

DESIGN AND IN VITRO TESTING OF A PERIPHERAL NERVE GUIDE

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DİLARA GÖKSU TAMAY

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submitted by **DİLARA GÖKSU TAMAY** in partial fulfillment of the requirements
for the degree of **Master of Science in Biotechnology Department, Middle East
Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences**

Assoc. Prof. Dr. Can Özen
Head of Department, **Biotechnology**

Assoc. Prof. Dr. Can Özen
Supervisor, **Biotechnology, METU**

Prof. Vasıf Hasırcı
Co-Supervisor, **Department of Biological Sciences, METU**

Examining Committee Members:

Prof. Dr. Aykut Özkul
Department of Virology, Ankara University

Assoc. Prof. Dr. Can Özen
Biotechnology, METU

Prof. Dr. Dilek Keskin
Department of Engineering Sciences, METU

Assoc. Prof. Dr. Pınar Yılğör Huri
Department of Biomedical Engineering, Ankara University

Assoc. Prof. Dr. Sreeparna Banerjee
Department of Biological Sciences, METU

Date: 03.09.2019



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Name, Surname: Dilara Göksu Tamay

Signature:

ABSTRACT

DESIGN AND IN VITRO TESTING OF A PERIPHERAL NERVE GUIDE

Tamay, Dilara Göksu
Master of Science, Biotechnology
Supervisor: Assoc. Prof. Dr. Can Özen
Co-Supervisor: Prof. Vasıf Hasırcı

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Regeneration after peripheral nerve injury (PNI) is limited as chondroitin sulfate proteoglycans (CSPGs) within the scar tissue inhibit axon outgrowth. Nerve guides (NGs) are alternatives to the “gold standard” autografting, which has limitations including donor site morbidity and limited tissue availability. NGs are tubular constructs designed to form a bridge between the transected nerve ends, provide physical support, and achieve axon guidance via topographical and/or chemical cues. This study aims to design and test the *in vitro* performance of a NG with topographical cues, capable of removing CSPGs via release of enzyme chondroitinase ABC (ChABC). A bisphenol A methacrylate (Dental SG)-based NG filled with crosslinked gelatin methacrylate (GelMA) hydrogel containing microchannels was constructed. For practical observation, a halved tube was used for *in vitro* testing. Cell adhesion and viability analyses were conducted *in vitro* using neuron-like PC12 cell line.

Optimum GelMA concentration was determined as 15% (w/v). *In vitro* studies showed hydrogel provides cells with the optimal conditions for attachment and growth. Enzyme release from alginate (using BSA as a model) was in accordance to Korsmeyer-Peppas model with a 100% release achieved in ~3 days. 3D printed Dental SG exterior was shown to significantly degrade in the presence of cells. The effectiveness of NG in supporting cell viability and growth was tested *in vitro*. Cells

showed good attachment and were viable along the microchannels with visible alignment during 3 days of incubation. However, due to possible toxic effect of Dental SG degradation products, cells did not survive until Day 7.

Keywords: Nerve guide, 3D printing, GelMA, chondroitinase ABC



ÖZ

PERİFERAL SİNİR YÖNLENDİRİCİ TASARIMI VE IN VITRO KOŞULLARDA DENENMESİ

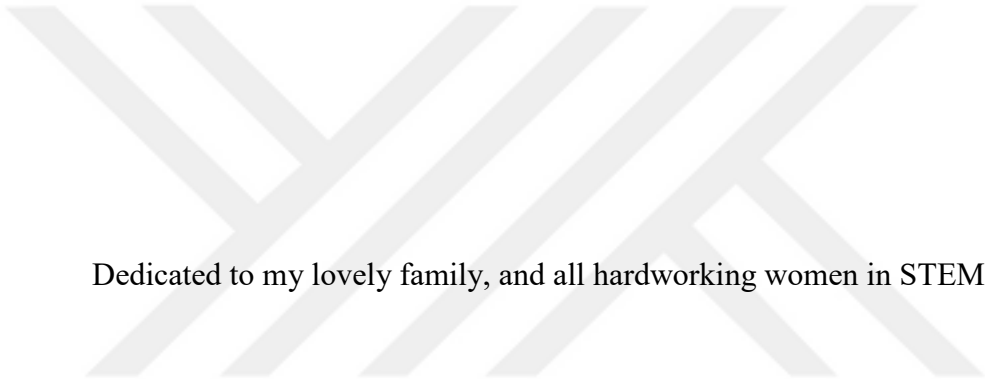
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Periferal sinir sistemi yaralanmalarında sinir rejenerasyonu yara dokusundaki yüksek miktarda kondroitin sülfat proteoglikanların (KSPGler) akson uzamasını baskılaması nedeniyle sınırlıdır. Sinir yönlendiriciler (SY), “altın standart” olarak kabul edilen fakat kısıtlı miktarda donör doku bulunması ve donör bölge morbiditesi gibi sınırlandırmaları olan otogrefte karşı önemli bir seçenektir. SYler proksimal ve distal sinir uçları arasında bir köprü görevi görmek, fiziksel destek ve topoğrafik ya da kimyasal belirteçler ile aksonun yönlendirilmesini sağlamak için tasarlanan tübüler yapılardır. Bu çalışma topoğrafik yönlendirme işaretleri içeren ve büyümeyi baskılayan yara dokunun enzimatik bozunma ile ortamdan uzaklaştırılmasını sağlayabilecek bir SY tasarlamayı amaçlamaktadır. Mikrokanallı, çapraz bağlanmış metakrile jelatin (GelMA) hidrojel ile doldurulmuş tübüler metakrile bisfenol A (Dental SG) temelli bir SY oluşturulmuştur. Yapının distal ucuna kondroitinaz ABC katılması ile distal sinir ucunda ifade edilen KSPGlerin enzimatik bozunmasının sağlanması amaçlanmıştır. Gözlem ve analizlerin kolaylaştırılması için *in vitro* testlerde yarım tübüler bir yapı kullanılmıştır. *In vitro* hücre yapışması ve canlılık analizleri nöron benzeri bir hücre hattı olan PC12 kullanılarak yürütülmüştür.

İdeal GelMA konsantrasyonu karakterizasyon testleri sonucunda %15 olarak belirlenmiştir. *In vitro* çalışmalar hidrojel hücre tutunması ve büyümesi için ideal koşulları sağladığını göstermiştir. Aljinat hidrojelden enzim salımı model olarak BSA kullanılarak, Korsmeyer-Peppas modeline uygun ve 100% salım 3 günde tamamlanacak şekilde gerçekleşmiştir. Dental SG ile 3B basılan ürünlerin hücre varlığında hızla bozunduğu görülmüştür. SYnin hücre canlılığı ve büyüme sağlamada etkinliği *in vitro* testler ile gözlenmiştir. Kültürün ilk üç gününde mikrokanallarda hücre yapışması, canlılık, ve kanal boyunca yönelme görülmüş, fakat Dental SG'nin bozunma ürünlerinin sitotoksik etki gösterme olasılığı nedeniyle kültürün yedinci gününde canlılık gözlenmiştir.

Anahtar Kelimeler: Sinir yönlendirici, 3B basım, GelMA, kondroitinaz ABC



Dedicated to my lovely family, and all hardworking women in STEM fields.

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LIST OF ABBREVIATIONS

ANS	Autonomous Nervous System
BBB	Blood-Brain Barrier
BSA	Bovine Serum Albumin
ChABC	Chondroitinase ABC
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
Dental SG	Dental Surgical Guide
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
ENS	Enteric Nervous System
FBS	Fetal Bovine Serum
HCl	Hydrochloric Acid
HS	Horse Serum
ID	Inner Diameter
IgC	Irgacure 2959
K ₂ HPO ₄	Dipotassium Hydrogen Phosphate
KH ₂ PO ₄	Potassium Dihydrogen Phosphate
MAA	Methacrylic Anhydride
Na ₂ CO ₃	Sodium Carbonate
NaCl	Sodium Chloride
NaHCO ₃	Sodium Bicarbonate

NaOH	Sodium Hydroxide
NGF	Nerve Growth Factor
OD	Outer Diameter
PDMS	Poly(dimethyl siloxane)
PNS	Peripheral Nervous System



CHAPTER 1

INTRODUCTION

1.1. The Nervous System

The nervous system is responsible for the maintenance of homeostasis in the body, working in coordination with the endocrine system (Farley et al., 2014). Changes in internal and external environment are controlled rigorously by the nervous system and countered with the appropriate response. Critical functions of the nervous system include acquisition and processing of sensory information (perception), initiation of voluntary movement (motor response), compiling and execution of emotional response (behavior), and organization and storage of information (memory).

The nervous system encompasses all neural tissues present in the body and is divided into two anatomical systems (Figure 1.1): (i) central nervous system (CNS), comprising the brain and spinal cord, and (ii) peripheral nervous system (PNS) including cranial nerves, spinal nerves, nerves that extend to and derive from skeletal muscle and skin, and components of autonomic nervous system (ANS) and enteric nervous system (ENS) (Hasirci and Hasirci, 2018). PNS transfers information that originates from or destined for the CNS through different regions of the body. The integration and coordination of this information occurs in the brain, which is also the center for initiation of the proper response (Scanlon and Sanders, 2018).

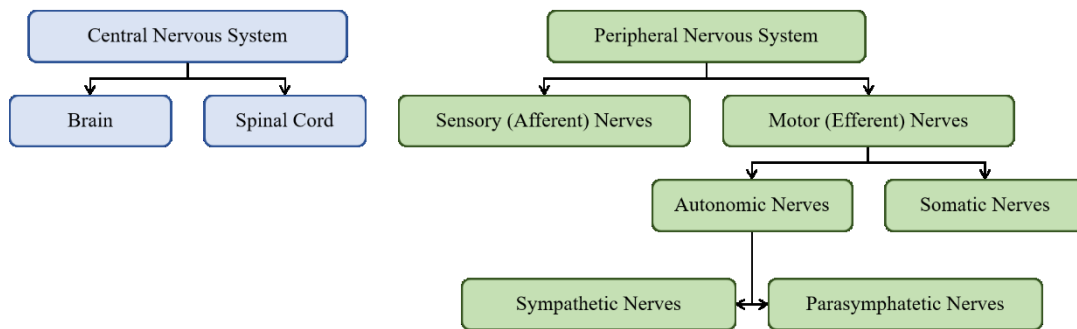


Figure 1.1. The nervous system.

1.1.1. Central Nervous System

The central nervous system consists of the brain and the spinal cord (Figure 1.2). Nerve cells (neurons) and glial cells (neuroglia) constitute the majority of the brain (Farley et al., 2014). The brain also houses connective tissue and blood vessels that provide physical support, protection, and nutrient supplement. The six major divisions of brain are medulla, pons, midbrain (the three referred collectively as the brain stem), cerebellum, cerebrum, and diencephalon (consisting of the thalamus, hypothalamus, optic chiasma, pineal gland and other small structures). These divisions are interconnected and operate synergistically to carry out vital processes, maintain homeostasis, and regulate higher functions such as intelligence, learning, memory, and emotion. The spinal cord continues from the brain stem and functions as a transmitter of impulses that contain sensory information to and from the brain, as well as a center for reflexive responses to various stimuli (Scanlon and Sanders, 2018). The vertebral canal in which the spinal cord is confined, protects the spinal cord from mechanical effects to the brain. Motor impulses are carried away from the brain via descending

tracts, and the central canal, which is continuous with the cavities in brain called the ventricles, holds the cerebrospinal fluid.

1.1.2. Peripheral Nervous System

Peripheral nervous system encompasses all neural tissue excepting the brain and the spinal cord (Figure 1.2). These include spinal nerves, afferent nerves, efferent nerves, and cranial nerves (Scanlon and Sanders, 2018). Spinal nerves originate in the brain stem, are connected to the spinal cord, and are in total 31 pairs named after their respective vertebra, whereas cranial nerves are those attached to the brain. Sensory and motor nerves transmit information to and from the CNS, and together constitute the somatic nervous system (SNS). Motor nerves control voluntary movement by communicating with skeletal muscle. Sensory nerves transmit impulses to the components of CNS from somatic receptors present throughout the body. Autonomous nervous system (ANS), on the other hand, coordinates the involuntary or unconscious actions in the body such as respiration and heart rate (Catala and Kubis, 2013). It has two subdivisions named sympathetic division and parasympathetic division. These two divisions exert their functions antagonistically to each other. An example would be the state of iris, a smooth muscle, which sympathetic division dilates, whereas parasympathetic division constricts it to normal. Enteric nervous system (ENS), a recently discovered subdivision of PNS, comprises intrinsic innervations of the gastrointestinal tract. ENS has also been referred to as the “brain of the gut” as it operates closely with the autonomous nervous system and regulates gastrointestinal function locally (Martini et al., 2012).

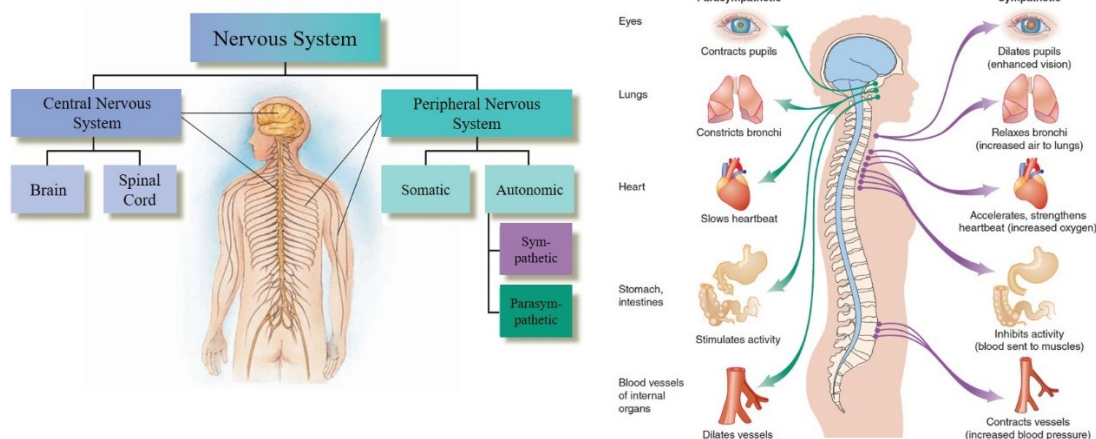


Figure 1.2. Central and peripheral nervous systems, their subdivisions, and functions. Adapted from Bernstein, D. (2008) and Feldman, R. S. (2015).

1.1.2.1. Anatomy of the Peripheral Nerve

The peripheral nervous system consists of spinal nerves, cranial nerves, afferent nerves, efferent nerves, and their branches. The nerve fibers in these PNS components can be autonomic or somatic, myelinated or unmyelinated, all enclosed by the supporting Schwann cells (Stewart, 2013). The individual nerve fibers in a peripheral nerve trunk are surrounded by the endoneurium, a collagen based supportive connective tissue that forms around each axon and its respective Schwann cells (Figure 1.3). Endoneurium serves to resist stretching with no elastin and a low number of fibroblasts in its structure. The fibers enclosed by the endoneurium are gathered together in nerve bundles called fascicles. The diameter of fascicles in human nerve can range from 200 to 800 μm (Delgado-Martínez et al., 2016). Fascicles are surrounded by the perineurium, a specialized tissue responsible for maintenance of the endoneurial fluid surrounding myelinated and unmyelinated fibers. The perineurium consists mainly of perineurial cell layers and collagen. The groups of nerve fascicles are enclosed by a connective tissue called the epineurium (Landers and Altenburger, 2003). Consisting of two different layers, this tissue comprises 25 to 75% of the cross-sectional area of the nerve. The inner layer, called internal epineurium, is made of

collagen fibers that fill the gaps between the individual fascicles, insulates them from the external pressure and promotes longitudinal motion. The outer layer, called external epineurium, retains the structural stability by holding all the fascicles together. The epineurium is denser in the proximal limb regions and joints. The mesoneurium is a loose layer of tubular tissue that surrounds and guides the epineurium along the nerve length. The vascular system enters the nerve through the mesoneurium and travels the length of the nerve within the epineurium, perineurium, and the endoneurium.

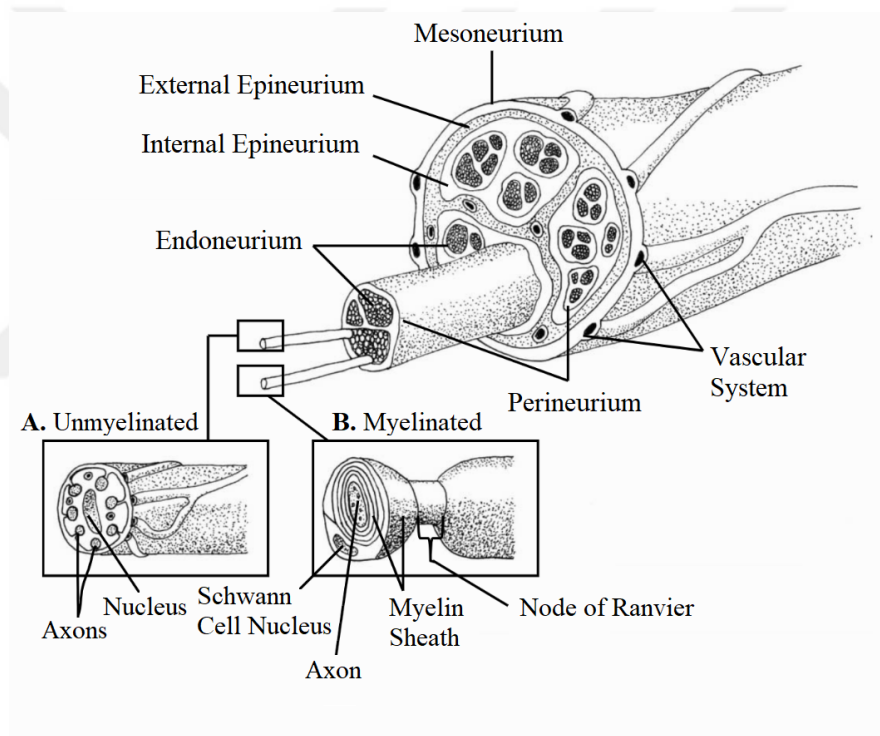


Figure 1.3. Anatomy of the peripheral nerve trunk. A) Unmyelinated and B) myelinated axons without the outer endoneurial tissue. Adapted with permission from Landers and Altenburger, 2003.

1.1.3. Neural Tissue

Neural tissue includes two main classes of cells: neurons (nerve cells) which are the basic functional units of the nervous system, and neuroglia (glial cells, from *glia*,

meaning “glue”) which are support cells that have crucial roles in the survival and functionality of nerve cells, and conservation of the physical and biochemical configuration of neural tissue (Martini et al., 2012). Nerve cells regulate all the communication and information processing in the neural tissue and oversee the functioning of the nervous system. Glial cells are higher in number in the nerve tissue compared to the nerve cells and are responsible for separation and protection of neurons, provision of neural tissue with a supportive structure, production of growth factors and neurotransmitters, local phagocytosis, development and regulation of synaptic transmission, distribution of nerve impulses, and regulation of the interstitial fluid composition (von Bernhardt et al., 2016).

