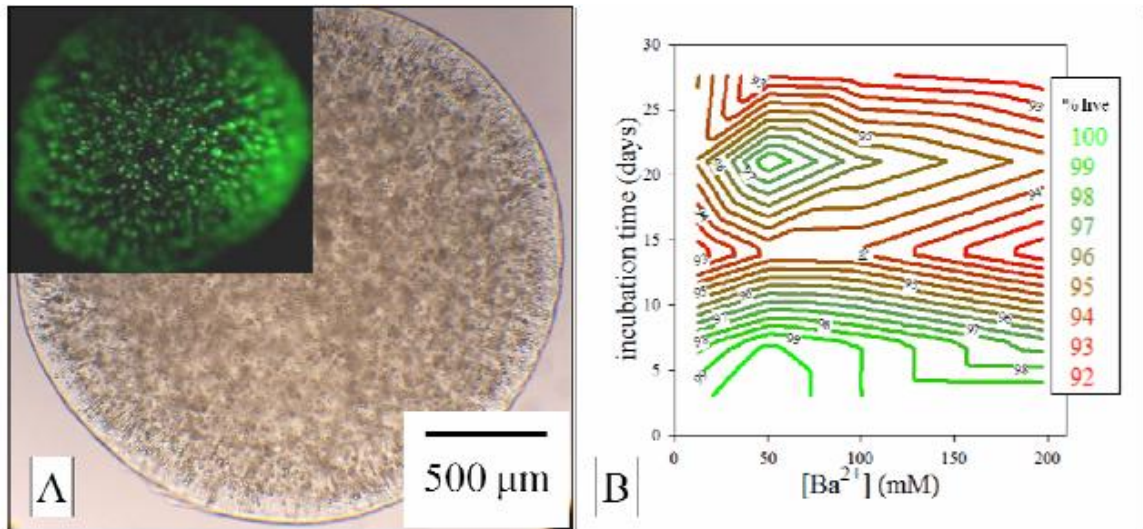
Figure 13. A) Dense RT4-GDNF cells in barium alginate microcapsule. Inset shows green fluorescence emitted by live cells. B) Contour plot with planar interpolation of survival ratios demonstrated that the 50 mM BaCl2 gelling solution gave the highest cell viability at > 92% at least 28 days in static culture.

Furthermore, microscopic images and MTT assay showed that cells within the capsule increased in number over time but only up to a certain saturation or confluency level, at approximately 8,000 cells/microcapsule; (Figure 15 A-B). It was observed that generally, the greater the barium ion concentration the slower the proliferation rate; suggesting that decreased Schwann cell migration and motility [42] due to more extensive alginate crosslinking negatively affects the ability of cells to multiply. [43]
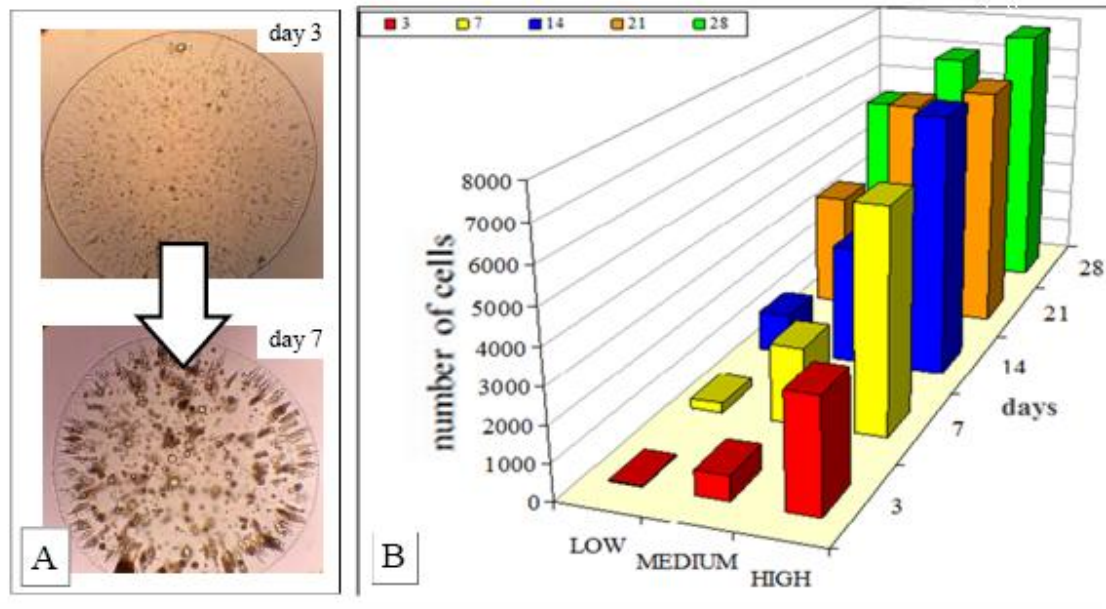
Figure 14. A) Microcapsule images of high seeded density capsules at day seven and B) MTT results depict that RT4-GDNF cell proliferation over the 28 day test period. High initial cell density (2000 cells/capsule) preparations reached confluency at around seven days in culture.

According to the MTT assay results, Rt4-GDNF cells were shown to proliferate within the barium-chloride microspheres throughout the period of the 28 day test. Both the low and medium seeded test groups displayed a steady increase in proliferation throughout the duration of the test, while the high seeded capsules show confluency at around day seven, which they appear to maintain the durance of the test. These results demonstrate that RT4-GDNF cells proliferate within the barium alginate microspheres.

GDNF-CELL LOADED CAPSULES CAN BE USED FOR IN VIVO NERVE REGENERATION EXPERIMENTS

Both non-encapsulated and encapsulated cells were tested for GDNF secretion afterwards. Samples were induced continuously via treatment of 10 μM of ponA. Moreover, other groups were evaluated for responses to removal and late addition of

inducer. Culture media were collected at 3, 7, 10, 14, 17, 21, 24, and 28 days post-incubation. ELISA was performed to quantify the amount of secreted GDNF. Results demonstrated that GDNF was released after ponA induction and the system was turned "off" when the inducer was removed. Induction led to a 5-fold increase in GDNF expression. Encapsulated cells however secrete 9× less GDNF compared to non-encapsulated cells (Figure 10).
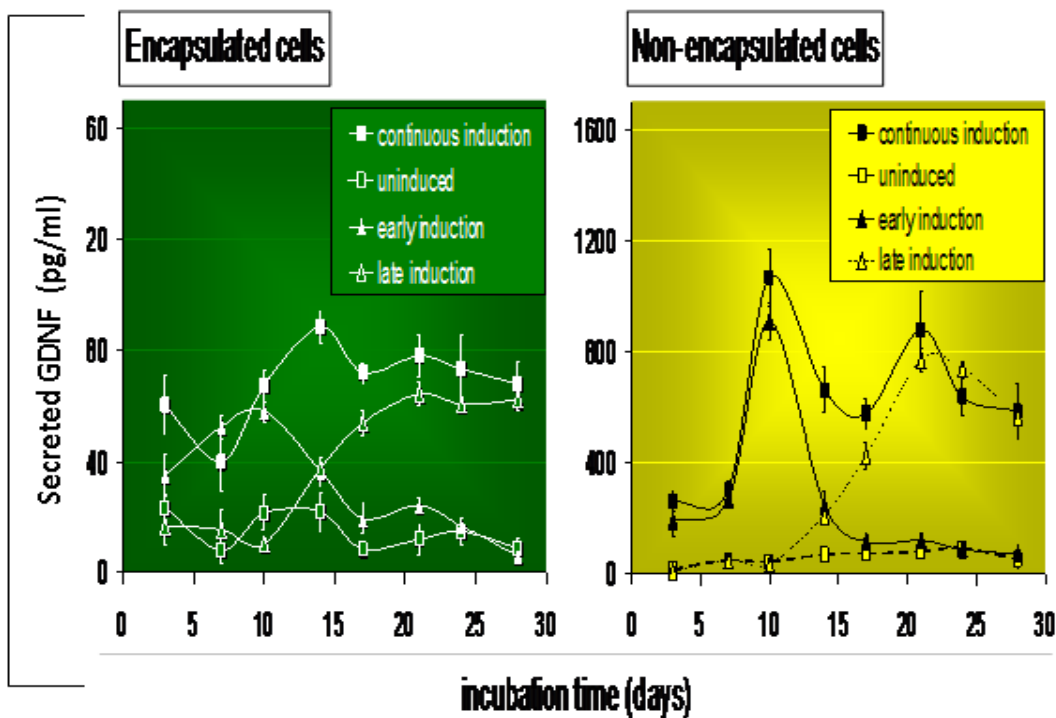
Figure 15. Encapsulated RT4-GDNF cells in 1% alginate (Alg) and non-encapsulated cells growing as monolayer on a tissue culture dish (Mono) release GDNF into the culture media upon induction. G1 samples that were continuously treated with 10 μM ponA every three or four days from day 0 to day 24 produce elevated levels of GDNF compared to G2 (left uninduced). G3 samples were induced only up to day seven, and removal of ponA led to decrease in culture medium GDNF similar to G2 baseline amounts. Conversely, induction was started at day 10 for the originally uninduced G4 groups, resulting in upregulation of GDNF. Induced GDNF level in Mono is approximately 9× higher than in Alg respectively.