1.1.3.1. Neurons (Nerve Cells)

Neurons are responsible of the signaling events of the nervous system, mainly transmission and processing of information (Scanlon and Sanders, 2018). The most common type of neuron, multipolar neuron, has four morphologically defined domains: (i) a cell body, (ii) short, branched dendrites, (iii) an axon, (iv) and presynaptic terminal branches (telodendria) (Figure 1.4).

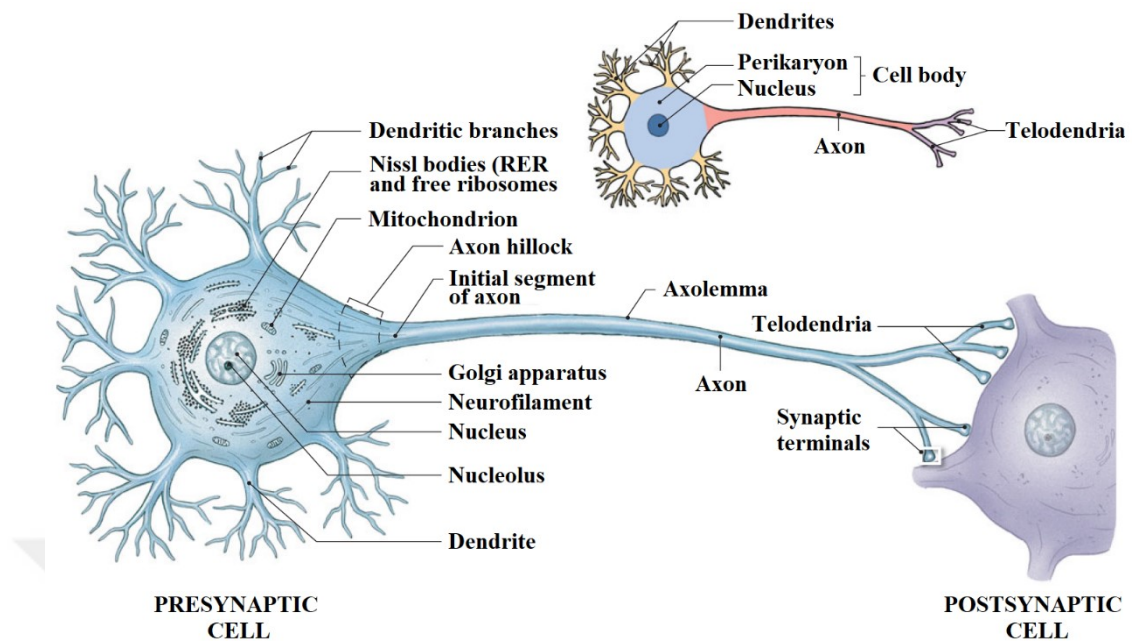


Figure 1.4. The anatomy of a multipolar neuron. Adapted from Martini et al., 2012. Copyright 2012 Pearson Education, Inc.

The cell body (soma), contains a large, round nucleus with a nucleolus, surrounded by a cytoplasm (perikaryon). The cytoskeleton is made up of neurofilaments and neurotubules, similar to the filaments and microtubules found in other cell types. Neurofibrils are bundles of these neurofilaments which extend into the dendritic branches and the axon, and function as an internal support structure. The organelles in perikaryon supply the nerve cell with energy and are responsible for the synthesis of various organic materials, most importantly the neurotransmitters, which play an important role in cell-to-cell communication. Dendrites are small branches that stretch out from the cell body and they are important contributors to the intercellular communication (Farley et al., 2014). Each dendritic branch contains 0.5-1 μm long processes referred to as dendritic spines which represent 80-90% of the total surface area of a CNS neuron. Dendritic spines are where a CNS neuron receives the information originating from other neurons. The axon is an extension of the cytoplasm and is capable of transmitting an electrical impulse named the action potential (Patton

and Thibodeau, 2017). The cytoplasm of the axon, called axoplasm, comprises neurofibrils, neurotubules, small vesicles, lysosomes, mitochondria, and various enzymes. Telodendria are the terminal branches that come after the main axon trunk and its collaterals. Synaptic terminals are located at the end of the telodendria, each a part of a synapse, the specialized site where a neuron communicates with another cell (Martini et al., 2012).

1.1.3.1.1. Classification of Neurons

Neurons can be grouped according to their structure or function (Farley et al., 2014). Structural subdivisions of neurons include: (i) anaxonic neurons, (ii) bipolar neurons, (iii) unipolar neurons, and (iv) multipolar neurons (Figure 1.5). Anaxonic neurons are small in cell body size and do not possess any morphological features that discern dendrites from axons. The processes of these cell types look all the same. They are found in the brain and sensory organs, but their functions are not well-known. Bipolar neurons are small neurons that exhibit two well-defined processes, a dendrite and an axon with the cell body positioned between the two. These types of neurons are quite rare, and they are located in the sensory organs where they receive and pass on sensory information from receptor cells to other neurons. Unipolar neurons have their dendrites and axons fused with the cell body located off to one side. The initial branched segment of their process is considered as the dendrite and the remaining segments are regarded as the axon. They constitute the majority of the neurons of the PNS with axons extending a meter or longer, ending at synapses in the CNS. The longest unipolar neuron is responsible for transmitting sensory information from the tips of the toes to the spinal cord. Multipolar neurons are the most common neurons of the CNS consisting of multiple dendrites and a single axon. The length of their axons can be similar to that of a unipolar neuron, the longest responsible for the relay of motor responses that move the toes (Patton and Thibodeau, 2017).

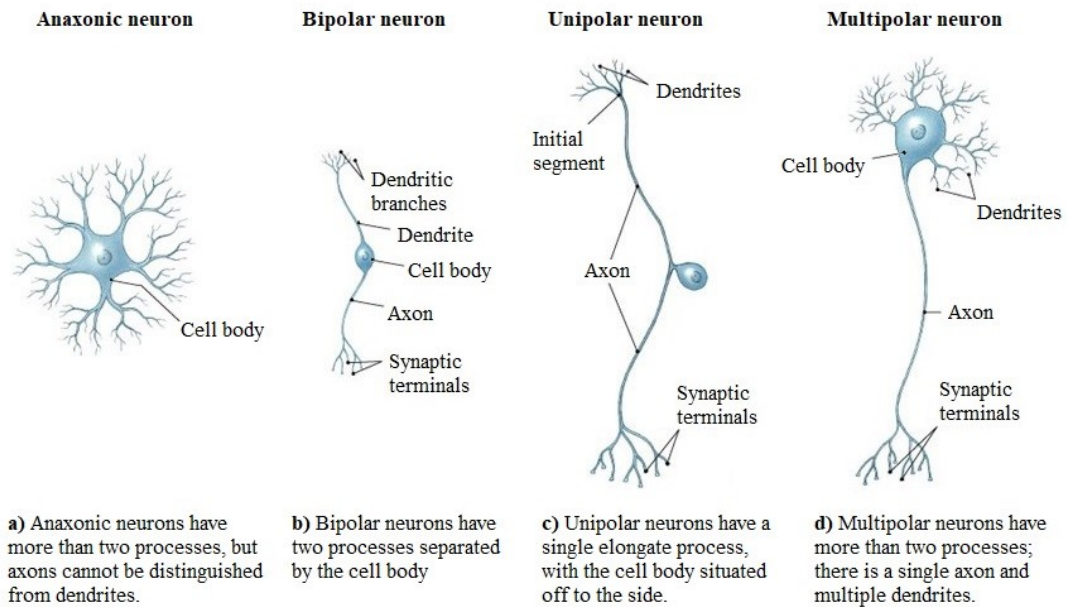


Figure 1.5. Structural classification of neurons. a) Anaxonic neuron, b) bipolar neuron, c) unipolar neuron, d) multipolar neuron. Adapted from Martini et al., 2012. Copyright 2012 Pearson Education, Inc.

Neurons are classified according to their function as (i) sensory neurons, (ii) motor neurons, and (iii) interneurons (Figure 1.6). Sensory receptors pass on the information regarding external or internal changes in the form of electrical impulses through the sensory (afferent) neurons to the CNS, where they are interpreted as a sensation. Sensory neurons are mostly unipolar neurons with processes (known as afferent fibers) extending between a sensory receptor and the CNS (Figure 1.7). Sensory neurons that relay information from the receptors in skin, skeletal muscles, and joints are called somatic sensory neurons, and those that relay information from the receptors in internal organs are called visceral sensory neurons (Scanlon and Sanders, 2018).

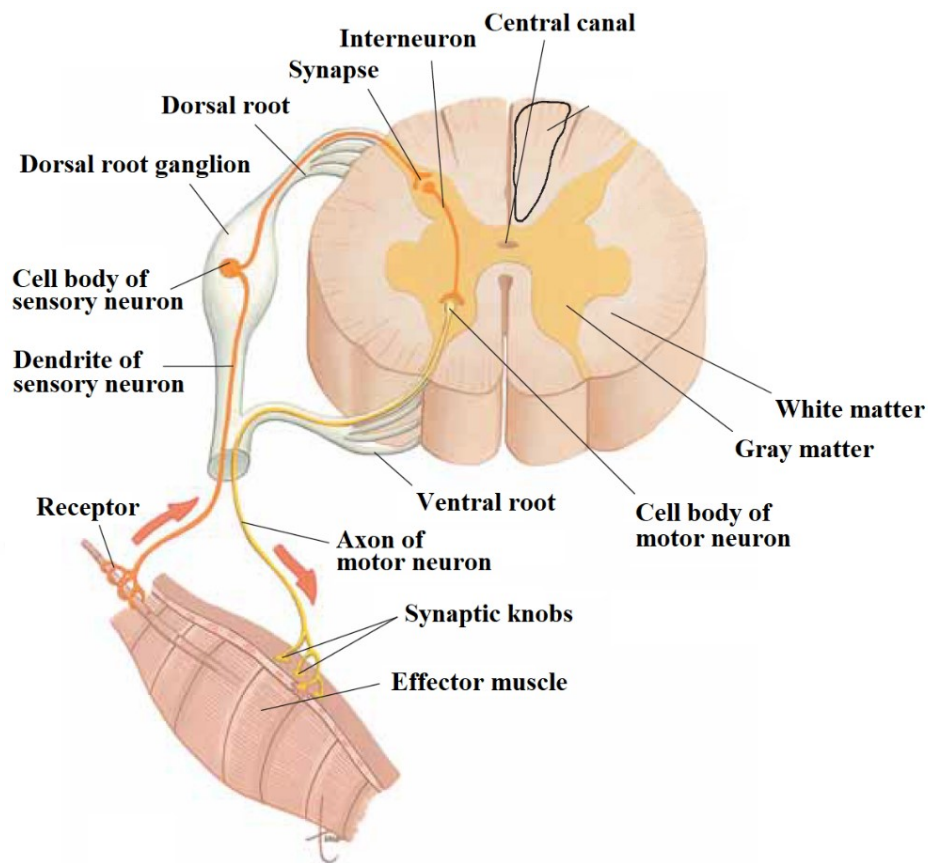


Figure 1.6. Cross-section of the spinal cord and the three types of neurons classified according to their function. Spinal nerve roots and their neurons (left), spinal nerve tracts in the white matter (right). All tracts and nerves are bilateral (located on both sides). Adapted from Scanlon and Sanders, 2018. Copyright 2018 F.A. Davis Company.

Motor (efferent) neurons carry information originating from the CNS to the peripheral effectors in a peripheral tissue, organ, or system (Figure 1.7). Muscles and glands are the two types of peripheral effectors. Muscles contract or relax and glands secrete chemicals in response to neural impulses. The axons that extend from the CNS to the peripheral effectors are called efferent fibers. Somatic nervous system (SNS) and autonomous nervous system (ANS) are the two efferent subdivisions of the PNS. Somatic motor neurons found in SNS originate from the CNS and extend to the neuromuscular junctions of the skeletal muscles. These neurons control the voluntary movement. Neurons of ANS, called the visceral motor neurons, regulate the

involuntary activities such as respiration, and heart rate. They innervate the smooth muscle, cardiac muscle, glands and adipose tissue; all peripheral effectors apart from the skeletal muscles. (Martini et al., 2012).

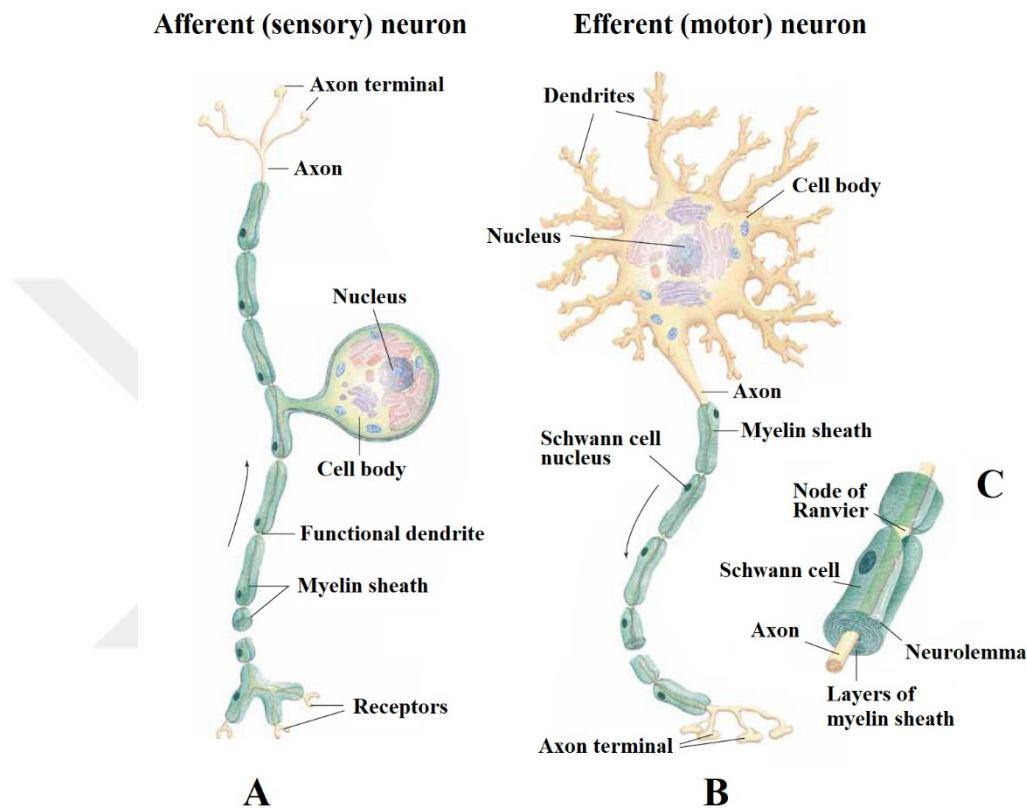


Figure 1.7. Structure of afferent (sensory) and efferent (motor) neurons. A) A typical sensory neuron, B) a typical motor neuron, C) details of the myelin sheath, nodes of Ranvier, and neurilemma formed by Schwann cells. Adapted from Scanlon and Sanders, 2018. Copyright 2018 F.A. Davis Company.

Interneurons (association neurons) are the most abundant of all three neuron types. Found mostly within the brain and the spinal cord, these neurons distribute the sensory information, monitor the motor responses, and are also involved in learning, thinking, memory, and planning. Interneurons are located between sensory and motor neurons, increasing in number in direct proportion to the level of complexity of the response to a certain stimulus (Scanlon and Sanders, 2018).

1.1.3.2. Neuroglia (Glial Cells)

Glial cells are more abundant in the neural tissue than the neural cells and are of various anatomy and functionality (Barha et al., 2016). Glial cells of the central nervous system (CNS) and the peripheral nervous system (PNS) differ, resulting in a different organization of neural tissue in the two systems (Martini et al., 2012). CNS also includes a larger variety of neuroglia than PNS (Figure 1.8).

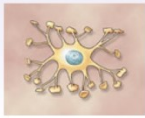
Neuroglia					
Central Nervous System				Peripheral Nervous System	
Ependymal cells	Astrocytes	Oligodendrocytes	Microglia	Satellite cells	Schwann cells
Line ventricles (brain) and central canal (spinal cord); assist in producing, circulating, and monitoring cerebrospinal fluid	Maintain blood–brain barrier; provide structural support; regulate ion, nutrient, and dissolved-gas concentrations; absorb and recycle neurotransmitters; form scar tissue after injury	Myelinate CNS axons; provide structural framework	Remove cell debris, wastes, and pathogens by phagocytosis	Surround neuron cell bodies in ganglia; regulate O ₂ , CO ₂ , nutrient, and neurotransmitter levels around neurons in ganglia	Surround all axons in PNS; responsible for myelination of peripheral axons; participate in repair process after injury
					

Figure 1.8. Neuroglia in CNS and PNS. Adapted from Martini et al., 2012. Copyright 2012 Pearson Education, Inc.

1.1.3.2.1. Glial Cells of the Central Nervous System (CNS)

Central nervous system (CNS) possesses four types of neuroglia: (i) astrocytes, (ii) oligodendrocytes, (iii) ependymal cells, and (iv) microglia (Scanlon and Sanders, 2018). Astrocytes are the most abundant and the largest neuroglia in the CNS (von Bernhardt et al, 2016). Cytoplasmic extensions of astrocytes encompass the endothelial cells that form the blood-brain barrier (BBB) by lining the CNS capillaries in brain. These astrocytes secrete chemicals that are responsible for regulation of the permeability of these endothelial cells, subsequently preventing hormones, amino acids, and any potentially detrimental waste products present in the blood from

diffusing into the brain tissue by isolating the CNS from the general diffusion. Another feature of the astrocytes is performing structural repairs in the event of a neural injury. They form a special type of scar tissue called “the glial scar” that fills the injury site, closing any gaps in the neural tissue. Interstitial fluid composition is also monitored by astrocytes (Martini et al., 2012). Oligodendrocytes have smaller cell bodies and fewer processes compared to astrocytes, and the processes that are in contact with axons expand their plasma membrane to a flattened thin pad, insulating the axons from the interstitial fluid. This wrapping of plasma membrane is called myelin, serving as an electrical insulator which enhances the propagation of action potential 10 to 100-fold along the axon it envelopes (Barha et al., 2016). Segments of several axons can be myelinated by a single oligodendrocyte, and the large myelinated segments of the axons are called internodes. The few micrometer long gaps between the internodes are called nodes of Ranvier, and an axon’s collaterals originate at these nodes. Ependymal cells line the central canal of the spinal cord and ventricles of brain, forming an epithelium called the ependyma (Martini et al., 2012). Ependymal cells in ventricles have cilia, which contribute to the circulation of the cerebrospinal fluid (CSF). In few parts of the brain, specialized ependymal cells secrete the CSF. Composition of the CFS is also monitored by the ependymal cells. Ependyma also contain stem cells that can generate additional neurons. Microglia are the smallest and fewest of CNS neuroglia, originating from the mesodermal stem cells that give rise to monocytes and macrophages (Barha et al., 2016). They constantly move through neural tissue, cleaning cellular debris, waste products, and pathogens through phagocytosis.

1.1.3.2.2. Glial Cells of the Peripheral Nervous System (PNS)

The two types of neuroglia present in the peripheral nervous system (PNS) are satellite cells (amphicytes), and Schwann cells (neurilemma cells). The processes of these neuroglia insulate the neuronal cell bodies and axons in the PNS from their immediate environment. Satellite cells surround the cell bodies in ganglia, the cluster of neural cell bodies in PNS. The function of satellite cells is similar to that of the astrocytes, their counterpart in CNS; they regulate the neuronal environment. Schwann cells, the

PNS counterpart of oligodendrocytes, are responsible for the myelin sheath wrapped around peripheral axons, insulating them from the interstitial fluid. Unlike the oligodendrocytes, a Schwann cell can myelinate only one segment of a single axon, requiring a series of Schwann cells to envelop an axon along its whole length. They can, however, enclose multiple segments of several unmyelinated axons. The spaces between adjacent Schwann cells are also called nodes of Ranvier. The outer surface of a Schwann cell covering an axon is called a neurilemma. If a peripheral nerve is transected and reattached, the axons and dendrites capable of regeneration extend through the tunnels formed by neurilemmas (Scanlon and Sanders, 2018). Schwann cells are responsible for the synthesis and secretion of growth factors that stimulate this regeneration. Due to the branched anatomy of the axons, nerve ends may not fully restore their precise connections, resulting with a partial recovery of sensation and movement.

1.2. Peripheral Nervous System (PNS) Injury and Cell Responses

Peripheral nerve injury is a common and critical health problem that affects the quality of life due to the loss of motor abilities in distal limbs, exposing the patient to an immense social and economic burden (Wang et al., 2017). The causes of neural trauma include penetrating trauma and subsequent transection, traction and compression, ischemia, electrocution and vibration injuries (Pangopoulos et al., 2017). Traction and transection related injuries to the upper extremities are the most common traumas in the civilian setting, while explosion or gunshot wounds are more frequently encountered in warfare or hostile settings. Seddon (1943) developed a classification system to categorize different histologies of peripheral nerve injury that depicted neurapraxia, axonotmesis, and neurotmesis. Sunderland (1951) expanded Seddon's classification into five categories by further dividing axonotmesis into three separate grades, obtaining a total of five grades, the last two requiring surgical intervention (Table 1.1).

Table 1.1. *Sunderland's grading system for peripheral nerve injury.*

Grade	Description
Grade I	- Neurapraxia: Temporary loss of sensory and motor function due to nerve conduction blockage - Focal demyelination without axonal degradation - Secondary to mild injury such as ischemia, compression, or toxicity
Grade II	- Axonotmesis: The axon, along with the myelin sheath, is disrupted irreversibly while neuronal stroma (endoneurium, perineurium, and epineurium) remains intact - Distal nerve undergoes Wallerian degeneration - Secondary to crush, over-stretching, and vibration injuries
Grade III	- Loss of axonal and endoneurial continuity
Grade IV	- Loss of axonal, endoneurial, and perineurial continuity
Grade V	- Neurotmesis: The axon, myelin sheath, and neuronal stroma are all damaged irreversibly - Secondary to severe lesions such as laceration, percussion or, neurotoxicity

These classifications are based on histology of specific injury models; however, most nerve lesions exhibit mixed pathology. Therefore, a 6th degree of nerve injury has been proposed to define this mixed pathology (Sullivan et al., 2016).

Peripheral nerve injury triggers a complex and tightly regulated sequence of degenerative and regenerative events focused on removal of the damaged tissue debris and induction of repair processes (Figure 1.9). Following axonotmesis, the proximal nerve end swells but damage is minimal with respect to the distal nerve end which undergoes Wallerian (anterograde) degeneration, a calcium-mediated protease activity named after its discoverer, August Waller. Wallerian degeneration is triggered by the granular fragmentation of the axon cytoskeleton and the myelin sheath of the distal end within the first few hours of injury. Neurotubules and neurofilaments are disorganized, subsequently affecting axonal contour which becomes irregular due to debris-induced swelling. This swelling is followed by chromatolysis (dissolution of Nissl bodies, granules of rough endoplasmic reticulum and ribosomes), and the migration of the nucleus towards the periphery of the cell body. Macrophages that

migrate to the injury site clear myelin and other debris to reinstate the regenerative capacity of axons as myelin-associated glycoprotein (MAG) inhibits the process. After phagocytotic activity, Schwann cells downregulate myelin-specific protein expression, align and form bands of Büngner, then start synthesizing and secreting signaling molecules that induce axon regrowth at the distal end. Reinnervation rate is very slow, approximating to 1-2 mm/day, thus severe injuries take months to repair and regain function in sensory and motor neurons.

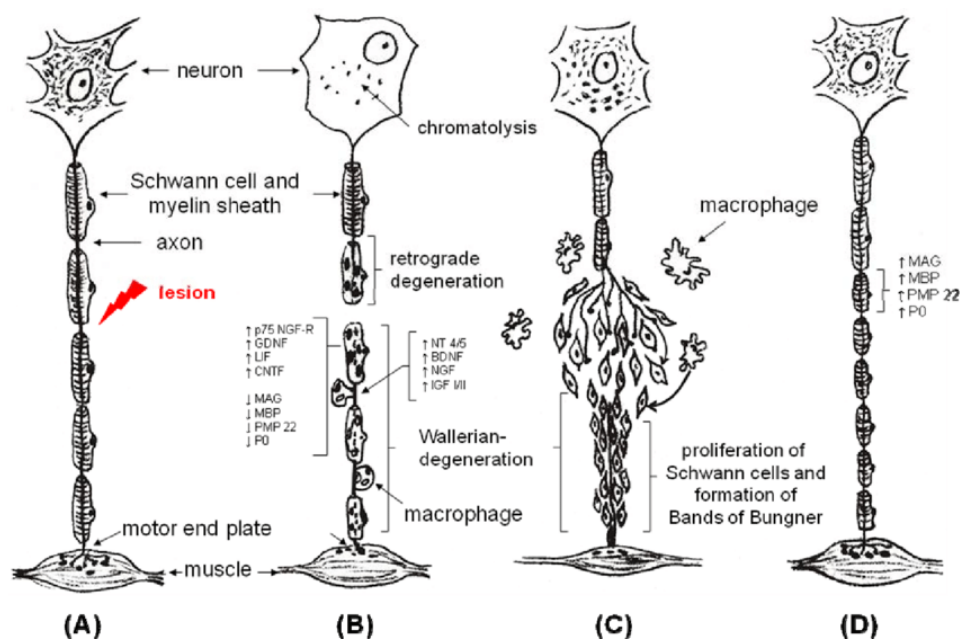


Figure 1.9. Injury to regeneration sequence in PNS. (A) State of the nerve prior to lesion, (B) initiation of retrograde and anterograde Wallerian degeneration upon injury and upregulation or downregulation of certain factors in Schwann cells switched to "myelinating" type, (C) invasion of macrophages clearing the cell debris, Schwann cell proliferation, and formation of bands of Büngner, (D) successful reinnervation upon guidance of axonal growth cones towards target muscle, remyelination of regenerated axons by Schwann cells, switching of Schwann cells to "non-myelinating" type and upregulation of certain factors. Adapted from Fansa and Keilhoff, 2003.

Scar tissue formation is a serious inhibitor of axonal growth cone migration in peripheral nerve injuries. Expression and secretion of inhibitory molecules such as myelin-associated glycoprotein (MAG) and chondroitin sulfate proteoglycans

(CSPGs) by macrophages and glial cells are upregulated surrounding and distal to the trauma site upon injury. CSPGs are significant contributors to axonal growth cone inhibition and regenerative failure in both CNS and PNS injuries. Abundant and rapidly accumulated at the injured distal endoneurial tissue, CSPGs act as chemorepellents to the axonal growth cones present at the proximal end, compelling the growth cones to retract themselves, resulting in a failed reinnervation. Zuo and colleagues reported first the inhibitory effect of CSPGs to neurite outgrowth and then the improvement of neurite elongation upon CSPG degradation (Zuo et al., 1998a; Zuo et al., 1998b; Ferguson and Muir, 2000). In their study in 2002, they found *in vivo* application of chondroitinase ABC (ChABC), a bacterial enzyme that acts on chondroitin sulfate glycosaminoglycans (CS-GAGs) of CSPGs, improved axonal regeneration in Sprague-Dawley rats. It was shown in later animal studies that ChABC was indeed promoting axonal sprouting across nerve coaptations (Graham et al., 2007; Lee et al., 2010) and was an important regeneration enhancing biomolecule that could be used in peripheral nerve repair treatments.

1.3. Tissue Engineering

With the publication of their research on design, fabrication and *in vivo* application of cell seeded, biodegradable, polymeric scaffolds, Dr. Robert Langer and Dr. Joseph Vacanti introduced for the first time the concept of “tissue engineering” (Vacanti et al., 1988). Later, in a paper published in *Science* they gave a definition to the concept as “an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” (Langer and Vacanti, 1993). Tissue engineering was based on the idea that a biocompatible and biodegradable scaffold that houses cells (preferably the patients’ own) and releases therapeutic agents (i.e. antimicrobials and growth factors) would aid and enhance the healing process by first integrating with the host tissue, inducing and providing a foundation for regeneration, then slowly eroding while being replaced with the newly formed tissue as wound healing process proceeded (Hasirci and Hasirci, 2018). This approach was superior to the conventional

methods of grafting and transplantation, because it lacked their serious drawbacks such as poor host or donor tissue availability, destructive immune response, and tissue rejection. Furthermore, tissue engineering surpasses cell-based therapies by providing the three-dimensional macro and microarchitecture of the native tissue critically required for proper regeneration (Zhang and Zhang, 2015).

As mentioned above, in order to realize a significant restoration and recovery of function, a tissue engineered construct should consist of three essential components: (i) the scaffold, (ii) the cells, and (iii) the bioactive compound(s) (Hasirci and Hasirci, 2018). The scaffold (or cell carrier) serves as a foundation for cells and tissues for attachment and growth, essentially mimicking the native extracellular matrix (ECM) whose integrity is compromised upon injury (Khorshidi et al., 2016). Scaffolds can be fabricated using natural or synthetic materials, or their blends to reproduce the intrinsic features of the native ECM (Asghari et al., 2017). The cells seeded on the scaffold either aid the host cells during healing process by releasing growth factors that induce tissue regeneration and revascularization or are actively involved in the regeneration process themselves. Their origin may be the patient themselves or a suitable donor, isolated either as fully differentiated cells or stem cells that can be manipulated to differentiate towards a desired lineage. The bioactive compounds may support cell attachment to the scaffold (e.g. integrin binding motifs), promote proliferation or differentiation (e.g. growth factors), guide cell migration to a target site (e.g. chemoattractants), or simply aid in the sterilization of the injury site (e.g. antimicrobials) (Harrison et al., 2014).

The process of fabrication of a tissue engineered construct begins with the isolation of cells either from the host or a donor, following culturing in an appropriate environment to increase cell number and/or induce desired function. The cells (along with bioactive agents) are then seeded onto a biocompatible, biodegradable scaffold with appropriate macro and microarchitecture and mechanical properties that would allow satisfactory cell adhesion and nutrient exchange. The final construct is either implanted

immediately or allowed to develop to a more mature state for days or weeks prior to implantation (Figure 1.10).

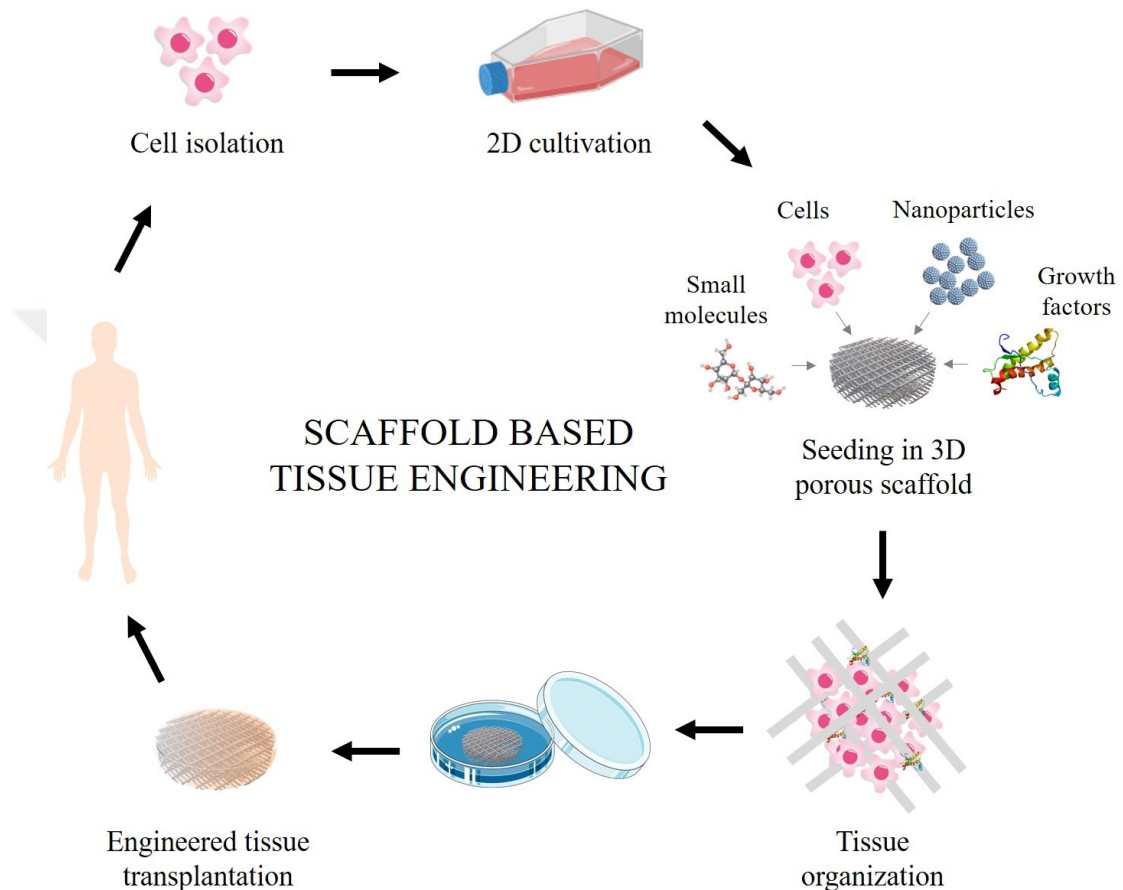


Figure 1.10. Scaffold based tissue engineering.

1.3.1. Scaffolds in Tissue Engineering

The ideal tissue scaffold demonstrates the intrinsic properties of the native tissue or the autograft as they are the foundation for cell attachment and growth in regeneration process (Hasirci and Hasirci, 2018). Thus, the physical and chemical form of the scaffold is crucial for recreating the ideal microenvironment. An ideal scaffold must first and foremost demonstrate biocompatibility, meaning the material from which the

scaffold was produced (and if degradable, its byproducts) must not show any adverse effects such as cytotoxicity, genotoxicity, hemotoxicity or carcinogenicity in the host. If required in the application, an ideal scaffold should exhibit a controlled rate of degradation that corresponds to the rate of regeneration. This harmony between scaffold erosion and new tissue formation allows the host tissue to slowly take over the injury site throughout the healing process, eliminating the need for another invasive procedure to remove the implant. To well accommodate the cells, the scaffold should possess high porosity, adequate pore size (200-300 μm), and good pore interconnectivity that allows cell penetration and tissue ingrowth, proper cell attachment, and sufficient flow of nutrients, growth factors, and waste products through the depths of the construct. The surface chemistry should also allow for or aid in cell attachment. Mechanical properties of the scaffold should match or closely represent that of the native tissue to promote growth and differentiation of cells. Moreover, the scaffold should maintain these physical, chemical and mechanical properties, without losing integrity, until the host tissue takes over.

1.3.1.1. Scaffold Types and Conventional Production Methods

The physical form of the scaffold depends on the type of tissue engineering application, driven by the tissue requirements. For example, in an application that requires cellular alignment, a fibrous scaffold with uniform aligned fibers would be preferred over a sponge with nonuniform porous architecture. Fibrous and sponge scaffolds for tissue engineering can be produced using different fabrication methods.

1.3.1.1.1. Fibrous Scaffolds

The most commonly used conventional techniques to produce fibrous scaffolds are electrospinning, wet spinning, and hot melt extrusion.

1.3.1.1.1.1. Electrospinning

Electrospinning is the production of nano or microfibers by pumping a polymer solution through a small diameter, metallic, and charged nozzle towards an oppositely

charged collector (Khorshidi et al., 2016). The three components of an electrospinning system are: (i) a high voltage power supply, (ii) a spinneret with an electrically conductive nozzle, (iii) and an electrically conductive collector. When a potential is applied to the tip of the nozzle and to the collector, the electrostatic force that forms within the polymer droplet at the tip overcomes the surface tension, resulting in a liquid jet to spray out. This charged polymer jet then undergoes a bending instability due to the electrical field formed between the nozzle and the collector, getting elongated and thinned as it is rapidly whipped in loops while the solvent it is dissolved in evaporates. The nano or microfibers deposited onto the collector (a static plate or a revolving drum or disc) forms a nonwoven fiber mat that can be used in the desired tissue engineering application. Depending on the solvent evaporation rate, the fibers could fuse into a lace-like network.

The drawbacks of this method is the poor cell penetration due to the narrow gaps between the fibers and thickness of the resultant mat (Kishan and Cosgriff-Hernandes, 2017), mechanical instabilities that arise from increased pore sizes and porosities (Kim, 2008), and limitation in geometry that does not allow a controlled 3D configuration (Ngadiman et al., 2017).

1.3.1.1.2. Wet Spinning

In wet spinning technique, a polymer solution is extruded from the nozzle of a spinneret directly into a coagulation bath where polymer fibers are formed due to chemical reaction or diffusion of polymer solvent to the coagulation solution (DeFrates et al., 2018). A scaffold is obtained by removing the fiber material from coagulation bath and drying. In this technique, fiber morphology and orientation is again affected by the properties of the polymer solution (i.e. concentration), the wet spinning setup (i.e. flow rate, nozzle diameter, and composition of coagulation solution), and the environmental factors (drying conditions).

The disadvantage compared to electrospinning is that wet spinning only produces microfibers with diameters in the range of 10-100 μm . Similar to electrospun

scaffolds, wet spun scaffolds do not allow for precise control of 3D microarchitecture (Alagoz et al., 2018).

1.3.1.1.3. Hot Melt Extrusion

In this method, a thermoplastic polymer is heated to a temperature between its glass transition temperature (T_g) and its melting point (T_m) where a decrease in viscosity allows for forced extrusion through a small nozzle resulting with fiber with the diameter of the nozzle. Pharmaceutical ingredients, plasticizers and surfactants, and polymers with high glass transition and melting points can be processed with this method (Repka et al., 2018).

1.3.1.1.2. Sponge Scaffolds

Sponge type tissue engineering scaffolds are conventionally produced by solvent casting, freeze drying (lyophilization), and gas foaming methods.

1.3.1.1.2.1. Solvent Casting

This method includes casting a polymer solution to form a film or mat by letting the solvent completely evaporate (Ye et al., 2018). When the polymer solution is mixed with a porogen insoluble in the solvent prior to casting, a porous structure can be obtained by washing out the porogens. A common porogen used in this method is sodium chloride, which can be dissolved simply by immersing the film in distilled water. The pore sizes can be controlled with the shape, dimensions, and the amount of porogen used.

While this method offers ease of performing and low cost, it is not scalable due to the large amounts of organic solvents required. The solvents used may also adversely affect the functionality of bioactive compounds during entrapment in production of controlled delivery systems or enzyme carrying membranes. Poor interconnectivity of pores is another limitation of this method.