The $9\times$ difference in the amount of GDNF secretion in the encapsulated versus the encapsulated cells is likely due to the fact that the GDNF molecule once secreted inside the capsule has to be able to find a way out of the capsule, hence through the pores of the capsule. Not only are the capsule loaded with 200 cells, but each of those cells are secreting GDNF molecules and they are all sharing this controlled space. These molecules are therefore limited to the amount of places from which they have to exit. At any rate, induction with ponA did result in secretion of functional GDNF that promoted the survival of more embryonic neurons. Removal of the inducer returned the GDNF level to baseline similar to uninduced samples.

RT4-GDNF IN BARIUM ALGINATE SECRETE GDNF UPON INDUCTION

As expected, both non-encapsulated and encapsulated RT4-GDNF cells subjected to continuous ponA induction (G1) secrete GDNF quantities greater than those produced by uninduced cells (G2) up to 28 days in vitro (Figure 16A-B). About 9% and 20% of the induced GDNF products account for constitutive expression by uninduced, unencapsulated and encapsulated cells, respectively; showing that the Schwann cell-transfected ecdysone system is not completely silent even in the absence of inducer. Removal of inducer led to GDNF level reversion back to baseline-uninduced levels after two weeks for encapsulated cells and ten days for cells growing on tissue culture plate (G3). In contrast, the previously uninduced RT4-GDNF cells produced relatively high amounts of GDNF after induction (G4); in which encapsulated cells generally respond slower than non-encapsulated cells.

RT4-GDNF IN BARIUM ALGINATE ARE EXPECTED TO RETAIN THEIR ABILITY TO SECRETE BIOACTIVE FACTOR AS EVIDENCED BY FITC-DEXTRAN RELEASE

Permeability of microcapsules was investigated by examining the diffusion of fluorescein isothiocyanae-labeled dextran (FITC-dextran) with the various molar masses (20, 40, and 70 kDa) from the microcapsules. At time points; 3, 18, 24, and 48 hours, samples of the FITC-dextran from the microcapsules into PBS were collected and analyzed in a microplate spectrofluorometer (SPECTRAmax GEMINI XS, Molecular Devices Corp, USA). A spectrophotometric scan of the samples was performed and the ideal reading wavelength was determined to be 490 Nm (Figure 17). The diffusion (%) is based on the percentage of the amount of released FITC-dextran over the initial amount of released FITC-dextran in the microcapsules (Figure 18).
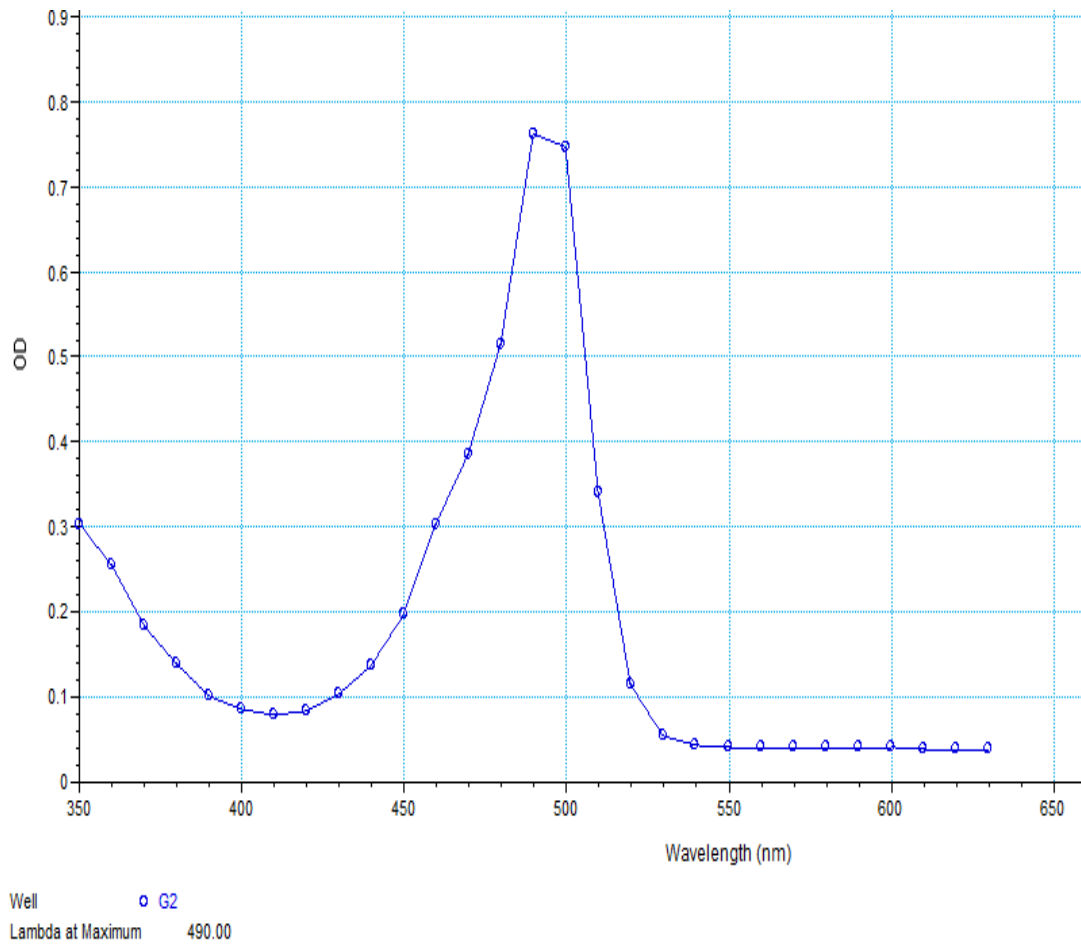
Figure 16. Spectrophotomic scan to determine optimal wavelength for analysis. The ideal wavelength was determined to be 490 nM.
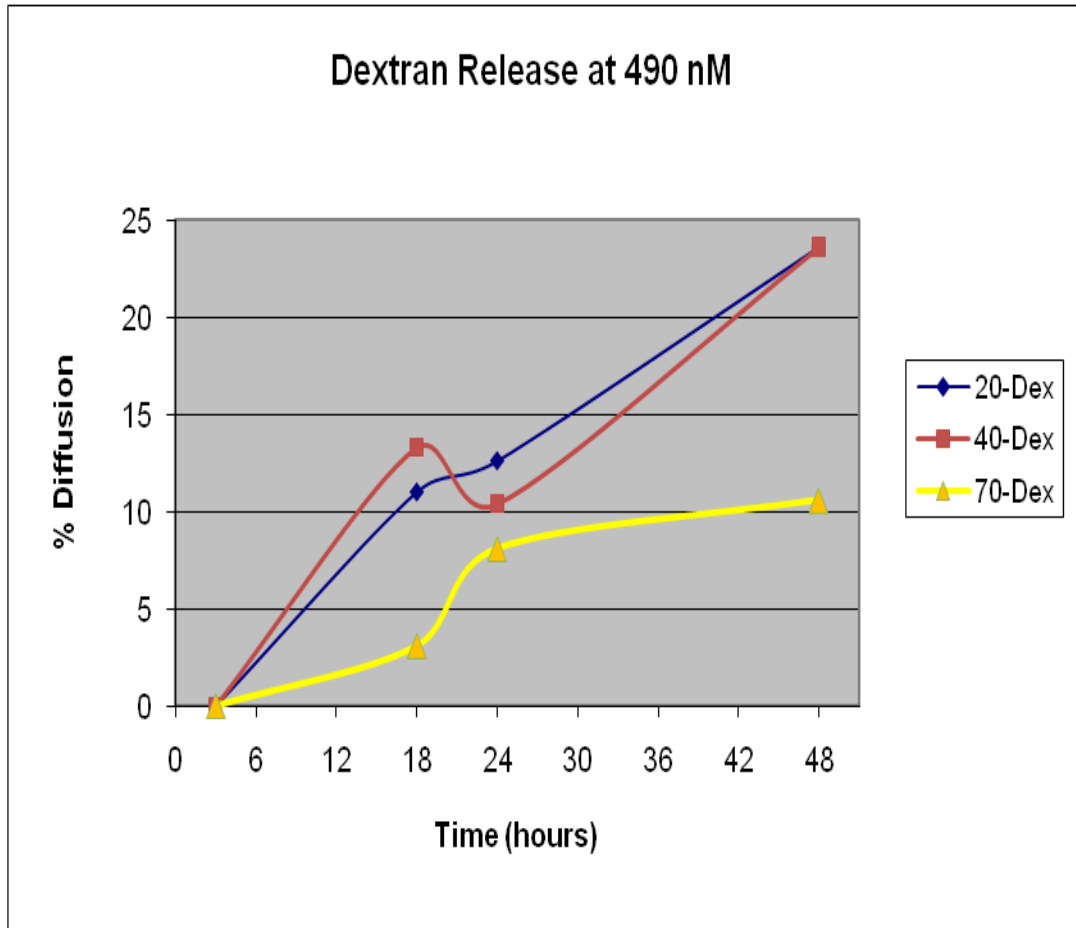
Figure 17. Percent diffusion of fluorescein isothiocyanae-labeled dextran (FITC-dextran) with the various molar masses (20, 40, and 70 kDa) from the microcapsules. At time points; 3, 18, 24, and 48 hours.

The fraction of the dextran which permeated the capsules increased with decreasing molecular weight and varied depending on time. Within 24 hours, 10.6% of the 70-Dex permeated the capsule, while for the 40 and 20-Dex's, 23.6% of the dextran permeated. By 48 hours, the percent of permeable 70-Dex is the same as at the 24 hour time point. While we found a decrease in the release of the 40 and 20-Dex's (13.7% and 12.9% respectively). The partial permeation of the dextran released from the microcapsules was

attributed to like-charge repulsion between the encapsulated polyelectrolyte and the charged dye molecules on the FITC-dextran.

The maximum diffusion of 70-Dex was 10.6% over a 48 hour time period. 40-Dex diffused 4.3% times more than 70-Dex initially and 2.2% more at 48 hours. 20-Dex diffused 3.5% more than 70-Dex initially and 2.2% more at 48 hours. Initially, 40-Dex diffused 1.3% quicker than 20-Dex, but in the time of 18 to 24 hours, 20-Dex continually diffused through the capsule pores and by 48 hours had diffused 23.6%. However, 40-Dex experienced a lag in diffusion over the 18 to 24 hour time period, but continued to increase over the 24 to 48 hour time frame and diffused similarly to 20-Dex at 23.6% at the end of the experiment.

RT4-GDNF IN BARIUM ALGINATE ARE ABLE TO WITHSTAND A FORCE OF AT LEAST 50 kPa

Compression testing was performed on RT4-GDNF cells on days 3, 7, 14, 17, and 21. Each capsule was observed until bursting point. The following succession of graphs displays the amount of force applied per area over 21 days (Figure 19). The capsules were measured in Newtons per millimeter; from which the amount of pressure at bursting point was measure in Pascals ($1 \text{ N/mm}^2 = 1$ kPa).

DAY 3

Load (N)

0.5
0
-0.5
-1
-1.5
-2
-2.5

-2.5   -2   -1.5   -1   -0.5   0   0.5

Displacement (mm)

— Alginate Blank
— Alginate Day 3 Low
— Alginate Day 3 Med
— Alginate Day 3 High

Day 7

Load (N)

0.5
0
-0.5
-1
-1.5
-2
-2.5

-3   -2   -1   0   1

Displacement (mm)