1.3.1.1.2. Freeze Drying (Lyophilization)

Highly porous scaffolds can be produced using this technique by first freezing a homogeneously mixed polymer solution according to the freezing point of the solvent (generally at -20 or -80 °C) and then eliminating the solvent through freeze-drying by placing the frozen solution into a lyophilizer (Raeisdasteh Hokmabad et al., 2017). Phase separation occurs under rapid cooling and the solvent is sublimated under vacuum, resulting with a structure with micropores. The porosity of freeze-dried scaffolds vary between 90-99% with pore sizes ranging between 20-200 μm .

Although freeze-drying method is advantageous over solvent casting particle leaching method with the elimination of porogen use, it creates smaller sized pores and has a closed pore structure. Use of organic solvents, similar to the solvent casting technique limits its use in tissue engineering applications where functionality of bioactive compounds is expected.

1.3.1.2. Additive Manufacturing or Three-Dimensional (3D) Printing

3D printing, otherwise known as additive manufacturing (AM) or rapid prototyping (RP) is a layer-by-layer fabrication process which generates structures of precise geometry and microarchitecture (Tamay et al., 2019). The technique replaces the randomized material deposition or pore formation experienced with conventional scaffold production methods, allowing design and creation of high complexity structures where many parameters such as dimension of details, distance, orientation, and even composition is under rigorous control of the operator.

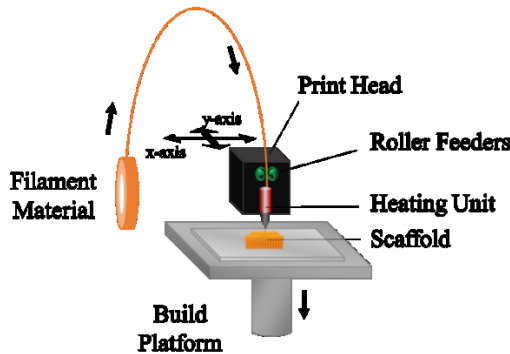
There are three main 3D printing categories that allow the use of solid and liquid polymers for scaffold fabrication: (i) fused deposition modeling (FDM), (ii) selective laser sintering (SLS), and stereolithography (SLA).

Fused deposition modeling (FDM) is the most commonly utilized 3D printing technique in various industrial areas including biomedical industry. The technique is based on melting a thermoplastic polymer in a heating chamber (cartridge) then

extruding the molten polymer through a nozzle onto a platform in fiber form, in a layer-by-layer fashion. The nozzle moves in x-y direction controlled by a computer and deposits the fiber in the predefined orientation. The nozzle is then moved in the z direction to a predefined height to print another layer (Figure 1.11). The precision and resolution is dependent on the printing parameters such as nozzle diameter, polymer viscosity, feed rate, and the angle, distance, orientation and number of fiber layers. FDM allows the use of multiple materials simultaneously, using several cartridges, to produce structures with multiple properties in each or subsequent layers. It is ideal for tissue engineering and especially for skin where different layers possess different mechanical properties and cellular composition. Another advantage of FDM is that it is free of organic solvents which makes them more biocompatible. The main disadvantage of FDM in tissue engineering applications is the limited range of biocompatible and biodegradable medical grade thermoplastic polymers as printing materials.

Selective laser sintering (SLS) technique that melts a thin layer of polymer or metal powder using a laser to create a 3D form. After a layer is finished, the platform moves down one-layer thickness and another layer of powder material is laid for the laser to fuse (Figure 1.11). SLS is superior to FDM in terms of resolution, precision and complexity because of the fineness of the powder. It is another solvent-free technique like FDM, although not suitable for biological compound or cell integration during printing as these would be harmed due to the high energy laser exposure.

A) Fused Deposition Modeling



B) Selective Laser Sintering

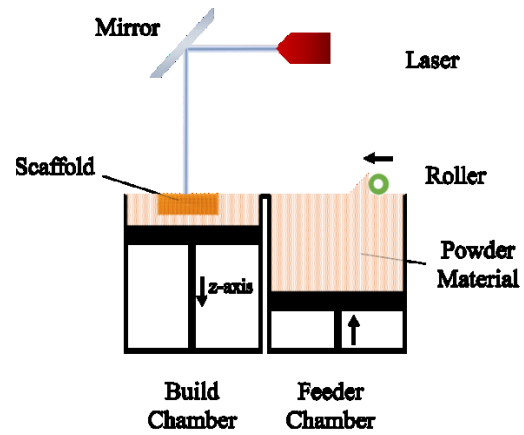


Figure 1.11. Schemes of (A) FDM and (B) SLS techniques. Adapted from Tamay et al., 2019.

Stereolithography (SLA) method is based on crosslinking of polymer resins using a light source such as UV or visible light. The narrow beam of light travels in the x-y platform, selectively polymerizing the resin on a platform that moves up in the z-axis a layer height after each layer is finished (Figure 1.12). When the whole structure is completed, it is washed extensively to remove uncrosslinked resin, and subjected to post curing with heat and/or light to finalize the polymerization reactions, ultimately increasing the stability of the product and decreasing its cytotoxicity.

SLA is superior to FDM in terms of the number of products that can be built simultaneously. The total print time of a single product does not differ significantly from 50; the printing time is only dependent on the height of the structure as every layer is built simultaneously. SLA is more refined in resolution and precision, being able to produce details as small as 5 microns (the width of the laser beam). The drawbacks of the technique are the limited number and range of biocompatible materials and large quantities of resin required to fill the container.

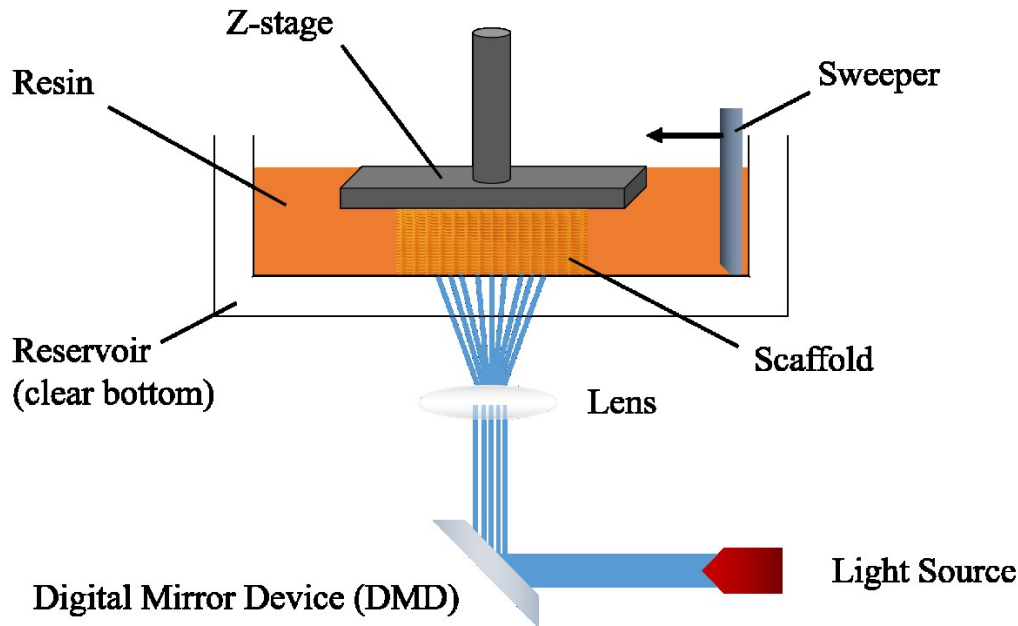


Figure 1.12. Scheme of SLA technique. Adapted from Tamay et al., 2019.

1.3.1.3. Materials Used in Scaffold Production

The biomaterials used in scaffold production could be of natural or synthetic origin, and preferably bioresorbable (Hasirci and Hasirci, 2018). As mentioned in section 1.3.1, scaffold is expected to gradually erode while the newly formed tissue takes its place, thus, it is important that its byproducts do not cause any adverse effect in the host system.

Natural polymers used in tissue engineering can be microbial, animal, insect, or plant-based. Some examples of these polymers used in tissue engineering applications are hyaluronic acid (HA), chitosan, chondroitin sulfate, collagen, gelatin, silk fibroin, alginate, agar-agarose, xanthan, cellulose, starch, and dextran (Bakhshandeh et al., 2017). Most natural polymers have some limitations such as low mechanical strength and integrity, and difficulty in processing. In order to improve the mechanical properties, they are modified with crosslinkable side groups or used as blends with synthetic polymers or inorganic compounds.

Synthetic polymers utilized in tissue engineering applications are always condensation polymers such as polyesters, polylactides, polyhydroxyalkanoates (PHAs), polyaminoacids, polyamides, and polyurethanes. Some examples frequently used in the biomedical field are poly(lactic acid) (PLA), poly(ϵ -caprolactone) (PCL), poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), poly(3-hydroxybutyrate) (P3HB), poly(3-hydroxyvalerate) (P3HV), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). Ceramics and biodegradable metals such as sintered bioglass powder and magnesium are, although rarely, used in tissue engineering applications. Ceramics such as hydroxyapatite (HAp) and tricalcium phosphate (TCP) are used as blends with synthetic or natural polymers as mechanical property enhancers or cell attachment and calcium deposition enhancers in bone tissue engineering, especially because the main mineral in the bone is very similar to hydroxyapatite, a calcium phosphate.

1.3.2. Cells Types in Tissue Engineering

Cells are the second essential component of tissue engineered products. Fully differentiated primary cells, xenogeneic cells, or stem cells can be used in tissue engineering depending on the application and the target tissue.

1.3.2.1. Primary Cells

These cells are fully differentiated, mature cells that have been isolated from donors. If the cells are isolated from the patient, they are called autologous cells. The advantage of using autologous cells is that there is no risk of an immune response, rejection, or disease transmission. However, harvesting creates a new injury, discomfort, risk of infection, and cost. Limited availability of tissue at the donor site is another disadvantage of using these cells. Allogenic cells are isolated from another human donor. Their use eliminates the discomfort and infection risk that an autologous tissue causes, but immune response, rejection and disease transmission are highly probable.

1.3.2.2. Xenogeneic Cells

Xenogeneic cells are isolated from non-human species. They are generally used in *in vitro* testing or off-the-shelf tissue engineering products as the risk of transmission of known and unknown disease pathogens, immune response, and rejection is very high. If immunosuppressives are used, the host system weakens and becomes more susceptible to pathogens. Moreover, it is difficult to maintain the growth and function of cells in the host system. As such, the use of these cells in tissue engineering applications are not preferred.

1.3.2.3. Stem Cells

Stem cells are unspecialized cells capable of differentiating into a variety of lineages when exposed to specific mechanical, chemical or topological cues (Zhang et al., 2013). They have the potential to grow organs or whole organisms, or act as an intrinsic regeneration system. Stem cells are able to divide without limit to form additional stem cells or differentiate into a specialized cell and assume a specific function. One of the stem cell sources in the body is the bone marrow, which produces bone marrow stem cells (BMSC); the progenitors of bone, cartilage, and skeletal tissue cells. The three main types of stem cells are: (i) totipotent, (ii) pluripotent, and (iii) multipotent. Totipotent stem cells are the first four cells of the embryonic stage, capable of differentiating into any cell type in the body. Pluripotent stem cells have the capability of differentiating into any type of cell in the body except those of the placenta. Embryonic stem cells (ESCs) are pluripotent stem cells that form the primary germ layers of the embryo (i.e. endoderm, ectoderm, and mesoderm). Multipotent stem cells are adult or somatic stem cells that have limited differentiation capability, and are found in the bone marrow, brain, and liver. Mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) are examples of multipotent stem cells.

1.3.3. Growth Factors in Tissue Engineering

Growth factors are the third main component of a tissue engineered product. They are soluble and diffusible polypeptides involved in signaling processes that regulate cell

survival, division, migration, and differentiation. They function as growth enhancers and inhibitors, chemoattractants, apoptotic initiators, and angiogenic factors. Some examples of growth factors used in tissue engineering are listed in Table 1.2.

Table 1.2. *Growth factors used in tissue engineering applications.*

Growth Factor	Abbreviation	Function
Fibroblast Growth Factor	FGF	Angiogenesis and embryonic development
Epidermal Growth Factor	EGF	Epithelial cell growth, proliferation, and differentiation
Nerve Growth Factor	NGF	Neuron survival and proliferation
Transforming Growth Factor- β	TGF- β	Osteoblast proliferation and differentiation, inhibition of epithelial cell proliferation
Vascular Endothelial Growth Factor	VEGF	Endothelial cell survival, migration, and proliferation
Platelet-Derived Growth Factor	PDGF	Embryonic development, proliferation, migration, endothelial cell growth

1.4. Nerve Tissue Engineering

1.4.1. Treatment Approaches for Peripheral Nervous System

PNS is intrinsically capable of regenerating its components, with the regeneration process being related with the age of the patient, the mechanism of injury, the extent of the damage, and most importantly the proximity of the injury to the nerve cell body (Faroni et al., 2015). For example, injury to the distal nerves in fingers result in loss of sensation of the fingertips and will regenerate well, while transection injury to the proximal brachial plexus in the neck impairs the sensation of the whole hand, hinders motor function and causes the patient to frequent pain and cold intolerance. Endogenous regeneration is possible for traction or crush injuries where the nerve remains in continuity, but it is limited to 12-18 months due to loss of neuromuscular junction endplates and fibrosis of the muscle (Sullivan et al., 2016; Kubiak et al., 2018).

Transection injuries, however, require surgical intervention to bridge the severed nerve ends. Neurorrhaphy, direct end-to-end matching of nerve ends with tension-free microsurgical epineurial suturing, is the gold standard of surgical treatment. However, this type of treatment is only possible with clean-cut transection injuries with minimal neural tissue loss and lowest possible gap between the nerve ends to ensure tension free suturing (Noaman, 2012; Pangopoulos et al., 2017). Although there are certain estimates in the literature for the maximal length of the nerve gap that can be treated via neurorrhaphy, they vary in their conclusions. Zachary (1954) argued that median and ulnar nerves could be sutured if the gap is 7 to 9 cm, while Wilgis (1982) cautioned against suturing gaps greater than 4 cm. Millesi (2000), on the other hand, regarded suturing of nerve ends with gaps greater than 2.5 mm extensive. Regardless, an ideal time span for end-to-end nerve repair is within 3 days with an injury zone that exhibits good vascularization and soft tissue availability. The advantage of these strict limits is that it is possible to perform intraoperative nerve stimulation, optimization of motor nerve recovery, and adequate exposure to and mobilization of nerve ends without the interference of any scar tissue (Wang, et al., 2017). Also, in this time period, the nerve ends still preserve their neurotransmitters, and symmetrical juxtapositioning of nerve fascicles require less effort than it would at later stages where Schwann cell proliferation, fibrosis, and angiogenesis significantly hinder the matching process.

1.4.1.1. Autologous Nerve Graft

Autologous nerve grafts are regarded as the gold standard with nerve transection injuries that have an end to end gap which does not allow tension-free neurorrhaphy (Faroni et al., 2015). The drawback of this approach is that it sacrifices a healthy segment of patient's nerves from another site, which is also in limited supply, and harvesting requires additional surgical intervention further distressing the patient. In addition, post-surgical complications such as donor site morbidity, neuroma formation (swelling and scar tissue development due to abnormal proliferation of proximal-end axons), and axonal misdirection (reinnervation of inappropriate sensory and motor

targets) further decreases the chances of a full recovery of motor function (Lee and Wolfe, 2000).

1.4.1.2. Allografts

Allografts have been studied as alternatives to autografts. They provide the patient with nerve tissue for reconstruction without creating any additional injuries on the patients themselves (Palispis and Gupta, 2017). However, allografts are likely to cause immune response and rejection in the host, which likely results in fibrosis and scarring, further complicating regeneration by blocking the regenerative path. Immunosuppressive treatment prior to the use of allografts decrease the likelihood of such negative responses with the cost of a weakened host immune system susceptible to any pathogen. Moreover, immunosuppressive agent use is linked with tumor formation. As pathogen transmission is another risk of using allografts, the method proves undesirable (Sedaghati et al., 2014).

1.4.1.3. Nerve Guides

In order to improve functional outcomes, nerve guides constructed of biocompatible materials of biological and synthetic origin, therapeutic agents (i.e. growth factors and chemoattractant molecules), and various cell types have been engineered by scientists and clinicians. Nerve guides (or nerve conduits) are tubular structures designed specifically to bridge otherwise untreatable gaps via neurorrhaphy and autografting, while also improving the outcome compared to these two treatment approaches. The advantage of nerve conduits over the gold standard grafts is based on a few key factors. Confining the proximal and distal nerve ends to a limited area in their lumen, nerve conduits prevent the abnormal proliferation and branching of regenerating proximal nerve ends. The resulting regenerative chamber also allows the integration of physical or chemical cues that provide directionality for the regenerating proximal nerves, subsequently aiding alignment of proximal and distal nerve ends or regenerating proximal axon with the target tissue. Control over the local tissue microenvironment is also improved due to the confinement, where an enhancement of neurotrophic factor

function is observed as a direct result of increase in local concentration (Wang et al., 2017).

An ideal nerve guide possesses properties such as biocompatibility, biodegradability, appropriate flexibility, high porosity, compliance, neuroinductivity and neuroconductivity, and mechanical stability (Arslantunali et al., 2014). The inner lumen of nerve guide can be designed to house a gel matrix and internal guidance elements such as aligned fibers and channels. The outer shell should be porous enough to allow nutrient and waste product exchange in order not to create a necrotic or hypoxic core. Outer shell may also house cells or bioactive molecules that would enhance axonal regeneration.

There are a number of FDA approved, commercially available nerve guides in the market that use a variety of materials such as collagen, poly(vinyl alcohol, and poly(glycolic acid) in their design (Table 1.3).

Table 1.3. *FDA approved commercially available nerve guides. Adapted from Kehoe et al., 2012.*

Nerve Guide	Material	Degradation (months)	Length (cm)	Company
Neurotube [®]	Poly(glycolic acid)	3	2-4	Synovis1 Micro Companies Alliance Inc.,
Neuragen [®]	Type I collagen	36-48	2-3	Integra Life Sciences Corp.,
Neurolac [®]	Poly(DL-lactide-ε- caprolactone)	16	3	Polyganics B.V.,
Neuroflex [™]	Type I collagen	4-8	2.5	Collagen Matrix Inc.,
NeuroMatrix [™]	Type I collagen	4-8	2.5	Collagen Matrix Inc.
AxoGuard [™] Nerve Connector	Porcine small intestinal submucosa (SIS)	3	0.1	Cook Biotech Products,
SaluTunnel [™] Nerve Protector	Poly(vinyl alcohol) (PVA)	Non- absorbable	6.35	Salumedica [™] L.C.C.

1.5. Aim of the Study

This study aims to design and test the efficacy of a nerve guide containing topographical cues and chemical axon elongation enhancers. The capillary channels constructed within the hydrogel inner matrix provide a regeneration pathway that aligns the proximal and distal nerve ends to promote successful reinnervation. In order to prevent hindrance on the function of axonal growth cones due to the presence of chondroitin sulfate proteoglycans at the distal end of the transected nerve, the enzyme chondroitinase ABC is released from the exterior of the NG from an alginate hydrogel matrix.