— Alginate Blank
— Alginate Day 7 Low
— Alginate Day 7 Med
— Alginate Day 7 High

Day 14

Day 17
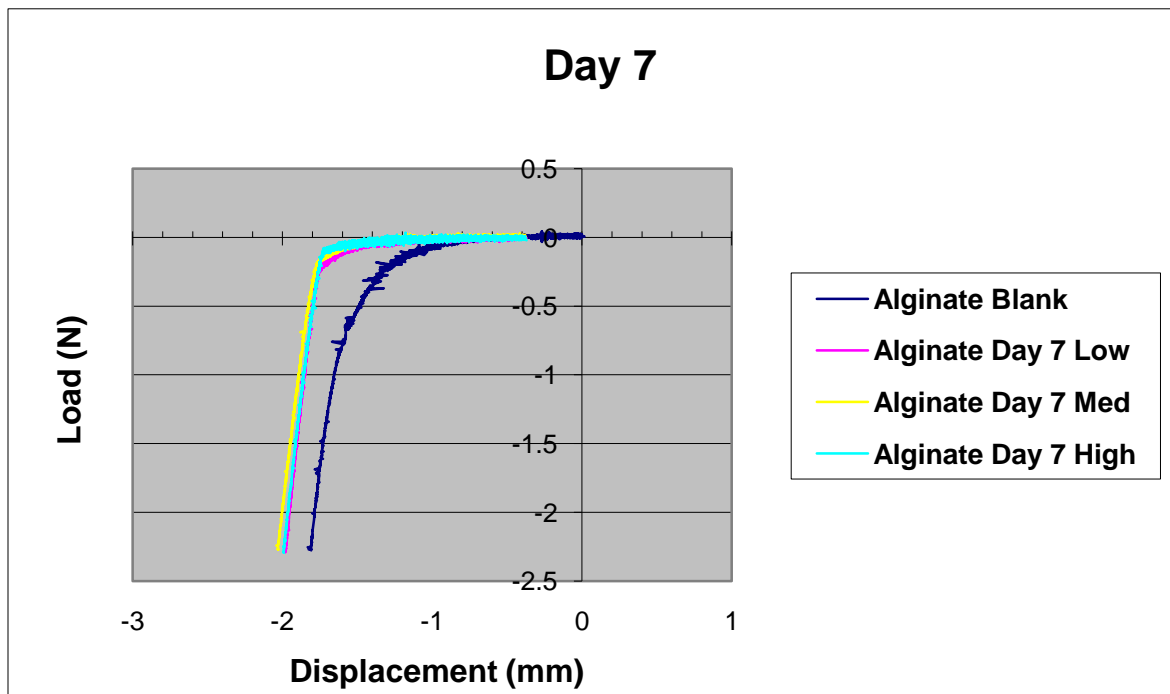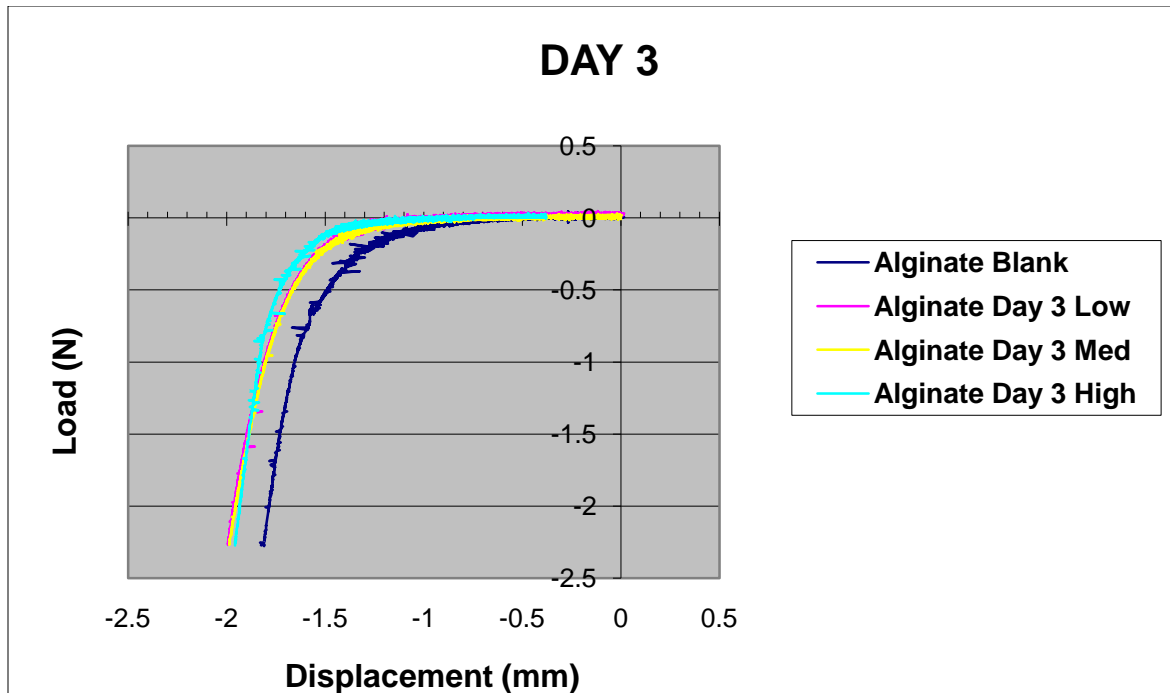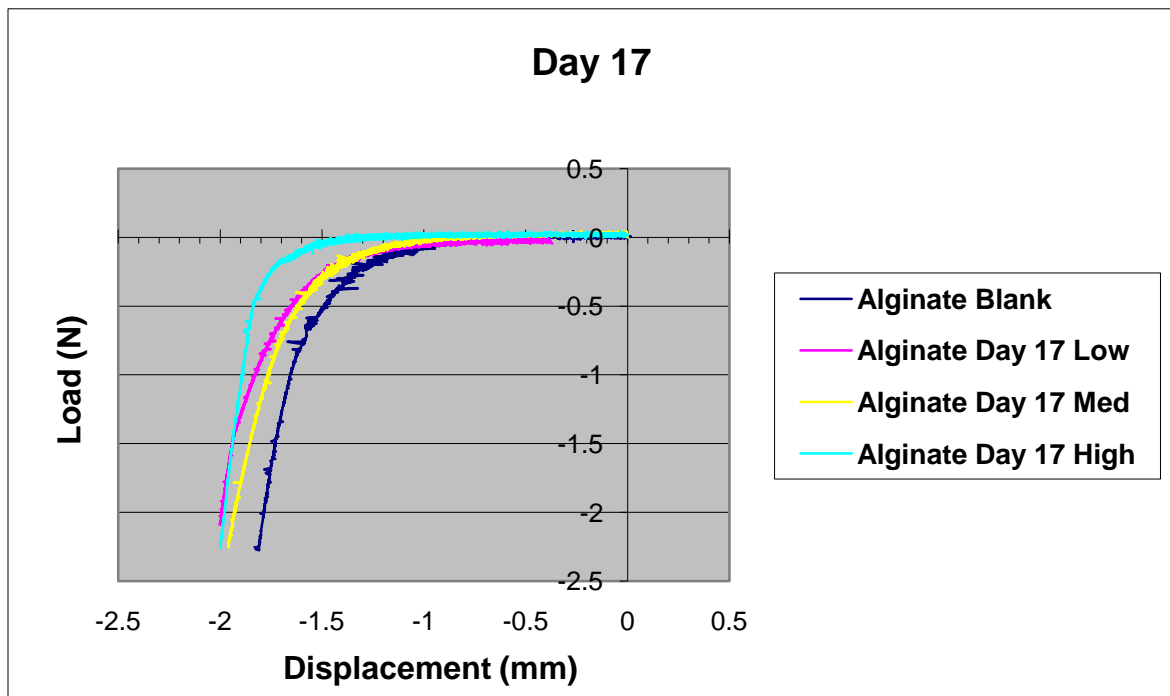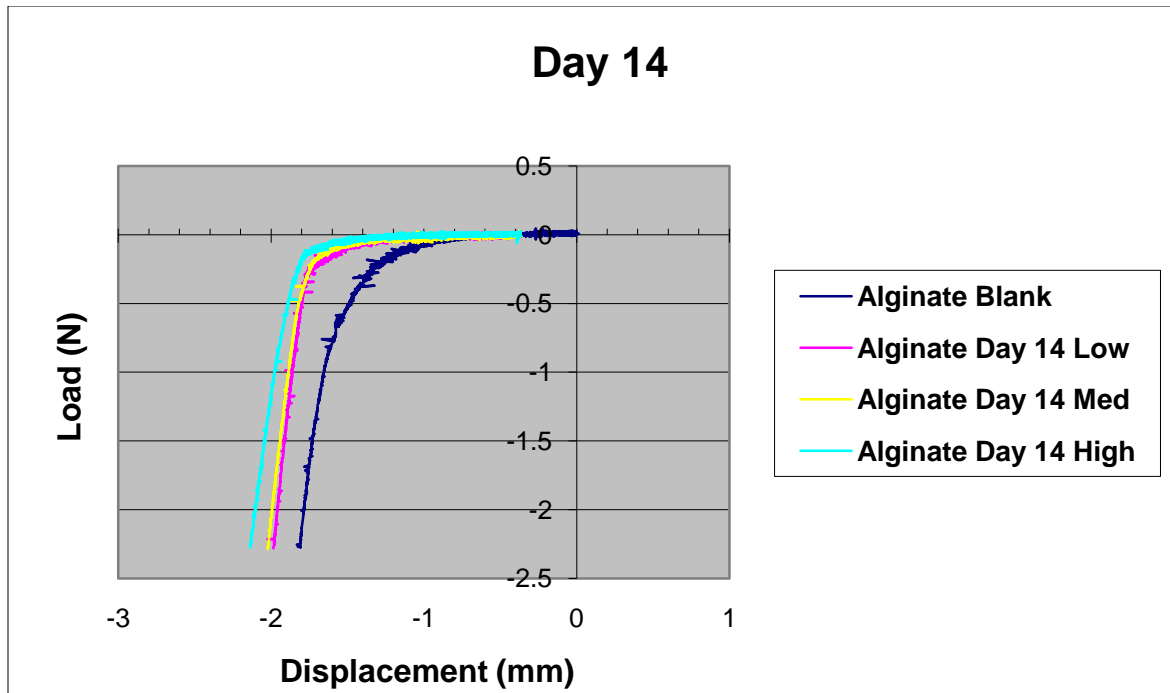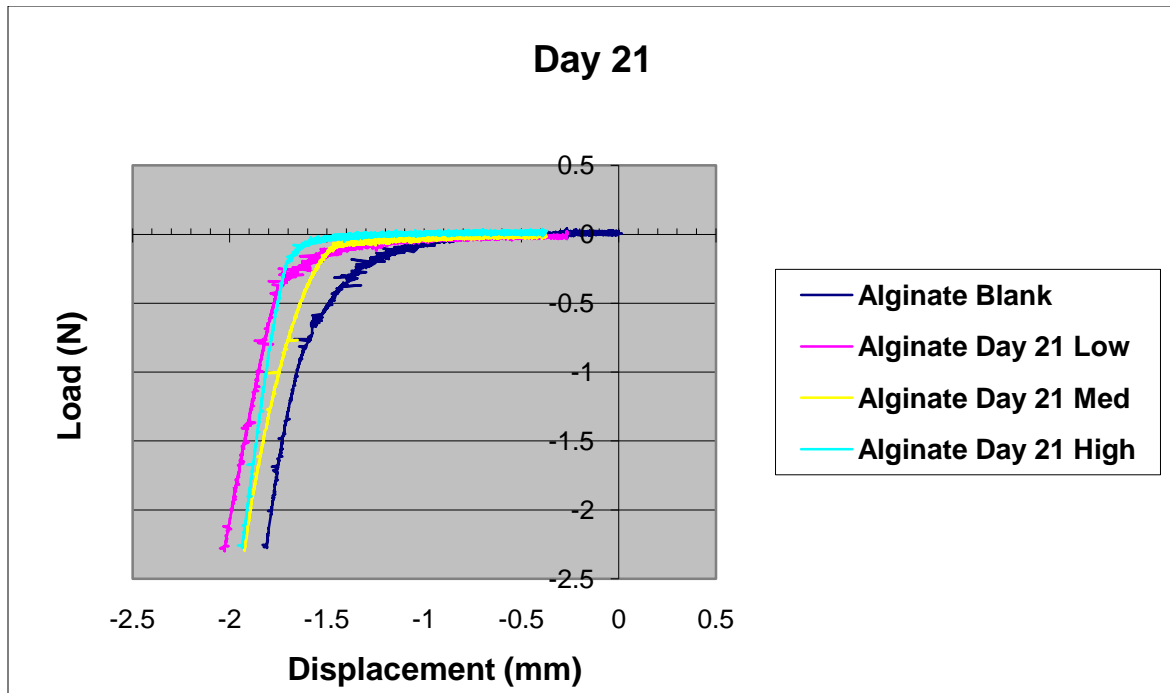
Figure 18. Compression testing on days 3, 7, 14, and 21 for Low, Med, and High Barium Alginate RT4-GDNF seeded capsules and unseeded (blank) capsules.

The following series of graphs displays comparative values in compression of each cellular group over the 21 day test period. The low seeded capsules were able to withstand more force per area over time. The high density seeded capsules withstood the lowest force per area over time. All densities sustain a loading force greater than 50 kPa over a 21 day test period (Figure 20).

LOW

Load (N) vs Displacement (mm)

Legend:
- Alginate Blank
- Alginate Day 3 Low
- Alginate Day 7 Low
- Alginate Day 14 Low
- Alginate Day 17 Low
- Alginate Day 21 Low

MEDIUM

Load (N) vs Displacement (mm)

Legend:
- Alginate Blank
- Alginate Day 3 Med
- Alginate Day 7 Med
- Algiante Day 14 Med
- Alginate Day 17 Med
- Alginate Day 21 Med
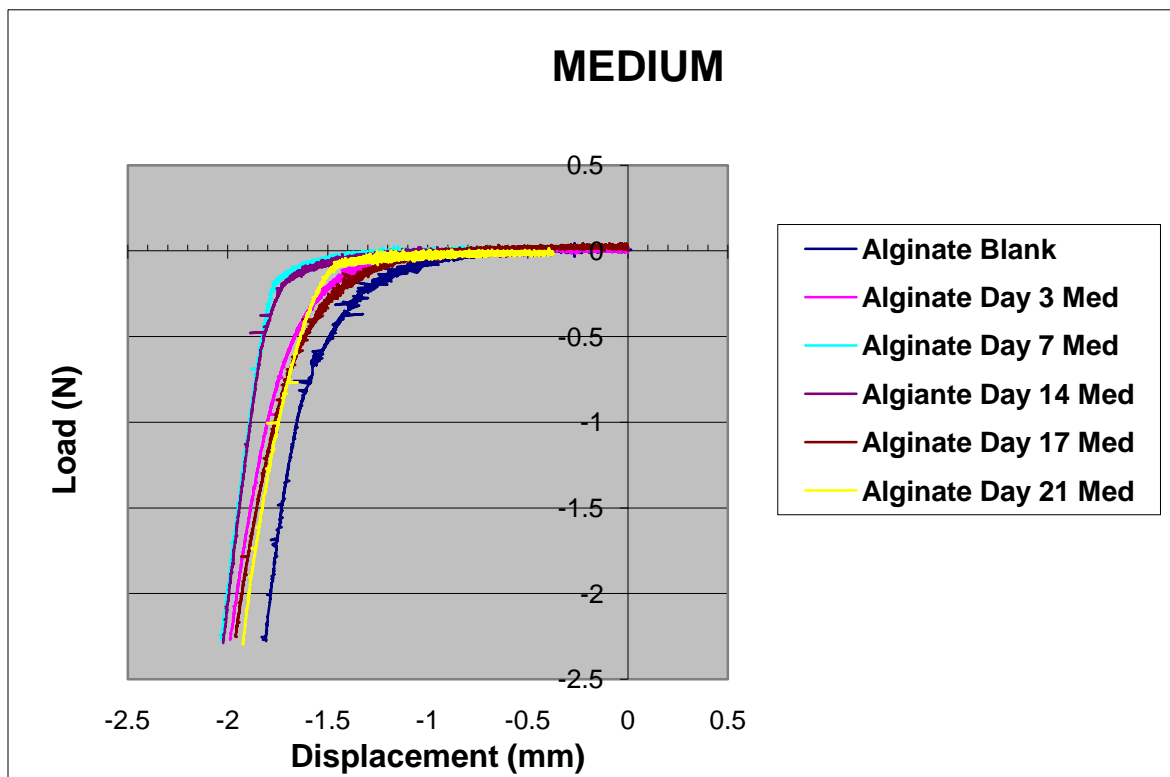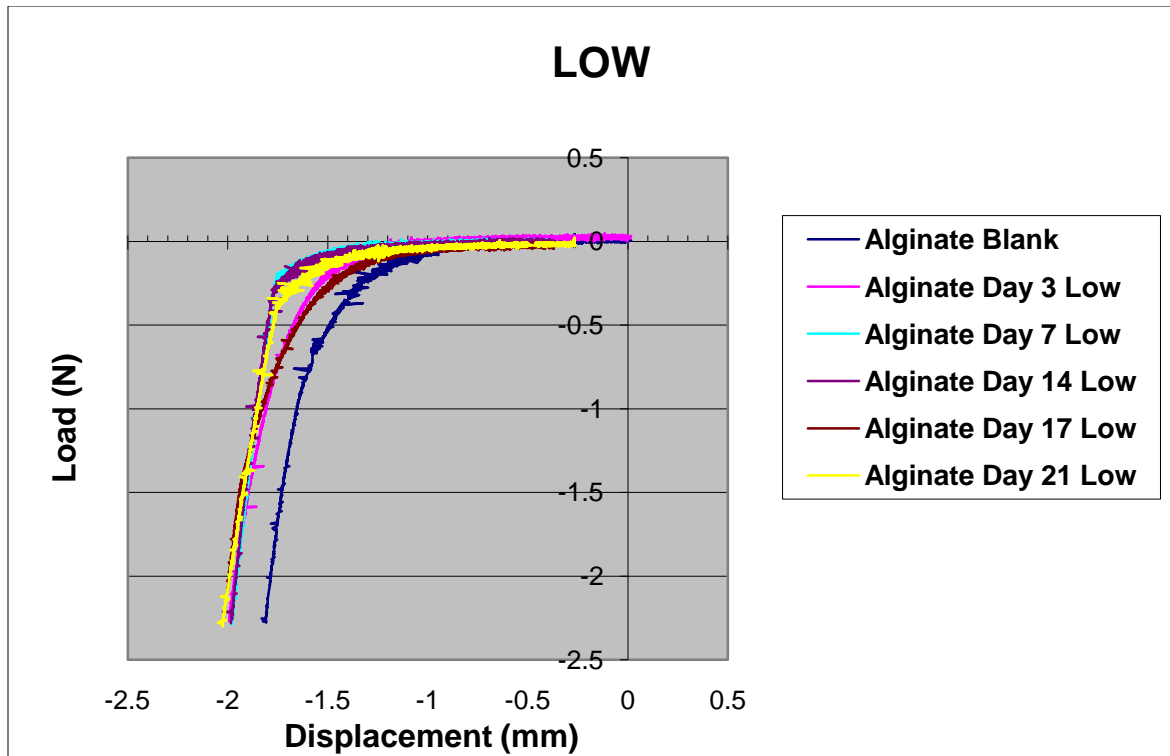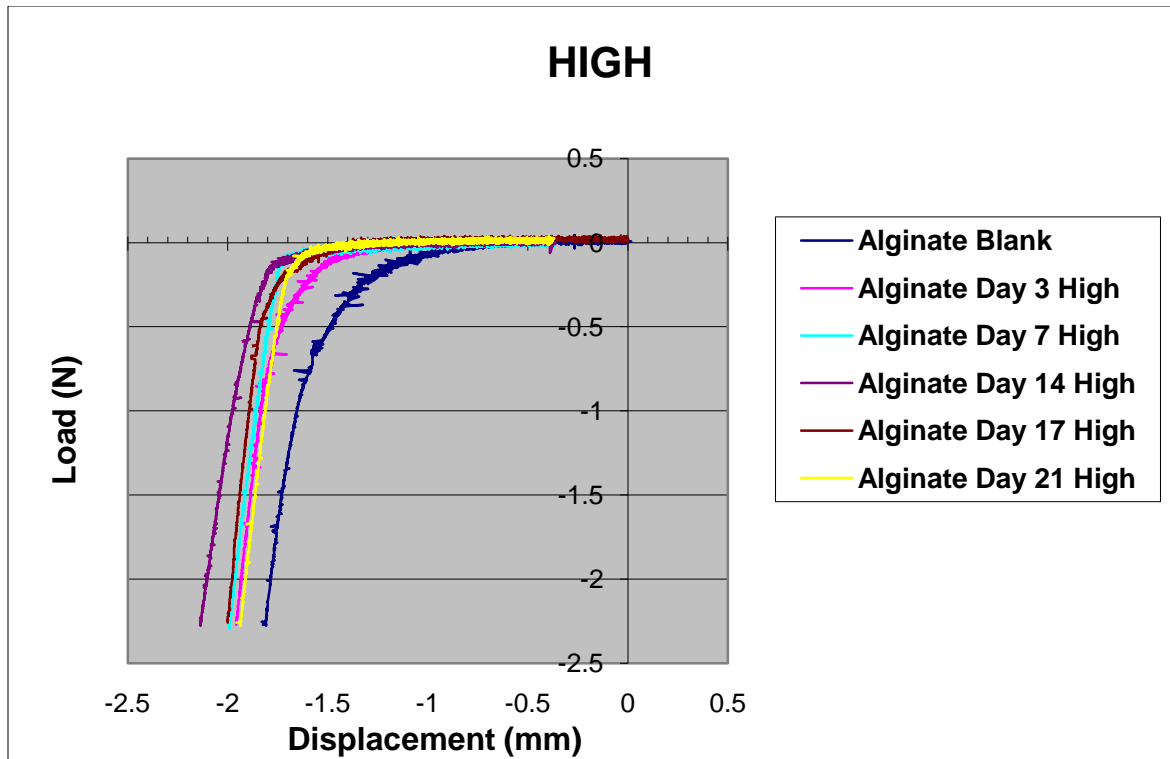
Figure 19. A comparison of compression loading for Low, Medium, and High density seeded capsules over the 21 day test period.