1.6. Novelty of the Study

There are many types of nerve conduits and treatment applications for peripheral nerve injury. In order to achieve a successful regeneration, both topographical and chemical cue integration in NG design is of great importance (Chiono and Tonda-Turo, 2015). Topographical cues have been used in NGs in many forms such as aligned nanofibers (Yucel et al., 2010) or internal channels (Sun et al., 2012). To mimic the fascicular anatomy of the peripheral nerve, a tubular NG filled with a GelMA hydrogel matrix that has microchannels was designed in this study. Chemical cues for axon regeneration include cell adhesion peptides such as RGD-motif of ECM proteins (Jenkins et al., 2015; Qian et al., 2018), or growth factors and enzymes that enhance axonal elongation such as NGF and chondroitinase ABC (Lin et al., 2016; Sivak et al., 2017; Oh et al., 2018). Zuo and colleagues published a paper in 2002 on the enhancement of nerve transection repair upon degradation of chondroitin sulfate proteoglycans present at the distal end using chondroitinase ABC enzyme (Zuo et al., 2002). Therefore, chondroitinase ABC was integrated into this study as a bioactive compound that promotes axonal regeneration. To the best of our knowledge, there is no study combining topographical cues and scar tissue dissolving enzymes in their design. In this study capillary channels formed in GelMA matrix were tested for their

effectiveness of maintaining cell viability and promoting migration as well as the extent of sustained release of chondroitinase ABC.



CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

L929 mouse fibroblast cell line and PC12 (adherent) mouse adrenal gland pheochromocytoma cell line were purchased from ATCC (USA). Dulbecco's Modified Eagle's Medium (DMEM) high glucose with L-glutamine and phenol red, penicillin/streptomycin, and F12 (HAM'S) nutrient mixture with L-glutamine were purchased from Biological Industries (USA). Donor horse serum (HS), fetal bovine serum (FBS), and goat serum (GS) were obtained from Biowest (France). Primary antibodies for β III-Tubulin (mouse) and NeuN (mouse) were obtained from Cell Signaling Technology (USA). Alamar Blue, Alexa Fluor 488 (phalloidin, and goat anti-mouse), Alexa Fluor 532-phalloidin, and Live/Dead viability/cytotoxicity kit for mammalian cells were purchased from Invitrogen Inc. (USA). Dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), Sodium Hydroxide (NaOH), and DMEM high glucose with sodium bicarbonate without phenol red were purchased from Merck Millipore (Germany). Bovine serum albumin (BSA), chondroitinase ABC, DAPI (4',6-diamidino-2-phenylindole), hydrochloric acid (HCl), Irgacure 2959 (2-(Hydroxyl)-4-(2-hydroxyethoxy)-2-methylpropiophenone), nerve growth factor (NGF), and type A porcine skin gelatin (~90-110 Bloom) were obtained from Sigma-Aldrich (USA). 4% paraformaldehyde (PFA) was purchased from Santa-Cruz (USA). L-glutamine was purchased from Lonza (Sweden). Poly(dimethyl siloxane) (PDMS) and PDMS pre-polymer were obtained from Dow Corning (USA). SnakeSkin dialysis tubing was obtained from ThermoFisher Scientific (USA). Dental Surgical Guide (Dental SG) was purchased from Formlabs (USA). Collagenase Type II was obtained from GIBCO (USA).

2.2. Methods

2.2.1. Methacrylation of Gelatin

Methacrylation of gelatin was done following the protocol of (Shirahama et al., 2016). Briefly, 20 g of type A porcine skin gelatin (~70-100 Bloom) was dissolved in 100 mL CB buffer (pH 9, 0.25 M) at 60 °C and pH was adjusted to 9 using 5M NaOH or 6M HCl. Subsequently, 2 mL methacrylic anhydride (MAA, 94%) was added at 50 °C at a MAA/gelatin feed rate of 0.1 mL/g. The reaction was allowed to proceed for 3 h with magnetic stirring. pH was readjusted to 7.4 to stop the reaction. Solution was filtered and dialyzed against distilled water for 7 days at 37 °C to remove unreacted methacrylic acid. The resultant solution was lyophilized (Labconco Freezone 6, USA), weighed, and stored at 4 °C until further use.

2.2.2. Analysis of Methacrylation Degree of GelMA

In order to determine the degree of Methacrylation (DM) of gelatin, proton nuclear magnetic resonance (¹H-NMR) method was used. Gelatin and lyophilized gelatin methacrylate (GelMA) were dissolved in deuterium oxide (D₂O) (30 mg/mL) at 40 °C. Bruker DPX 400 spectrometer was used to obtain ¹H-NMR spectra at ¹H resonance frequency of 400 MHz. Sixteen scans were conducted to average the signal-to-noise ratio.

2.2.3. Preparation of GelMA Hydrogel Slabs

To create hydrogel slabs from GelMA, first a silicone mold was prepared by coating the base of a 48-well plate with poly(dimethylsiloxane) (PDMS). Briefly, PDMS prepolymer was homogeneously mixed with the catalyst, degassed in a vacuum oven to remove bubbles, poured in wells of a 48-well plate in equal amounts of 500 µL, and cured at 70 °C for 3 hours. In order to prepare GelMA hydrogels, GelMA solutions with varying GelMA concentrations (7.5, 10 and 15% GelMA w/v) were prepared in PBS (pH 7.4, 10 mM) containing 1% (w/v) Irgacure 2959 photoinitiator. 200 µL of each solution was transferred to the PDMS coated wells and exposed to UV (365 nm,

20 J/cm²) for 1.5 minutes in a UV curing cabinet (BIO-LINK™ UV Crosslinker DLX-365, Germany). Both the top and bottom of the gel slabs were exposed to UV to achieve sufficient crosslinking.

2.2.4. Preparation of Alginate Hydrogel Slabs

Alginate hydrogel solutions with varying alginic acid concentrations (0.5, 1, and 2% w/v) was prepared in PBS (pH 7.4, 10 mM) to create hydrogel slabs. 200 µL of each solution was transferred to a PDMS coated 48-well plate and left to set at 4 °C. 5% (w/v) calcium chloride (CaCl₂) solution was prepared in distilled water (dH₂O) using 6.67 g of calcium chloride dihydrate (CaCl₂·2H₂O). Alginate hydrogels were then crosslinked by immersion in CaCl₂ solution for 15 min.

2.2.5. Characterization of Hydrogels

2.2.5.1. Equilibrium Water Content of GelMA Hydrogels

GelMA hydrogel slabs were incubated in PBS at 37 °C for 24 h in a shaking incubator. Wet weigh of hydrogel slabs (W_{wet}) were recorded after gently removing the excess water using filter paper. Hydrogels were immersed in distilled water to remove the salts accumulated in the gel due to PBS immersion, and then lyophilized. Dry weight of lyophilized hydrogels (W_{dry}) were then recorded. Equilibrium water content (EWC, %) was calculated using the following equation:

$$EWC (\%) = \left[1 - \frac{W_{dry}}{W_{wet}} \right] \times 100 \quad (1)$$

2.2.5.2. Degradation of GelMA and Alginate Hydrogels in PBS

GelMA and alginate hydrogel slabs (n=3 and n=6, respectively) were rinsed with distilled water (dH₂O) and lyophilized to record their initial dry weights. Lyophilized slabs were then incubated in PBS (pH 7.4, 10 mM) at 37 °C in a shaking incubator for 14 days. Samples were rinsed with distilled water, lyophilized and weighed on days and 1, 3, 5, 7, and 14. Percent final weights (W_F , %) of the corresponding time points were calculated using the following equation:

$$W_F (\%) = \left[\frac{W_{dry}}{W_0} \right] \times 100 \quad (2)$$

where

W_0 : Initial dry weight of the hydrogel slabs

W_{dry} : Dry weight of samples at each time point after incubation.

2.2.5.3. Enzymatic Degradation of GelMA Hydrogel in Collagenase Type II Solution

Collagenase type II was used to determine the susceptibility of GelMA hydrogels to enzymatic degradation (n=3). Initial wet weights of hydrogel slabs were recorded (W_0), then hydrogels were incubated in varying concentrations of collagenase type II (2.5, 5, and 10 U /mL PBS, pH 7.4, 10 mM) for 4 h. Wet weights of hydrogels (W_{wet}) were determined at 1 h intervals. Degradation (%) was calculated using the following equation.

$$Degradation (\%) = \left[1 - \frac{W_{wet}}{W_0} \right] \times 100 \quad (3)$$

2.2.6. Nerve Guide Preparation

The nerve guide design consisted of a 3D printed tubular exterior with a hydrogel-based core bearing hollow microchannels inside functioning as a topographical guidance cue (Figure 2.1). The distal end of the exterior was filled with an alginate hydrogel matrix for sustained release of chondroitinase ABC to digest the axonal growth inhibitory scar tissue component chondroitin sulfate proteoglycans (CSPGs).

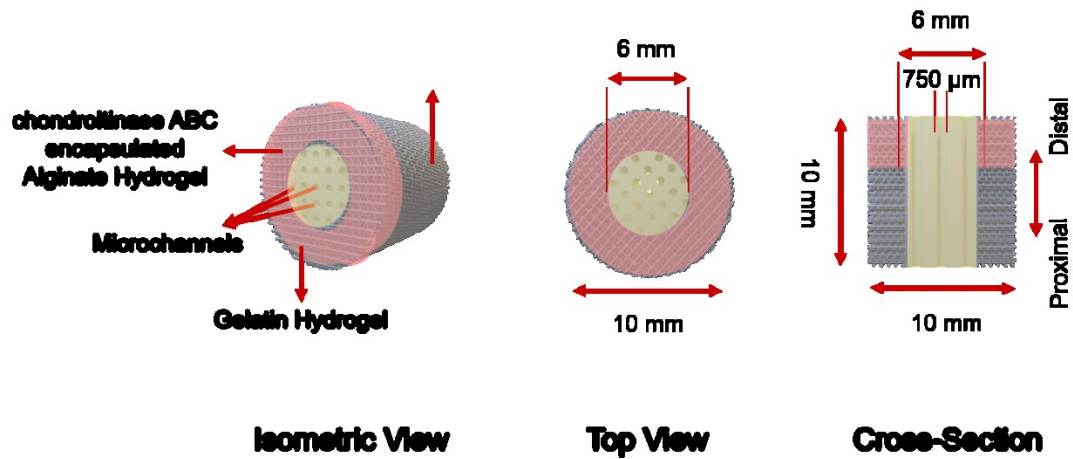
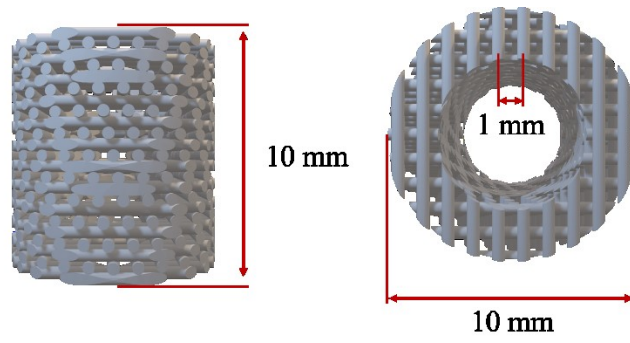


Figure 2.1. Nerve guide design.

2.2.6.1. Preparation of 3D Printed Nerve Guide Exterior

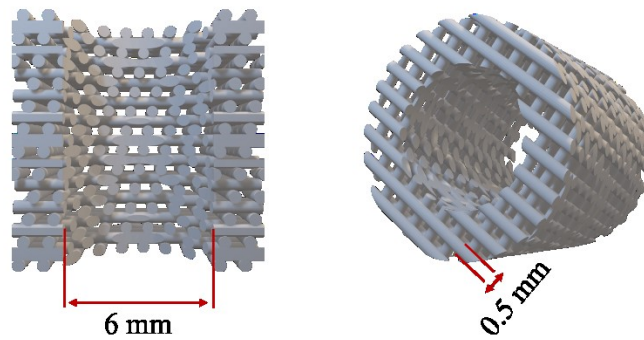
A 3D model of the tubular exterior of the nerve guide (NG) was drawn in Autocad 2019 (Autodesk Inc., USA) for printing. The NG model prepared was 10 mm in height and diameter, had 6 mm inner diameter, and consisted of 500 μm diameter fibers distanced 1 mm from each other in each layer, with a log pile laydown pattern and a layer shift of 200 μm (Figure 2.2). The model was loaded to PreForm software (Formlabs, USA) for pre-print processing and printjob creation for Form 2 printer (Formlabs, USA). Form 2 is a stereolithography (SLA) type 3D printer where a precisely controlled laser cures high-resolution 3D products from liquid photopolymer resins in a layer by layer fashion. NGs were printed with Form 2 SLA printer using an ethoxylated bisphenol A based photocurable resin labeled by its producers as “Dental Surgical Guide (Dental SG)” (Formlabs, USA).

After printing, NGs were immersed twice in 96% isopropanol for 15 min to wash out the unreacted resin remaining in the porous structure. After air drying, constructs were subjected to post curing under UV 395 nm for 8 h at 60 °C in an oven. Post-cured NGs were stored at room temperature under sterile conditions until further use.



SIDE VIEW

TOP VIEW



SIDE VIEW SLICED

ISOMETRIC VIEW

Figure 2.2. 3D model of nerve guide exterior.

2.2.6.2. Preparation of Nerve Guide Channel Mold

3D model of a mold intended to create nerve guidance channels was designed in Autocad 2019 (Autodesk Inc., USA). The mold was 12 mm in diameter with a 10 mm inner diameter that allows for the fitting of NG exterior, with 750 μm diameter holes at the base for glass capillary insertion (Figure 2.3).

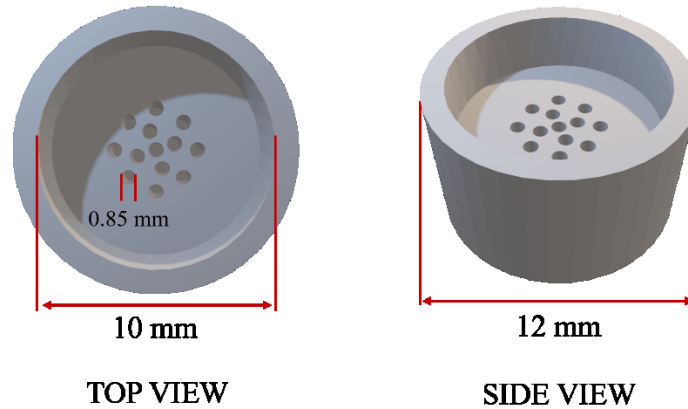


Figure 2.3. Channel mold model that holds glass capillaries.

The mold models were printed with Form 2 SLA printer using Dental SG resin, immersed in 96% isopropanol twice for 15 minutes after printing, and post-cured (365 nm UV exposure for 8 h at 60 °C in an oven). Molds were kept in a 24-well plate at room temperature under sterile conditions until further use.

2.2.6.3. Preparation of Nerve Guide Inner Matrix from GelMA and Channel Production

GelMA (15%, w/v) was dissolved in PBS including 1% w/v Irgacure 2959 at 60 °C and sterilized using 0.45 μ m syringe filters. Glass capillaries were sterilized by autoclaving and inserted into the 3D printed channel molds. NG exteriors were then fitted into the molds and their lumens were filled with the GelMA solution prepared. GelMA was allowed to set at 4 °C, then the NG exteriors containing GelMA cylinders with microchannels were detached from the channel molds. The GelMA was then crosslinked for 90 s in a UV curing cabinet at 20 J (top and bottom curing, 3 min in total). The resultant NGs were kept in phosphate buffered saline (PBS, pH 7.4, 10 mM) containing 1% (v/v) penicillin/streptomycin solution at 4 °C until further use. The NGs were immersed in complete PC12 media containing 1% (v/v) penicillin/streptomycin solution prior to cell seeding to condition the GelMA matrix.

2.2.6.4. BSA loading of Alginate for Controlled Enzyme Release

The NG exterior was selected to house an alginate hydrogel matrix that would release the enzyme “chondroitinase ABC” (ChABC) which acts on chondroitin sulfate proteoglycans (CSPG) present at the scar tissue site encapsulating the distal end of the transected nerve. For initial release studies, bovine serum albumin (BSA) was used as a model for ChABC. Alginic acid (0.03, 0.06, or 0.12 g) was dissolved in PBS (6 mL, pH 7.4, 10 mM) containing BSA (0.255 g, 4.25% w/v) to prepare 0.5, 1, and 2% (w/v) alginate hydrogel solutions. These solutions were then loaded into a cylindrical PDMS mold with Teflon base (n=6, 200 μ L solution loaded on each sample) and left to set at 4 °C. After setting, alginate hydrogels were crosslinked by immersing in CaCl₂ solution (5%, w/v) for 15 min. The final structure was rinsed with PBS (pH 7.4, 10 mM) to remove excess CaCl₂, and used in the release studies.

2.2.6.4.1. Release Studies

2.2.6.4.1.1. Drug Release from Alginate Hydrogel Matrix

0.5, 1, and 2% alginate hydrogel solutions were prepared in 6 mL PBS (10 mM, pH 7.4). 0.255 g BSA was mixed into these solutions yielding 8.5 mg BSA in 200 μ L aliquots to be loaded into the NG exterior. 200 μ L of hydrogels were loaded into cylindrical PDMS mold with Teflon base and left to set at 4 °C. The hydrogels were then immersed in CaCl₂ solution at room temperature for 15 min for gelation.

BSA laden alginate hydrogels were incubated in PBS at 37 °C (n=6) in 24 well-plates. Absorbance values of PBS collected from the well plates at 1, 2, 3, 4, 5, 6, 12, and 24h of incubation were recorded at 280 nm in a spectrophotometer, and the amount of BSA released was calculated using the previously generated BSA absorbance calibration curve.

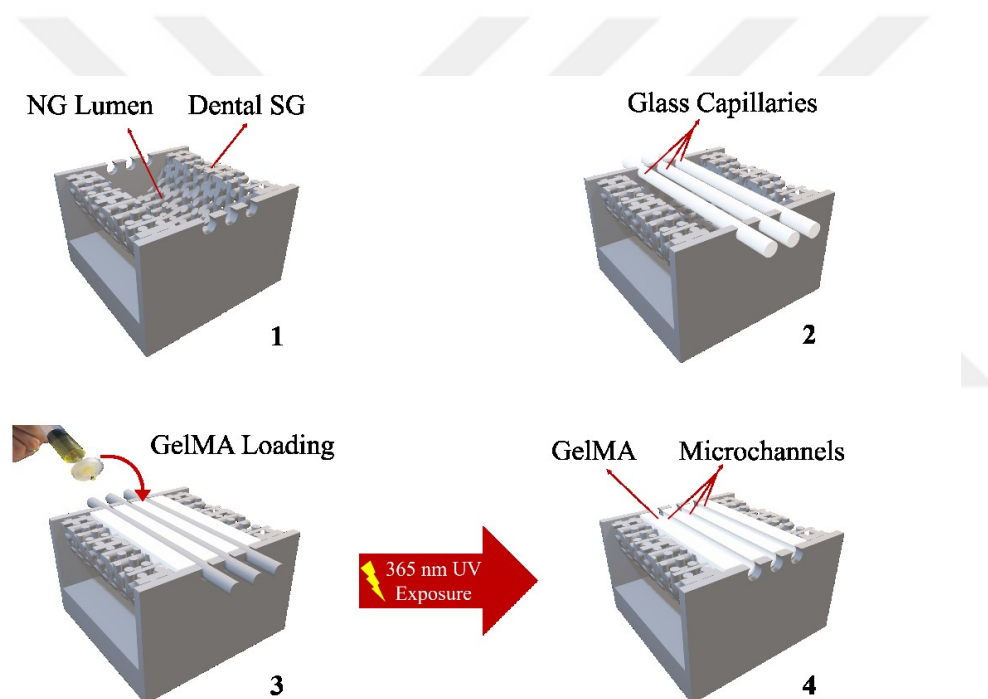
2.2.6.5. Preparation of Vertical and Horizontal Nerve Guide Setup and Sterilization

Two NG setups were prepared in this study: (i) a Vertical Setup which consisted of the whole NG structure with microchannels and alginate matrix loaded with ChABC, and (ii) a Horizontal Setup which consisted of a half cylinder cut along the axis of the whole NG, produced for better visualization of cell behavior under microscope. Components of the NGs were produced and sterilized individually, stored and assembled under sterile conditions to construct both setups.