Varying the initial number of seeded cells did effect the maximum loading force which the capsules could sustain before bursting over the testing period. On each test day all of the cellular capsules (low, med, and high) required more force to break than the non-cellular capsules (Figure 21).
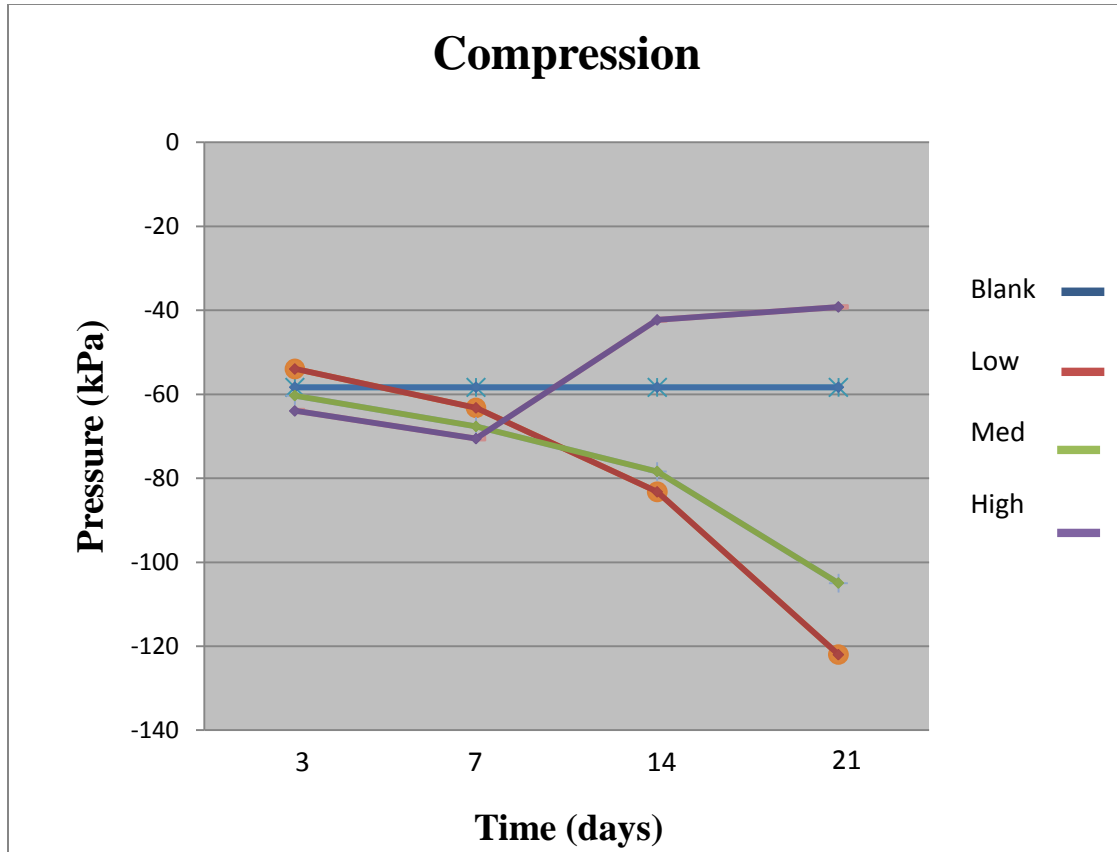
Figure 20. Compression testing displaying the bursting pressures of the non-encapsulated and encapsulated microcapsules over 21 day test period.

The following table (Table 4) displays the true amounts of pressure in KDa, which each cell group could sustain before bursting on each test day over 21 days.

Table 4. Pressures of the non-encapsulated and encapsulated microcapsules at bursting point in kiloPascals, over 21 day test period.

Test Day			
3	7	14	21
Blank (kPa)			
-58.32	-58.32	-58.32	-58.32
Low (kPa)			
-59.35	-63.21	-83.22	-122.00
Med (kPa)			
-60.30	-67.65	-78.35	-104.96
High (kPa)			
-63.93	-70.53	-42.28	-39.79

At day three, both cellular and non-cellular capsules demonstrate a compressive resistance of about 60 kPa. At day seven all cellular groups show a slight increase in resistance. At day 14, low and medium seeded densities continue to display an increased capacity for resistance, however, the high seeded system appears to fail at day 14 and lose all compressive resistance for the rest of the test. At day 21, the low seeded group shows about a 30% increase in strength, proving to withstand the highest amount of pressure of 122 kPa, while medium seeded densities show a 25% increase in resistance to almost 105 kPa. It appears that cellular proliferation adds to the compressive strength of the capsule when seeded at a low to medium initial density due to the fact that these capsules were able to withstand the highest amount of force per area before bursting.

The initial number of cells seeded per capsule did have a noticeable effect on the success of the capsule. A low number of cells per capsule allowed for ECM production and increased strength, while a higher numbers of cells potentially caused unfavorable conditions and possibly cell death, which would ultimately lead to a weaker capsule and in our experiment with complete failure at 2000 cells per capsule by day seven.

Overall, low seeded densities withstood the highest amount of force per area in 28 days before bursting and appears to be an optimal system for cell transplantation experimentation; however, medium densities proved to be a potentially optimal system as well.

CHAPTER 4

DISCUSSION

Schwann cells have a pivotal role in response to PNS injury. The PNS regenerative powers are partially due to intrinsic properties of Schwann cells that encourage a spontaneous and favorable environment for nerve regeneration. PNS axonal regeneration occurs through the initiation of signaling cascades that activate Schwann cells. Schwann cells represent the glial cells of the PNS and support axons by sheathing them with insulating myelin layers and producing neurotrophic factors, cytokines, extracellular matrix and adhesion molecules, which aid in regrowth of the injured nerve. [44] Upon injury, Schwann cells synthesize surface cell adhesion molecules (CAMs) and dividing Schwann cells remain in the basement membrane (BM) tube that surrounds the original nerve fiber. [65] Even in the presence of high levels of degradative enzymes released after injury, Schwann cell-derived BM and supporting endoneurial connective tissue is preserved and not degraded for the support and guidance of the sprouting axons, which promote the survival and regeneration of the neurons.[44] The BM not only offers a structural support for regenerating axons but also provides a favorable substrate for axonal regrowth.[65] Due to these reparative qualities, Schwann cells are considered a viable option for transplantation. There are many advantageous of using Schwann Cells as transplantable cells; such as they are not a target of immune attack in CNS autoimmune demyelinating disease, [22] they can be cultured within the laboratory and they can be expanded from adult biopsies for autologous engraftment. [66] In addition, and they can remyelinate and provide several other vital factors that are proven to promote nerve

fiber regeneration. [66] However, these cells can be destroyed by the host immune system, thus making encapsulation a valuable technique to promote survival.

Controlled release of neurotrophic factors is a challenge in current nerve regeneration strategies. Glial cell line derived neurotrophic factor (GDNF) has been shown to be a potent survival protein for motor neurons. [4] In order to test its temporal delivery, we have engineered a Schwann cell line to produce GDNF upon induction through the ecdysone mammalian expression system (RT4-GDNF cells). Prior to use *in vivo*, we must protect these cells from the host immune system through a procedure called microencapsulation. This describes a procedure where cells are enclosed within a semi-permeable membrane. Key characteristics of microcapsules include biocompatibility, adequate resistance to environmental constraints, appropriate membrane stability, and permeability. [67] In particular, preservation of the structural integrity of microcapsules is crucial in many applications such as immunoisolation in cell transplantation. Previous research has suggested that mechanically strong and durable capsules were less likely to rupture, thus prolonging *in vivo* functions of the encapsulated cells. Thus, transfected cells were cultured *in vitro* in 1% alginate solution at 5×10^5 cells/mL using 50 mM $BaCl_2$ gelling buffer. Both non-encapsulated and encapsulated cells were tested for GDNF secretion upon induction. Samples were induced continuously via treatment of 10 μM of ponasterone A (ponA). Moreover, cells were evaluated for responses to removal and delayed addition of ponA inducer. Culture media were collected at several days post-incubation. ELISA was performed to quantify the amount of secreted GDNF. Results demonstrated that GDNF was released after ponA

induction and removal of the inducer returned the GDNF level to baseline similar to uninduced samples. Induction with ponA resulted in secretion of functional GDNF that promoted the survival of more embryonic neurons. However, the encapsulated cells secrete 9× less GDNF compared to non-encapsulated cells. Initially, the number of RT4-GDNF cells seeded were the same in all treatment groups; however, the proliferation rate of cells growing as monolayer is faster compared to those within the alginate capsule (Figure 16). The presence of higher number of cells over time could partially account for the increased GDNF expression in non-encapsulated versus encapsulated cells. The inducer ponA, a lipophilic compound that binds to nuclear receptors, has been shown in an earlier study by Xu *et al.* to activate secretion of nitric oxide (NO) from calcium alginate-microencapsulated cells. [56] Removal of ponA led to reduction of NO and NO's tumor suppressing ability. [56] Maximum NO levels were detected three days after ponA addition, [56] while in this study, we found that the GDNF secretion plateau at approximately two weeks. The delayed reaction is possibly due to ponA's slow diffusion in alginate gel; while the response time probably varies depending on how the ecdysone system elements was integrated into the cell line and the alginate M-G ratio and arrangement. We found ≥ 88% cell viability while Pannunzio *et al.* obtained 73 to 76% survival in 1% alginate using 100 mM $CaCl_2$ as gelling solution *in vitro*. [68] Generally, adhesion-dependent cells undergo apoptosis when detached from the substrate; and we should therefore determine the encapsulated Schwann cell mode of survival. RT4-GDNF cells in alginate seeded at a higher density have faster proliferation rate compared to

those with lower initial cell counts; suggesting that viability is due to cell-to-cell interaction and possibly mediated by cell adhesion molecules (CAMs).

In our microencapsulation study, Schwann cells were isolated from the external environment by a semi-permeable alginate membrane, which should allow for entry of oxygen and nutrients, and exit of waste products and therapeutic molecules. Proper encapsulated cell functions require strict control over permeability of the microcapsule membrane. Permeability measurements were carried out with individual microcapsules by examining the diffusion of fluorescein isothiocyanae-labeled dextran (FITC-dextran) with the various molar masses from the microcapsules. In a study (Chen *et al.*) found that the dextran ingress was significantly reduced with increasing molecular weights (MWs) of fluorescent markers. Irrespective of microcapsules, low MW dextran (4 KD) infiltrated to the interior of the microcapsules at a great extent (diffusion ratio > 70%), whereas permeation of larger dextrans, 40 KD and 70 KD, was greatly restricted, with the inflow ratio around 20% and below 5%, respectively. [67] Likewise in our study, dextran release was observed to be molecular weight-dependent. Release of a lower molecular weight dextran is mainly governed by the diffusion through the barium–alginate gel matrix. With increasing molecular weights, dextran release was strongly influenced by the dissolution of alginate matrix through the exchange of Ba^{2+} ions which act as a cross-linker. These results suggest that the 50 mM $BaCl_2$-alginate gelling solution is a useful vehicle for controlled release of GDNF delivery. However, it appears that of the three dextrans, both the 20 kDa and 40 kDa dextrans could be used to measure the permeability accurately. While the 70 kDa dextran capsule permeabilities were often less than 5% and therefore

an unstable source for studying the permeability of GDNF. There may be several possibilities to account for these permeability results. Since the FITC-dextran was incorporated into the seeded capsules, this could have an effect on the amount of dextran that was released, initially and over the 48 hour test period. Our MTT assay shows that our cells are indeed proliferating throughout the duration of the test. So as the amount of cells inside the capsule increased, the amount of permeating dextran could have a more difficult time finding access to the pores of the capsule by which to exit; especially the dextrans with a higher molecular weight. This does correlate with our permeability results. At 24 hours when the 70 kDa dextran appears to plateau around 10.6% release, this may suggest that the failure may not entirely be contributed to the dextran weight, but may be due to the increased amount of cells within the capsule, which restricted further permeation from occurring. Conversely, as proliferation was shown to be increasing within the capsules, this may have contributed to the alginate capsule itself becoming effected in such ways as the breaking down of the capsule, which could have led to possible holes in the capsule which allowed for a higher release of dextran; such as seen in the lower weighted dextrans. These are some possibilities which should be explored in further research to determine that our alginate capsules are stable.

To determine if microspheres with cells are strong enough to withstand the pressure inside the body, mechanical testing was performed. Mechanical properties of microcapsule membranes are of key importance for their integrity preservation and *in vivo* behavior. It was previously reported that microcapsules with strong membranes were more durable and less likely ruptured, which allows for prolonged functions of the

encapsulated cells.[67] Despite being crucial; precise determination of the microcapsule mechanical strength is difficult because of the size (generally $100\,\mu m$ to $2\,mm$ in diameters) [68] and fragile nature of the microcapsule. Alginate microcapsules loaded with RT4-GDNF cells, were compressed and forces were measured as a function of displacement; (mm) at various loading forces; Newtons (N). A typical peripheral nerve at relaxed position has a compressive pressure of 0.7 kPa. Movement into different functional positions can lead to increase in compressive stress up to 4 kPa. [69] Compressive stress values were determined in our testing to be above 50 kPa and therefore, in theory, our RT4-GDNF cell line, encapsulated in alginate solution can withstand implantation without being irreversibly damaged inside the body.

It was noticeable during testing that the microcapsules had changed into an elliptical shape under mechanical force before breaking, indicating the elasticity of the cross-linked capsules and showing that covalent cross-linking by alginate adds stabilization to the microcapsules. This cross-linking however may have been affected by the density of cells within the capsule. As we shown, the cells are proliferating throughout the duration of the test period. We see for the high seeded density group that at day seven, there is complete failure of the system. This could be attributed to the fact that the cells were seeded initially at 2,000 cells per capsule and that over time as they continued to proliferate that the capsule just could not hold that many cells and therefore the capsule became so weakened that at 70 kPa of force, the capsules burst. Alternatively, we could also suggest that due to an overwhelming amount of cells within the capsule, that the cells themselves became too populated that the cells on the inside of the capsule

were deprived from essential nutrients to grow and undergone apoptosis. The dead cells could have created so much debris that became toxic to the rest of the system, including the alginate capsule itself and evoked a breakdown in the capsule material itself, which would also explain the system failure and capsule weakness. These are also parameters which should be further investigated to ensure that our system is ideal for cell encapsulation and biodelivery applications.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

This engineered construct will ultimately provide a foundation for developing neurological therapies. By using a controlled system such as this, we are able to control the amount of protein release to a specified target area. Additionally, we acknowledge that the use of clonal cell lines for human implants is unlikely. Thus, our future research will include the use of modified primary Schwann cells or precursor stem cells. It is thought that pre-Schwann cells are contained within peripheral nerves, thus differentiation and maturation occurs locally. Recently, studies have been published in which stem cells from bone marrow and hair follicles have been shown to differentiate into Schwann-like cells *in vitro*.[65,66] The use of these precursor Schwann cells would facilitate translation of the current research proposed and will ultimately be a direction for our research. However, we need to first establish the optimal time delivery of GDNF, which is the focus of our current research. We believe that the proposed research will help overcome a problem that has eluded physicians for many years, ultimately reducing the morbidity and mortality associated with peripheral nerve degeneration and traumatic nerve injuries.