The vertical setup consisted of a cylinder with 10 mm height, 10 mm OD, 6 mm ID, and 2 mm wall thickness (Figure 2.1). This tubular exterior was fit to a mold that contained glass capillaries in order to create microchannels along the axis of the cylinder. Both the tubular exterior and the mold were printed with Form 2 SLA printer using commercially available Dental SG resin. After printing, both structures were subjected to post-curing as previously explained and sterilized under UV (365 nm) in a sterile laminar flow cabin for 15 min. GelMA hydrogel solution was syringe filtered (0.45 μ m) and poured inside the tube. After cooling at 4 °C, the mold was removed and NGs were exposed to UV for 3 min (1.5 min top and bottom) in a curing cabinet (BIO-LINK™ UV Crosslinker DLX-365, Germany) to crosslink the GelMA hydrogels. NGs were stored at 4 °C in PBS (pH 7.4, 10 mM) with 1% (v/v) penicillin/streptomycin solution until further use. Prior to cell seeding, NGs were immersed in PC12 complete media containing 1% (v/v) penicillin/streptomycin solution to condition GelMA hydrogel matrix.

The horizontal setup of the microchannelled nerve guide was designed to be able to efficiently observe cell behavior in microchannels under microscope. The setup consisted of a horizontally sliced nerve guide fitted inside a stand that would hold three glass capillaries just above the surface of the NG inner lumen that would be filled with GelMA hydrogel (Figure 2.4). These models were printed with Form 2 SLA printer using Dental SG resin. After printing, NGs were subjected to post-curing as

previously explained and sterilized under UV (365 nm) in a sterile laminar flow cabin for 15 min. GelMA hydrogel solution was sterilized using a syringe filter (0.45 μm). Glass capillaries were fitted to the NGs and their lumens were filled with GelMA solution. After setting GelMA at 4 °C, glass capillaries were removed, and NGs were exposed to UV for 1.5 min in a curing cabinet (BIO-LINK™ UV Crosslinker DLX-365, Germany) to crosslink the GelMA hydrogels. NGs were stored at 4 °C in PBS (pH 7.4, 10 mM) with 1% (v/v) penicillin/streptomycin solution until further use. Prior to cell seeding, NGs were immersed in PC12 complete media containing 1% (v/v) penicillin/streptomycin solution to condition GelMA hydrogel matrix.



HORIZONTAL NERVE GUIDE SETUP

Figure 2.4. Preparation of horizontal nerve guide.

2.2.7. Nerve Guide Characterization

2.2.7.1. Nerve Guide Dimensions

2.2.7.1.1. Stereomicroscopy Analysis

NGs were observed under a stereomicroscope (Nikon SMZ 1900, Japan) and NG height, diameter, and inner lumen diameters were determined using the micrographs and NIH Image J software (USA).

2.2.7.1.2. Scanning Electron Microscopy (SEM) Analysis

NG exteriors sliced vertically and horizontally and coated with gold under vacuum were studied with a scanning electron microscope (SEC, Mini-SEM, South Korea). Micrographs obtained from SEM were processed with NIH Image J software to determine the fiber diameter and fiber distance values of NGs.

2.2.7.2. Nerve Guide Mechanical Properties

Compression testing is a technique used in determining the mechanical properties of materials including hydrogels. Briefly, the material or a product made of the material is placed between two plates and compressed under a constant pressure until a predetermined extent of strain is achieved. Using the pressure applied to a unit area of the material and the extent of material compression, the mechanical properties can be calculated. This compression test of a viscoelastic material yields a stress strain plot that shows an elastic region where the slope gives the Elastic Modulus value (Figure 2.5).

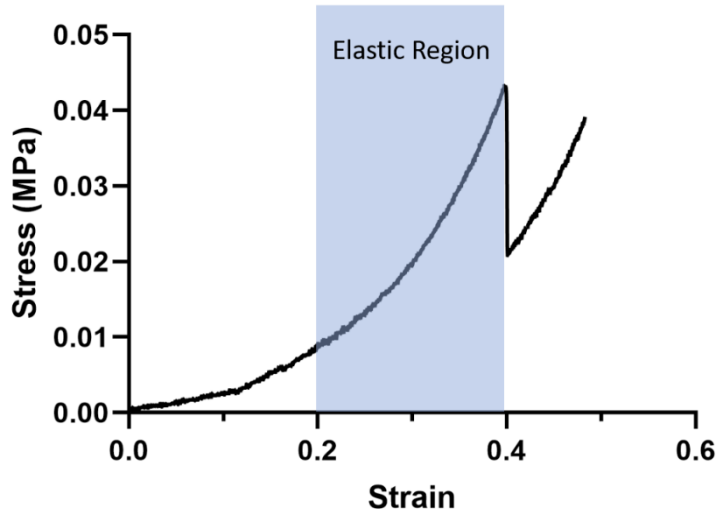


Figure 2.5. Stress versus strain plot for GelMA15. Elastic region is highlighted in blue.

The Elastic Modulus (E) or Young's Modulus is an intrinsic property of the material that enables the users compare materials quantitatively. The stiffer the material, the higher is E , and the lower is the strain under an applied load. Elastic Modulus is calculated from the stress and strain values obtained after a compressive testing. Stress is defined as the force applied to a unit area and is represented with the following formula:

$$\text{Stress } (\sigma) = \frac{\text{Force (N)}}{\text{Area (m}^2\text{)}} \quad (4)$$

The SI unit for stress is N/m^2 or Pascal (Pa)

Strain on the other hand is the change in the measured and original length of the sample while a constant load is being applied. Strain value is obtained using the following formula:

$$\text{Strain } (\epsilon) = \frac{\text{Length Measured (m)}}{\text{Original Length (m)}} \quad (5)$$

Strain is a unitless value and may sometimes be expressed as the percent deformation ($\epsilon\%$).

The relationship between Elastic Modulus, Stress, and Strain is explained analogous to the Hooke's Law for springs:

$$F = -kx \quad (6)$$

where F is the force applied, k is the Spring constant, and x is the displacement.

When applied to viscoelastic materials, which behave like an ideal spring, the formula becomes:

$$\sigma = -E\epsilon \quad (7)$$

where σ is stress, ϵ is strain, and E is the Elastic Modulus.

2.2.7.2.1. Mechanical Properties of GelMA

GelMA slabs prepared were tested mechanically under compression with a 10 N load cell (Shimadzu AGS-X, Japan) at a displacement rate of 2 mm/min at room temperature until a strain of 80% is achieved. Compressive moduli of the slabs were calculated from the resulting Stress-Strain plot.

2.2.7.2.2. Mechanical Properties of Dental SG

NG exteriors printed with Form 2 SLA printer from Dental SG resin were subjected to compressive testing a 200 N Load Cell at a displacement rate of 2 mm/min at room temperature until a strain of 80% is achieved. Compressive moduli of the NG exteriors were calculated from the resulting Stress-Strain plot.

2.2.7.3. Cytotoxicity Testing of Nerve Guide Exterior Material Dental SG Resin

In order to evaluate the cytocompatibility of Dental SG for *in vitro* and *in vivo* use, indirect and direct cytotoxicity tests were performed with Alamar Blue cell viability assay.

For direct cytotoxicity testing 5x5x2 mm meshes with 140 μm fiber diameter, 1 mm fiber distance, and 200 μm layer shift were printed using Form 2 SLA printer. The meshes were post cured by exposure to UV 365 nm at 60 $^{\circ}\text{C}$ in an oven as explained previously. For sterilization, the meshes were immersed in 70% (v/v) ethanol followed by UV 365 nm exposure in laminar flow cabinet. Mouse fibroblast cell line L929 was suspended by trypsinization and diluted to a concentration of 1×10^6 cells/mL in DMEM-high glucose tissue culture medium containing 1% (w/v) penicillin (100 U/mL)/streptomycin (100 $\mu\text{g}/\text{mL}$) solution, and 10% FBS. 50 μL aliquots of the cell suspension were seeded onto the meshes so that a cell density of 5×10^4 cells/scaffold was achieved. The cell seeded meshes were incubated under standard conditions (37 $^{\circ}\text{C}$, 5% CO_2) for 4 h to achieve sufficient cell attachment. Fresh medium (DMEM-high glucose containing 1% (v/v) penicillin (100 U/mL)/streptomycin (100 $\mu\text{g}/\text{mL}$) solution, and 10% FBS) was then added onto the meshes in a 24-well plate and incubation was continued for 3 weeks while replacing the medium every 2 days. Cell number was determined via Alamar Blue Cell Viability Assay on days 1, 3, 14 and 21.

For indirect cytotoxicity testing, Dental SG structures printed for horizontal NG setup were sterilized by immersion in 70% v/v ethanol followed by UV 365 nm exposure, then immersed in PC12 complete media (HAM'S F12 media supplemented with 15% donor horse serum, 5% fetal bovine serum, 50 ng/mL NGF, and 1% (w/v) penicillin (100 U/mL)/streptomycin (100 $\mu\text{g}/\text{mL}$) solution) for 1 day. This extract was then used for indirect cytotoxicity. PC12 cells were suspended by trypsinization and diluted to a concentration of 1×10^6 cells/mL in PC12 complete media. 100 μL aliquots of the cell suspension were seeded into the wells of a 24-well plate so that a cell density of 10×10^4 cells/well was achieved. The cell number in wells were quantified with Alamar Blue cell viability assay prior to extract exposure. The cells were then incubated in Dental SG extract for a day, and cell number in wells were quantified again with Alamar Blue cell viability assay.

Alamar Blue, a water soluble, cell permeable, non-toxic, blue-colored weak fluorescent dye called resazurin, is an alternative to the commonly used toxic counterpart MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] cell viability reagent. Resazurin is in oxidized form and upon internalization is reduced in the cytosol by mitochondrial enzyme activity. The redox reaction results in a change in color visible in the culture medium from indigo blue (resazurin) to fluorescent pink (resorufin), proportional to the aerobic respiration activity. By proportioning the change in UV-visible absorbance or fluorescence intensity of the dye in culture medium measured by a UV-visible spectrophotometer or a fluorimeter to cell number, cell viability in a culture can be quantified.

Cells seeded on Dental SG meshes and their unseeded counterparts were washed twice with DMEM-high glucose colorless tissue culture medium. Then the samples, positive controls (cell-seeded tissue culture well-plates), and negative controls (unseeded tissue culture well-plates), were incubated in 1 mL of Alamar Blue solution (10% in DMEM-high glucose colorless with 1% (v/v) penicillin (100 U/mL)/streptomycin (100 µg/mL) solution) at 37 °C for 1 hour. After incubation, 200 µL of supernatant was transferred into 96 well plates (3 technical replicates for each sample). Absorbance values were obtained at 570 and 600 nm. Percent reduction (PR, %) was calculated from absorbance values according to the reagent manufacturer's (Invitrogen Inc., USA) instructions using the following equation:

$$PR (\%) = \frac{((\epsilon_{ox})_{\lambda_2} * A_{\lambda_1}) - ((\epsilon_{ox})_{\lambda_1} * A_{\lambda_2})}{((\epsilon_{red})_{\lambda_1} * A'_{\lambda_2}) - ((\epsilon_{red})_{\lambda_2} * A'_{\lambda_1})} \quad (8)$$

$$\lambda_1 = 570 \text{ nm} \quad \lambda_2 = 600 \text{ nm}$$

$$(\epsilon_{ox})_{\lambda_1} = 117.216 \quad (\epsilon_{ox})_{\lambda_2} = 155.677$$

$$(\epsilon_{red})_{\lambda_1} = 80.586 \quad ((\epsilon_{red})_{\lambda_2} = 14.652$$

where,

A_{λ_1} = Absorbance of the test well at $\lambda_1 = 570 \text{ nm}$

A_{λ_2} = Absorbance of the test well at $\lambda_2 = 600$ nm

A'_{λ_1} = Absorbance of the negative control (blank) or unseeded scaffold at $\lambda_1 = 570$ nm

A'_{λ_2} = Absorbance of the negative control (blank) or unseeded scaffold at $\lambda_2 = 600$ nm

A calibration curve was prepared using known concentrations of cells (PC12 and L929) to relate percent reduction values of the dye to the cell number.

2.2.7.4. Degradation of Dental SG in PBS

Initial dry weights of Dental SG NG exteriors (n=3) were recorded. NG exteriors were then incubated in PBS (pH 7.4, 10 mM) at 37 °C in a shaking incubator for 14 days. Samples were rinsed with distilled water, lyophilized and weighed on days 0, 3, 5, 7, and 14. Percent weights remaining (W_F , %) of the corresponding time points were calculated using Equation 3 given in Section 2.2.5.2..

2.2.8. In Vitro Testing

2.2.8.1. Cell Culture

Rat pheochromocytoma cell line PC12 (passages 5-7, P5-7) and mouse fibroblast cell line L929 were incubated in F12 (HAM's) media supplemented with 15% donor horse serum (HS), 5% fetal bovine serum (FBS), 50 ng/mL nerve growth factor (NGF) and 5% penicillin/streptomycin, and DMEM high glucose media supplemented with 10% FBS and 5% penicillin/streptomycin, respectively, under standard conditions (37 °C, 5% CO₂) until confluency. Cells were passaged once before seeding onto the scaffolds or NGs.

2.2.8.2. Live/Dead Cell Viability Assay

To observe the viability of cells (%) seeded on nerve guide microchannels, Live-Dead cell viability assay was performed on days 1, 3, and 7. Briefly, culture medium was discarded, and samples were washed twice with PBS (pH 7.4, 10 mM). The samples were then double stained with Calcein-AM (2 μ M in PBS) and ethidium

homodimer-1 (4 μ M in PBS) for 20 min at room temperature. After staining, samples were rinsed twice with PBS (pH 7.4, 10 mM) and examined under Zeiss LSM 800 (Germany) confocal laser scanning microscope (CLSM) with z-stack and tiles imaging modules.

2.3. Statistical Analysis

Statistical analyses were performed with GraphPad Prism (v6.01) using Two-way ANOVA or Student's t-test with a minimum confidence level of 95%, where p-values equal to or smaller than 0.05 were considered statistically significant. All values were reported as the mean $\pm$ standard deviation of the mean (s.d.m.) and plotted using GraphPad Prism.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, a nerve guide (NG) with microchannels capable of controlled delivery of scar tissue dissolving chondroitinase ABC (ChABC) enzyme was produced. This was done by 3D printing the exterior of the NG, and methacrylated gelatin (GelMA) hydrogel interior mold with glass capillaries. This study proceeded in four phases: (i) GelMA production by reacting gelatin and methacrylic anhydride, subsequent NMR characterization, and determination of optimum concentration for hydrogel production, (ii) 3D printing of NG exterior using commercial Dental Surgical Guide (Dental SG) resin, and *in vitro* cytotoxicity testing of Dental SG, (iii) preparation of NG by filling the lumen of tubular exterior with GelMA in the presence of capillary tubes to create microchannels and loading with ChABC loaded alginate hydrogel to the distal end of the NG, and finally (iv) *in vitro* testing of NG for cell viability and sustained enzyme release.

3.1. Methacrylated Gelatin (GelMA) Synthesis and Characterization

Methacrylated gelatin (GelMA) was synthesized by reacting methacrylic anhydride (MAA) and type A porcine skin gelatin (~70-100 Bloom) (Section 2.2.1). The resultant GelMA, when dissolved in distilled water, produced a UV photocrosslinkable polymer solution which would crosslink and produce a hydrogel in the presence of a photoinitiator (Irgacure 2959). Crosslinked gel was stable under physiological conditions and provides cells with the optimal conditions for attachment due to the presence of RGD motifs (Bektas and Hasirci, 2018; Pepelanova et al., 2018; Bahcecioglu et al., 2019). In the first phase of the study, GelMA concentration was optimized for NG interior environment.

3.1.1. Determination of Degree of Methacrylation (DM) of GelMA

Degree of Methacrylation (DM) is defined as the ratio of amine groups (lysine, hydroxylysine) in unreacted gelatin to the methacrylate functionalized amine groups in gelatin following the reaction. DM is determined by two methods: (i) Proton nuclear magnetic resonance (^1H -NMR), and (ii) 2,4,6-Trinitrobenzene sulfonic acid (TNBS) assay. In this study, ^1H -NMR method was used to determine the DM of GelMA using MestreNova NMR analysis program (v6.0.2, Mestrelabs Research, Spain) in the calculations.

^1H -NMR spectra was normalized to the phenylalanine signal (6.9-7.5 ppm) corresponding to the concentration of gelatin following integration of lysine methylene signals (2.8-2.95 ppm) of unreacted gelatin and GelMA spectra to calculate the peak areas (A_{Gelatin} and A_{GelMA}). The DM of GelMA was then calculated according to the following equation:

$$DM (\%) = \left(1 - \frac{A_{\text{GelMA}}}{A_{\text{Gelatin}}}\right) \times 100 \quad (9)$$

where,

A_{GelMA} : Peak area of lysine methylene signal of GelMA spectrum

A_{Gelatin} : Peak area of lysine methylene signal of unreacted gelatin spectrum

^1H -NMR results showed that the synthesis method used in this study was able to produce methacrylated gelatin with a DM of 81% (Figure 3.1); this indicates a 81% efficiency, and is in agreement with the results in the literature (Nichol et al., 2010; Yue et al; 2015).

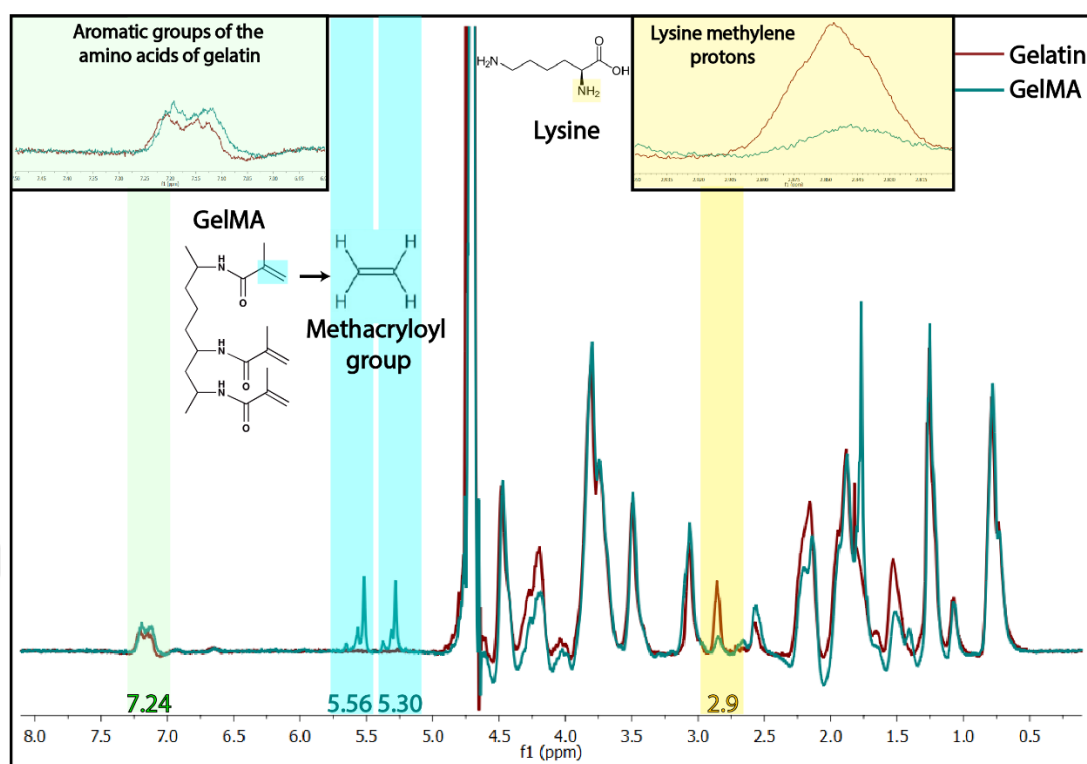


Figure 3.1. ^1H -NMR spectra of gelatin and methacrylated gelatin superimposed. Inserts show the expanded regions between 6.90-7.50 ppm and 2.80-2.95 ppm corresponding to peaks of aromatic groups used in normalization of peaks and methylene protons of methacryloylated lysine groups in GelMA and hydroxyl lysine groups of unmethacryloylated gelatin. The peaks highlighted in blue between 5.30-5.56 ppm region correspond to the acrylic protons of methacryloyl groups.

3.1.2. Equilibrium Water Content of GelMA Hydrogels

Hydrogels are crosslinked 3D networks of hydrophilic polymer chains that can absorb water in amounts thousands of times greater than their own dry weight. They are of great interest for biomaterials and tissue engineering research due to their hydrophilicity, tunable physical and chemical properties, and potential biocompatibility (Zhang and Khademhosseini, 2017). In this study, the effect of methacrylated gelatin concentration on the equilibrium water content was investigated to select an appropriate GelMA concentration for the innertube hydrogel production. GelMA concentrations of 7.5% (GelMA7.5), 10% (GelMA10), and 15% (GelMA15) (in PBS, 10 mM, pH 7.4) were tested for their water absorption capacity. The results

showed that as the uncrosslinked GelMA polymer concentration increased, the water content decreased (Figure 3.2). The crosslink density is highly influenced by the reaction conditions such as the polymer concentration, initiator concentration, duration of crosslinking, which in turn affect the water absorption capacity of the hydrogel produced. The crosslink density is increased with higher concentrations of polymer, tightening the inner 3D polymeric network, subsequently causing the polymer to absorb less water. The results indicate that GelMA15, with the highest GelMA concentration, retains significantly less water than GelMA7.5 and GelMA10. However, GelMA7.5 and GelMA10 do not show a significant difference in their water retention capabilities. The main reason for the low statistical significance of the difference in the water contents originates from the small changes in the methacrylated gelatin concentrations (7.5%, 10%, and 15%).

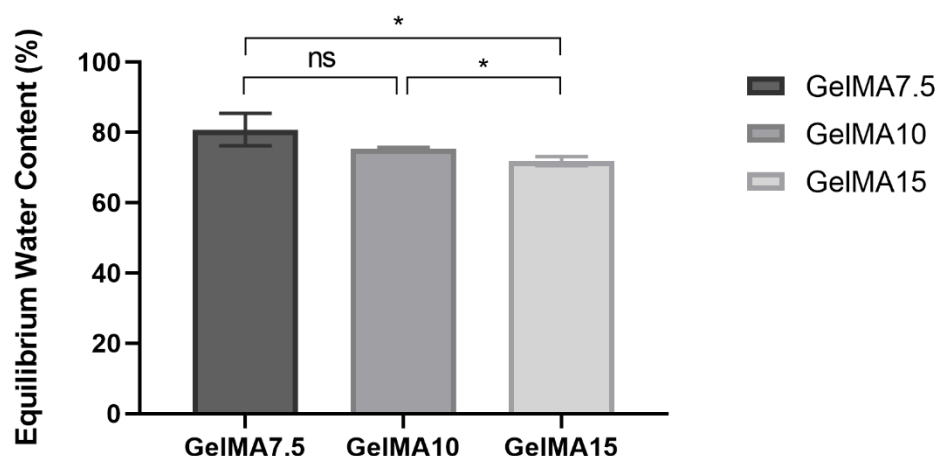


Figure 3.2. Water content of GelMA hydrogels after 24h of incubation in PBS (10 mM, pH 7.4) at 37 °C. GelMA concentrations of 7.5%, 10%, and 15% are abbreviated as GelMA7.5, GelMA10, and GelMA15, respectively. (n=3, ns: not significant, * : $p < 0.05$)

3.1.3. Degradation of GelMA Hydrogels

GelMA hydrogels with different uncrosslinked GelMA polymer concentrations were incubated under physiological conditions in PBS (4mM, pH 7.4) at 37 °C for 14 days. Figure 3.3 shows that GelMA7.5 and GelMA10 showed a greater amount of weight loss (15% and 25% respectively) on the first day of incubation compared to GelMA15, which retained almost 95% of its initial dry weight Figure 3.3. Weight loss of GelMA7.5 and GelMA10 was significantly more than that of GelMA15, especially in the first week. This difference in the extent of degradation is attributed to the difference in the initial polymer concentration of the hydrogels. The greater availability of reactive groups in higher polymer concentrations in a unit volume creates a denser interconnected polymer network, impeding the expansion of the network upon absorption of water into the structure and decreasing the degradation. This result correlates with the low water absorption capability of GelMA15 with respect to GelMA7.5 and GelMA10 (Nichol et al., 2010; Hoch et al., 2012; Zhao et al., 2016).

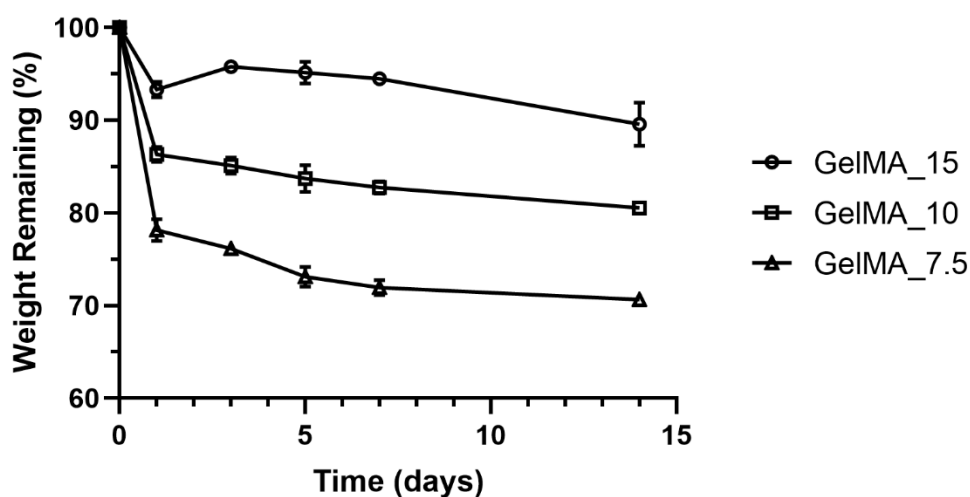


Figure 3.3. Degradation of GelMA hydrogels for 14 days in PBS (10 mM, pH 7.4) at 37 °C (n=3).

3.1.4. Enzymatic Degradation of GelMA Hydrogels in Collagenase Type II

Gelatin is obtained by thermal or chemical partial hydrolysis of collagen and is enzymatically degradable in *in vivo* conditions (Van Vlierberghe et al., 2014; Zhang et al., 2014; Deshmukh et al., 2017). Thus, the stability of GelMA hydrogels under physiological conditions were investigated in this study by incubating GelMA7.5, GelMA10, and GelMA15 in 2.5, 5, and 10U collagenase type II solutions (Figure 3.4).

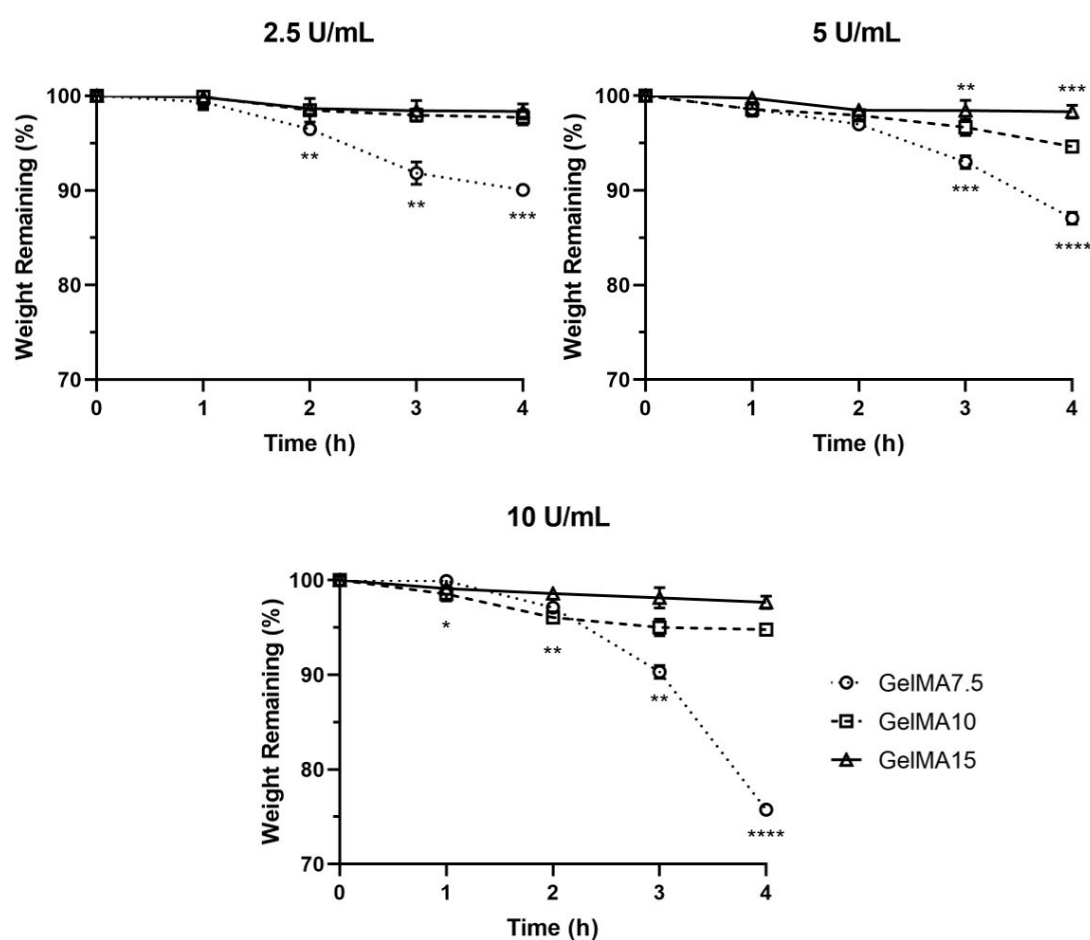


Figure 3.4. Percent weight remaining in GelMA hydrogels as a result of enzymatic degradation using three different collagenase type II concentrations (2.5 U/mL, 5 U/mL, and 10 U/mL). Statistical analyses were performed between samples at each time point (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, and **** : $p < 0.0001$).

Results show that the extent of degradation of GelMA7.5 was significantly greater than GelMA10 and GelMA15, consistent with the results of degradation in PBS. GelMA7.5 lost stiffness substantially in the first hour of incubation for all collagenase type II concentrations, gradually becoming softer as incubation period extended. GelMA10 stiffness was affected within 3 hours of incubation in 10 U/mL collagenase type II, but otherwise was stable. Stiffness of GelMA15 was the least altered by collagenase type II even after 4h of incubation, retaining 97% of its original weight. In conclusion, GelMA15 was the most stable under enzymatic degradation conditions out of the three GelMA concentrations selected. Results are consistent with changes in degradation profile with respect to GelMA concentration reported in literature. Zhao et al. (2016) reported complete degradation of 7.5%, 10%, and 15% GelMA hydrogels after 4, 13 and 21 days of incubation, respectively, in 2 U/mL collagenase solution. Hutson et al. (2011) incubated 10% and 15% GelMA in 2.5 U/mL collagenase type II and reported complete degradation in 24 and 36 hours, respectively. Both the results in this study and the literature indicate a relationship between polymer concentration and extent and rate of degradation where higher concentrations of polymer remain stable for longer periods of time. Presence of enzyme substantially increases degradation; higher concentrations of enzyme degrades hydrogels more rapidly and extensively.

In tissue engineering applications, in most cases, scaffold degradation is a requirement in tandem with the synthesis of new extracellular matrix by local cells during healing process (Hasirci and Hasirci, 2018). In peripheral nerve injuries, the time required for functional recovery can range between weeks and months, thus, producing a stable structure that will support axon growth cones and myelinating Schwann cells is a primary objective in NG design (Griffin et al, 2014; Tezcan, 2017). Accordingly, controlled degradation of the GelMA hydrogel under physiological conditions is an important aspect of this study due to both overall NG stability and the stability of cell seeded microchannels in the GelMA hydrogel. The microchannels were designed as topographical cues for axonal elongation and should hold their shape without

collapsing during the axonal regeneration process. Within the three investigated GelMA concentrations in this study, GelMA15 emerged the most suitable for further implementation with lesser water retention that will prevent alterations in channel dimensions, and greater stability under physiological conditions that will ensure structural integrity throughout the regenerative process.

3.2. Nerve Guide Characterization

The NGs consisted of a stereolithography (SLA) printed 3D cylindrical exterior with microchannelled GelMA matrix filling the inner lumen. Dimensions of the NG exterior and the microchannels were recorded as SEM, CLSM, and stereomicroscopy micrographs. Stability of Dental SG exterior was investigated by gravimetry under *in vitro* conditions and SEM imaging.

3.2.1. Nerve Guide Dimensions

The structure of nerve guide exterior was examined under a scanning electron microscope (SEM) (Figure 3.5). The micrographs show that the NG exterior consists of fibers with diameters of $418 \pm 2 \mu\text{m}$ and distance of $870 \pm 20 \mu\text{m}$. The exterior was 10 mm in height and diameter, with an inner diameter of 6 mm for the lumen.

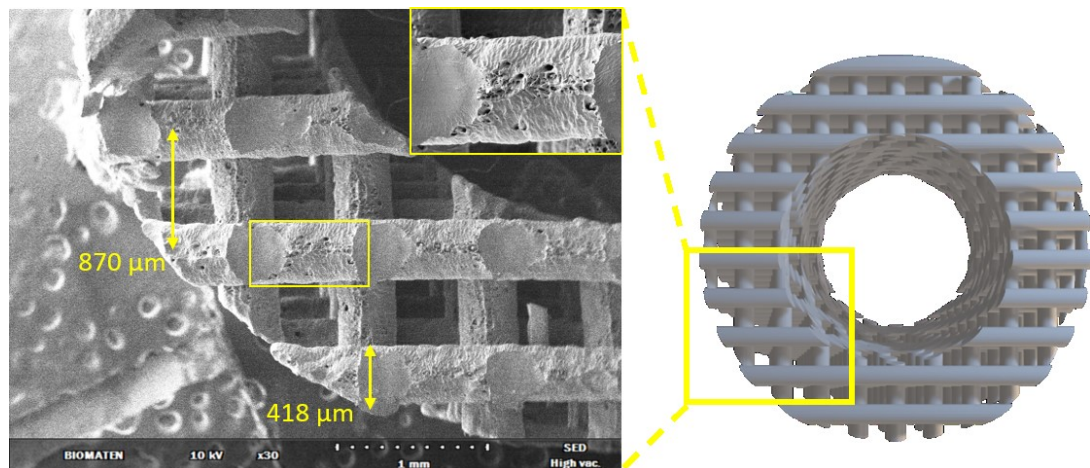


Figure 3.5. SEM micrograph of NG exterior produced from Dental SG via SLA printing. Fiber distance and diameter measurements marked on the SEM micrograph. 3D model for reference on the right. Scale bar: 1mm.

The nerve guide designs in the first studies in the field were hollow cylindrical tubes attempting to bridge the proximal and distal ends of a transected nerve. Nerve fibers in their undisturbed anatomy are bound in fascicles along the length of the nerve reaching the appropriate sensory or motor target. Without this fascicular constraint, regenerating axons disperse while trying to reach their target within the NG, subsequently reinnervating inappropriate targets. Therefore, NGs that have multiple microchannels were designed as candidates to mimic the fascicular anatomy of uninjured nerve, achieving targeted regeneration by bringing together the nerve ends. The NG designed in this study had multiple microchannels created inside a GelMA hydrogel filling the lumen of an SLA printed NG tube. These channels were observed using a stereomicroscope after assembly (Figure 3.6A), and a CLSM after immersing the construct to Calcein-AM for 15 min (Figure 3.6B). CLSM was especially useful in imaging of the construct topography since GelMA rapidly absorbs any dye and the gel-free regions are distinctly visualized. ImageJ analysis of the micrographs has yielded a channel diameter of $760 \pm 135 \mu\text{m}$.

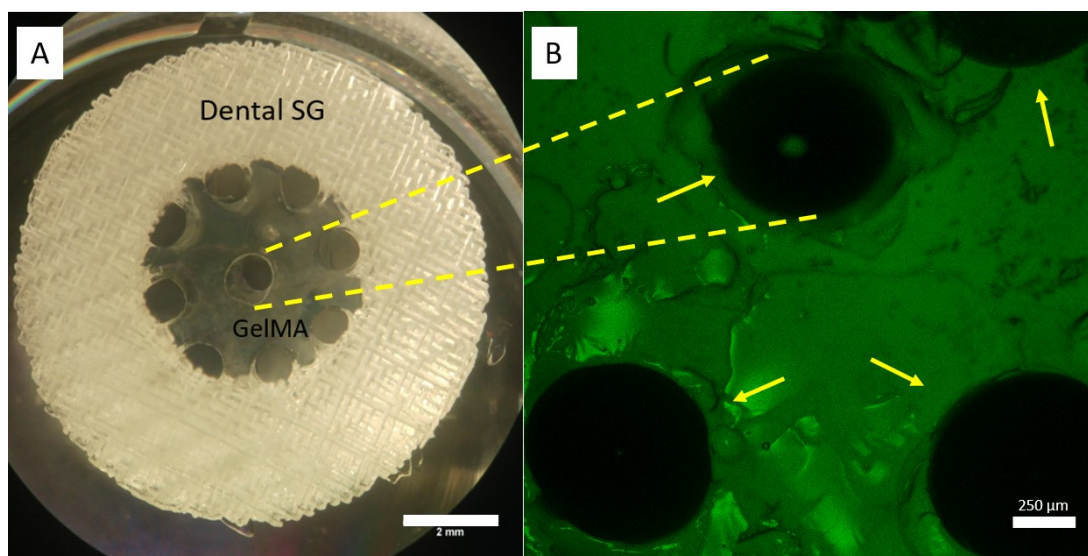


Figure 3.6. Microchannels in GelMA filled NG. A) Stereomicrograph of the NG designed with an interior hydrogel with multiple microchannels, B) CLSM micrograph of microchannels. Arrows indicate the microchannel positions. Scale bars: A) 2 mm, B) 250 μm .