APPENDIX A

GLOSSARY

Allogenic - Being genetically different although belonging to or obtained from the same species.

Astrocytes - are characteristic star-shaped glial cells in the brain. They perform many functions, including biochemical support of endothelial cells which form the blood-brain barrier, the provision of nutrients to the nervous tissue, and a principal role in the repair and scarring process in the brain.

Axotomy - The process of cutting or to otherwise sever an axon.

Axon - Is a long, slender projection of a nerve cell, or neuron, that conducts electrical impulses away from the neuron's cell body or soma.

Axonotrophic molecules - Molecules which act as survival factors to prevent neuronal cell bodies from undergoing apoptosis and improve axonal growth.

Basement Membrane (BM) - is a thin sheet of fibers that underlies the epithelium, which lines the cavities and surfaces of organs, or the endothelium, which lines the interior surface of blood vessels.

Blood-Brain Barrier (BBB) - is a separation of circulating blood and cerebrospinal fluid (CSF) maintained by the choroid plexus in the central nervous system (CNS).

Cell Adhesion Molecules (**CAM**s) - are proteins located on the cell surface involved with the binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion.

Cation- A positively-charged ion, which has fewer electrons than protons.

Cross-links - Are covalent bonds linking one polymer chain to another.

Central nervous system (CNS) - The portion of the vertebrate nervous system consisting of the brain and spinal cord.

Cytoskeleton - It is a dynamic structure that maintains cell shape, and also has been known to protect the cell, enables cellular motion, and plays important roles in both intracellular transport and cellular division.

De novo synthesis – Anew; Refers to the synthesis of complex molecules from simple molecules.

Dopaminergic neurons - Functions to enhance the effects mediated by dopamine in the central nervous system.

Dorsal root ganglion (DRG) - Is a nodule on a dorsal root that contains cell bodies of neurons in afferent spinal nerves.

Extracellular matrix (ECM) - Is the extracellular part of animal tissue that usually provides structural support to the cells in addition to performing various other important functions.

Glial cell – Are non-neuronal cells that provide support and nutrition, maintain homeostasis, form myelin, and participate in signal transmission in the nervous system

Glial cell-line derived neurotrophic factor (GDNF) – A small protein that promotes the survival of neurons, especially it supports the survival of dopaminergic and motor neurons.

Glycoproteins - are proteins that contain oligosaccharide chains covalently attached to their polypeptide backbones.

Immunoisolation - The process of protecting implanted material such as biopolymers, cells, or drug release carriers from an immune reaction.

Intrathecal - Is an injection into the spinal canal.

In vitro - Refers to the technique of performing a given experiment in a test tube, or, generally, in a controlled environment outside a living organism.

In vivo - Means that which takes place within a living organism.

Lipophilic -- refers to the ability of a chemical compound to dissolve in fats, oils, lipids, and non-polar solvents.

Matrix -- The intercellular substance of a tissue or the tissue from which a structure develops.

Messenger Ribonucleic Acid (**mRNA**) **--** Is a molecule of RNA encoding a chemical "blueprint" for a protein product.

Microencapsulation -- Is a process in which tiny particles or droplets are surrounded by a coating to give small capsules with many useful properties.

Molecular weight cut out (MWCO) -- The size designation in Daltons for ultrafiltration membranes. The molecular weight of the globular protein that is 90% retained by the membrane. No industry standard exists, hence the MWCO ratings of different manufacturers are not always comparable.

Myelin sheath -- The insulating envelope of myelin that surrounds the core of a nerve fiber or axon and facilitates the transmission of nerve impulses. In the peripheral nervous system, the sheath is formed from the cell membrane of the Schwann cell and, in the central nervous system, from oligodendrocytes.

Nerve conduit-- An artificial means of guiding axonal regrowth to facilitate nerve regeneration and is one of several clinical treatments for nerve injuries.

Neuroma – A benign tumor from nervous system.

Neuron - Electrically excitable cells in the nervous system that process and transmit information.

Neurotrophic growth factors -- Is a small secreted protein which induces the differentiation and survival of particular target neurons (nerve cells).

Neurotrophic factors -- Are a family of proteins that are responsible for the growth and survival of developing neurons and the maintenance of mature neurons.

Newtons (N) -- is the unit of force derived in the SI system; it is equal to the amount of force required to accelerate a mass of one kilogram at a rate of one meter per second per second.

Nodes of Ranvier -- Are regularly spaced gaps in the myelin sheath around an axon or nerve fiber.

Oligodendrocyte – Neuroglial cell of the central nervous sytem (CNS) in vertebrates, whose function is to myelinate CNS axons.

Peripheral nervous system (PNS) -- The part of the vertebrate nervous system constituting the nerves outside the central nervous system and including the cranial nerves, spinal nerves, and sympathetic and parasympathetic nervous systems.

Polymer -- Is a substance composed of molecules with large molecular mass composed of repeating structural units, or monomers, connected by covalent chemical bonds.

Reinnervation -- Restoration of nerve function after it has been lost.

Satellite cell -- Are mononuclear progenitor cells found in mature muscle between the basal lamina and sarcolemma.

Schwann cell – Are a variety of glial cell that mainly provide myelin insulation to axons in the peripheral nervous system of jawed vertebrates.

Wallerian degeneration -- Is the process of degeneration of the axon distal to a site of transection.

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ABSTRACT

CHARACTERIZING THE CONTROLLED RELEASE OF GLIAL CELL-LINE DERIVED (GDNF) NEUROTROPHIC FACTOR FROM ENCAPSULATED SCHWANN CELLS

by

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Major: Biomedical Engineering

Degree: Masters of Science

Controlled expression of glial cell line derived neurotrophic factor (GDNF) can be integrated in the development of a system for repair of injured peripheral nerves. This delivery strategy was demonstrated via inducible GDNF from microencapsulated cells in barium alginate. The Schwann cell line RT4-D6P2T was initially modified utilizing an ecdysone-based stable transfection system to produce RT4-GDNF cells. During construct preparation, it was found that C6 cells (where GDNF cDNA was isolated) make three GDNF transcript variants. Additionally, the importance of 5' untranslated region to drive biologically-functional GDNF synthesis was shown. Encapsulation of RT4-GDNF in 1% alginate was then performed. It was determined that cells were able to survive at least 1 month *in vitro* using starting densities of 20, 200 and 2000 cells/capsule and in a barium ion concentration of 50 mM. Most importantly, our results demonstrate that encapsulated Schwann cells continuously secreted exogenous GDNF at all time points and increased

GDNF secretion upon ponasterone A induction. Compressive stress values were determined in our testing to be above 50 kilopascals of force. Finally, permeability results with FITC-Dextran strongly suggest that microcapsules conformed from 50 mM $BaCl_2$-alginate gelling solution is a useful vehicle for controlled release of GDNF delivery. Thus, we expect this encapsulation system can be utilized for optimizing the release of GDNF for improved nerve regeneration and regulated GDNF release from these microcapsules *in vivo* may potentially aid in the regeneration of damaged nerves.

AUTOBIOGRAPHICAL STATEMENT

KRISTY BROADRICK

I graduated from Eastern Michigan University with a Bachelor's degree in Biochemistry and Toxicology, with a group minor, in Biology, Physics, and Math. After graduating, I knew that I wanted to continue with my education which is when I became familiar to the field of Biomedical Engineering. I applied to Wayne State University Master's program and began this journey in August of 2006. After my first year in the program, Dr. VandeVord offered me a Graduate Research Assistant position in the Bioengineering Department and I couldn't have been more excited to have my first job in the industry. Under her superb guidance, our research laboratory group has published two scientific papers entitled; "Characterizing the controlled release of glial cell line derived neurotrophic factor from alginate-matrigel microencapsulated Schwann cells." In the Journal of Microencapsulation (2008 October) and "A Comparative Study Evaluating the *In Vivo* Incorporation of Biological Sling Materials." In the Urology Journal (2009 September). During my time at Wayne State University, our projects were also awarded at the Society for Biomaterials Annual Meeting and Exposition in Chicago, Illinois, 2007, oral presenter for the abstract entitled, "*In Vivo* Responses to Urological Biomaterials as Utilized for Urological Reconstruction" and in Los Angeles, California, 2007, poster presenter for the abstract entitled "The Controlled Release of GDNF from Microencapsulated Schwann Cells." My experience at Wayne State University has been a true learning experience both on an educational and personal level.