3.2.2. Mechanical Properties of NG components

3.2.2.1. Mechanical Properties of GelMA15 Hydrogel

Compressive Modulus of the hydrogel is important because cell adhesion is strongly affected by substrate mechanical properties. Compressive Modulus of GelMA15 was examined by compression testing with a 10 N load ($n=3$). Elastic Modulus was found to be 147 ± 22 kPa with an ultimate compressive strength of 396 ± 27 MPa. It is a stiffer hydrogel compared to the various other examples reported in the literature. For example, Nichol et al. (2010) reported a 15% GelMA with an Elastic Modulus of ~ 30 kPa while Zhao et al. (2016) reported an Elastic Modulus of 89 ± 9 kPa for the same GelMA concentration. This difference in reported E values is a result of the differences in the processing parameters. Shuurman et al. (2013), and Wu et al. (2019) reported that the Elastic Modulus of GelMA hydrogels increase with increasing concentration of GelMA or extended duration of UV exposure, supporting our results. For example, Wu et al. (2019) reported a ten-fold increase in the Elastic Modulus when GelMA concentration is increased from 5% to 10%.

3.2.2.2. Mechanical Properties of DentalSG

Compressive mechanical properties of DentalSG exterior were also investigated as the flexibility of a NG is critical as it is not desired to be too soft to allow kinking, and not too rigid to mechanically irritate the neighboring tissue. Two tubular exterior structures were printed with SLA technique, the main difference being the dimension of the fibers. The first (denoted by DSG1) had a much thinner fiber diameter of $418 \pm 2 \mu\text{m}$ and the second (denoted by DSG2) had a fiber diameter of $100 \pm 9 \mu\text{m}$. Fiber distances were close; they were $774 \pm 31 \mu\text{m}$ and $870 \pm 20 \mu\text{m}$, respectively. Both structures had the same length (height) of 10 mm, constructed with different numbers of consecutive layers in the z-direction due to the difference in fiber thickness. The compressive modulus of each structure was also examined by compression testing with a 200 N load ($n=3$). The Elastic Moduli were found to be $55 \pm 3 \text{ MPa}$ for DSG1 and $0.335 \pm 0.060 \text{ MPa}$ for DSG2. As indicated by the compressive moduli measurements, the material mechanical properties vary with varying fiber thicknesses of the structure built with the material.

3.2.3. Cytotoxicity Testing of Dental Surgical Guide (Dental SG)

Dental SG is a bisphenol A-based photocurable resin with photoreactive side groups. Various studies show that substances that leach out of acrylic resin-based products can cause irritation and inflammation in tissues (Kedjarune et al., 2018). In order to evaluate the cytocompatibility of Dental SG structures produced by stereolithography, direct cytotoxicity testing using Alamar Blue cell viability assay was performed on Dental SG fiber meshes after seeding with L929 cells, the recommended cytotoxicity testing cell line standard.

Day 1 results of Alamar Blue viability assay on meshes seeded with L929 cells show very low cell numbers possible because of poor cell attachment to the scaffold and also due to leakage through the gaps of the printed structure (Figure 3.7). Contact angle values for Dental SG indicate that the material is hydrophobic ($\theta=104.5$) and in general, cells are known to display lower affinity to hydrophobic surfaces compared

to hydrophilic ones (Lampin et al., 1997). Moreover, pore size and interconnectivity of the substrate also affect cellular adhesion and viability (Zeltinger et al., 2001).

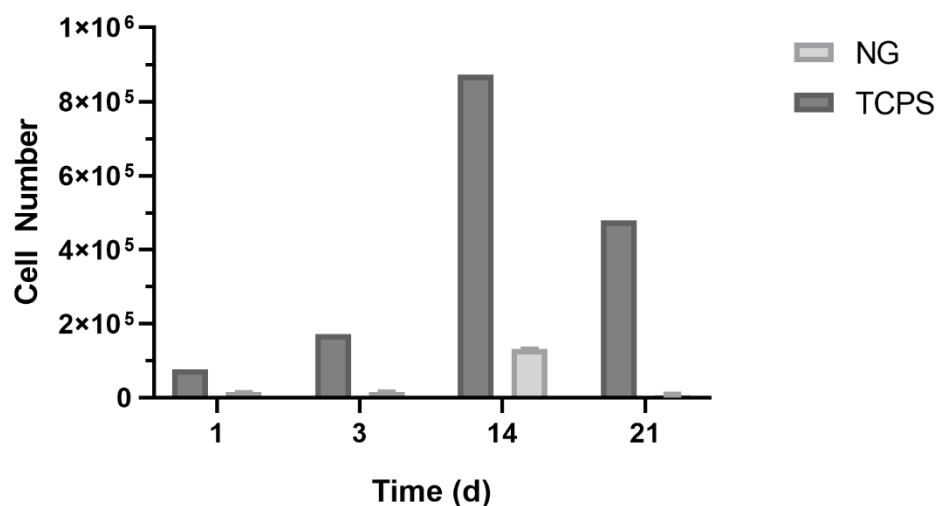


Figure 3.7. L929 viability on Dental SG meshes (n=3).

An increase in the cell numbers on the seeded meshes were observed for 14 days. A sharp decrease was observed on Day 21 for both Dental SG and the control TCPS'. SEM analysis of cell seeded meshes on day 21 of incubation has shown a significant degradation compared to their unseeded counterparts (Figure 3.8). The extent of degradation in meshes corresponds to the sharp decline in cell number on day 21, indicating that continuous change of substrate surface topography plays a role in cell proliferation. The decrease in cell number for TCPS control, however, is due to the high confluency of L929 cells and subsequent cell sheet formation that led to loss of cells while handling.

Due to the extensive degradation of fibers of Dental SG meshes in the presence of cells, the fiber thickness of Dental SG was increased to 500 μm for the NG tubular exterior for further studies to produce a more stable structure.

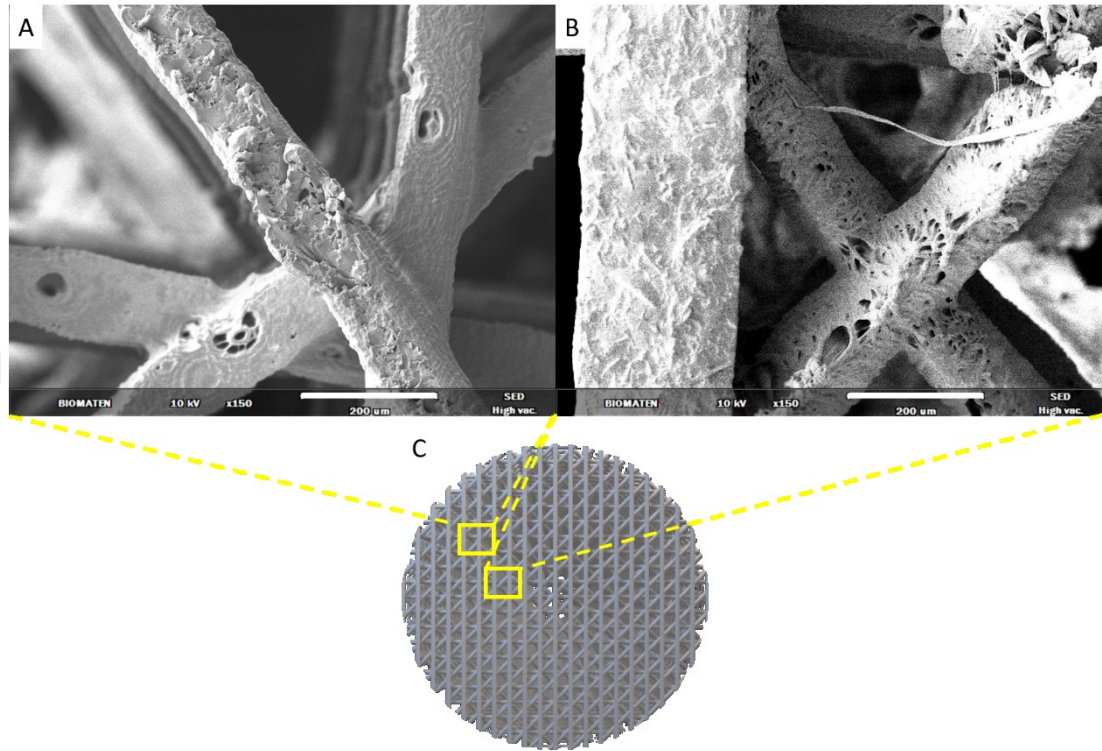


Figure 3.8. SEM micrographs of Dental SG meshes incubated in L929 complete media on Day 21. A) Unseeded, B) L929 seeded mesh. C) 3D model for reference. Scale bars: 200 μm .

3.2.4. Degradation of SLA Printed Dental SG NG Tubes in PBS

The exterior tube of the NG SLA printed in this study was designed to provide mechanical stability to prevent any mechanical damage to the GelMA hydrogel that would jeopardize the integrity of the microchannels. Besides, fluid exchange with the medium was allowed due to the printing approach and structure design. Therefore, the exteriors should retain their structural integrity throughout the regenerative process with a stable rate of degradation. After recording their initial dry weights, Dental SG tubular exteriors with $418 \pm 2 \mu\text{m}$ fiber diameter were incubated in PBS (10 mM, pH 7.4) for 14 days at 37 $^{\circ}\text{C}$. On days 3, 5, 7, and 14, DSG1 samples were rinsed with

dH₂O, lyophilized and their weights were recorded. Percent final weight ($W_F\%$) was calculated according to Equation 3 given in Section 2.2.5.2.. The morphology of fibers on days 0, 3, and 14 were investigated under SEM.

Results show that the material has a very slow rate of degradation for 14 days with a final weight retention of 90% (Figure 3.9). This is compatible with the rate of degradation of GelMA15, indicating the structural integrity of the whole NG will be preserved without one material being absorbed before the other.

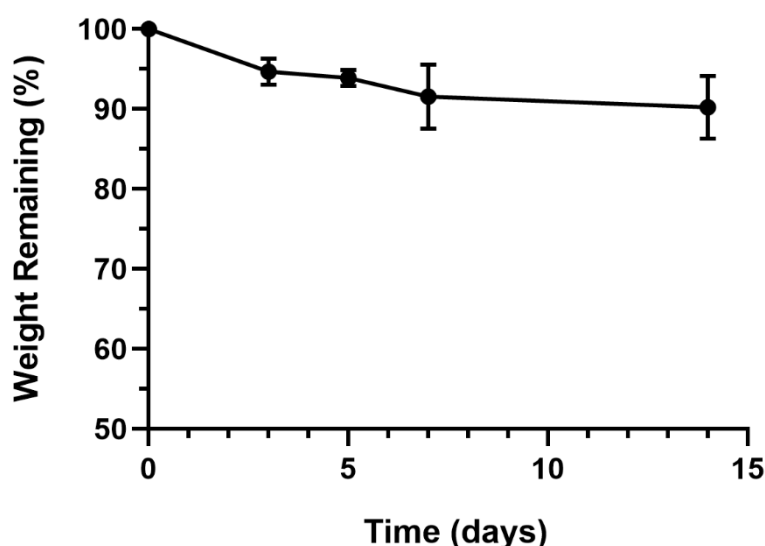


Figure 3.9. Degradation profile of DSG1 exterior in PBS (10 mM, pH 7.4) at 37 °C.

SEM micrographs taken on incubation days 0, 3, and 14 show the extent of degradation on DSG1 (Figure 3.10). The structure shows minimal number of pores in the fiber structure immediately after printing and post processing (Figure 3.10A). As incubation time proceeds, fiber morphology is changed significantly with larger and higher number of pores formed within the fibers. Fiber diameter is also affected by incubation. The initial value of $418 \pm 2 \mu\text{m}$ in day 0 becomes 412 ± 13 on day 3 and 353 ± 18 on day 14.

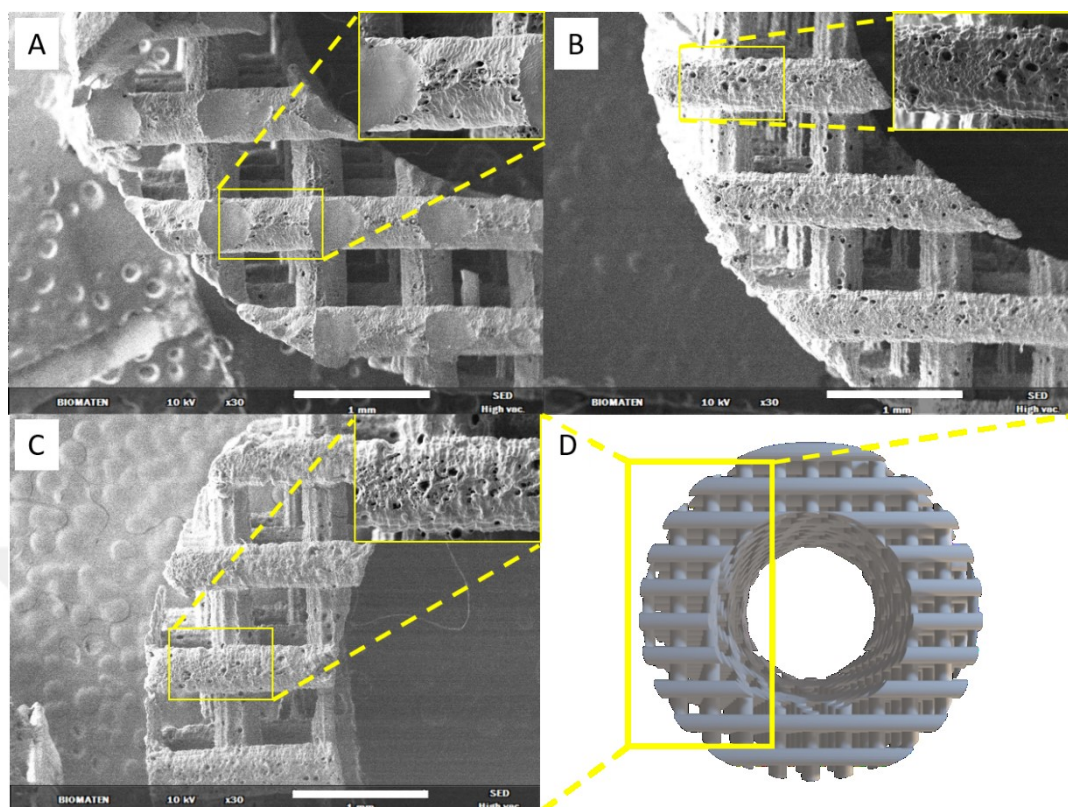


Figure 3.10. SEM micrographs of DSG1 samples incubated in PBS (10 mM, pH 7.4) at 37 °C. A) Day 0, B) day 3 and C) day 14. Insets show the fiber morphology at higher magnification (150x). D) 3D model for reference. Scale bars: 1 mm.

3.3. *In Vitro* Tests

3.3.1. BSA Release from Alginate Hydrogel

The release kinetics of BSA was assessed by recording the absorbance values of PBS collected at different time points at 280 nm in a spectrophotometer and calculating the amount of BSA using a previously generated absorbance calibration curve. The resultant data was fit to Korsmeyer-Peppas, Higuchi, and Baker-Lonsdale release models using DDSolver software to determine best fitting kinetic model for the release.

An initial release of ~30 was observed in the first 4 hours of release. At the 24 h mark, 1% alginate had released ~60% of its BSA content while 0.5% alginate released ~80%

(Figure 3.11). From the kinetic release analysis, the best fit was obtained with Korsmeyer-Peppas model (Table 3.1). This model explains the release of a drug molecule from a polymeric matrix such as a hydrogel by establishing an exponential relationship between the amount of drug released from a polymer and the time:

$$F(\%) = \frac{M_t}{M_\infty} = k_{KP} t^n \quad (11)$$

where F is the percent amount of drug released, M_∞ is the amount of drug present in the polymer at the equilibrium state (in some cases close to the amount of drug loaded into the polymer), M_t is the amount of drug released at time point t , k_{KP} is the release velocity constant, and n is the exponent of drug release in function of time t (Bruschi, 2015, Gouda et al., 2017). A good fit with an r^2 value of 0.924 was observed with BSA release from 1% alginate hydrogel (Table 3.1).

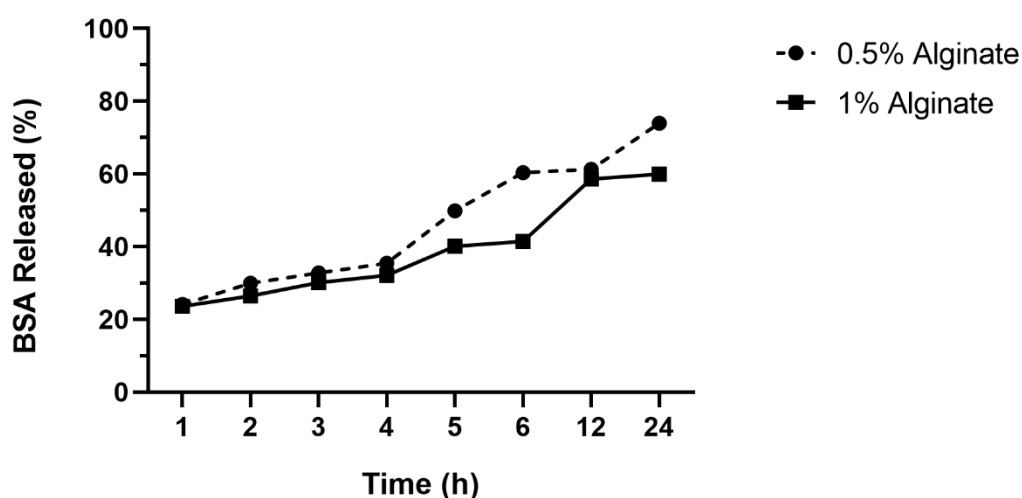


Figure 3.11. BSA release from alginate hydrogels. A) Release from 0.5% alginate, B) release from 1% alginate. At each time point the difference between samples were statistically significant ($n=6$, $p < 0.0001$).

Table 3.1. Kinetic analysis of BSA release from 0.5% and 1% alginate hydrogels.

Samples	Release Models, Rate Constants, and r^2 Values						
	Korsmeyer-Peppas			Higuchi		Baker-Lonsdale	
	k _{KP}	n	r^2	k _H	r^2	k _{BL}	r^2
0.5% Alginate	23.875	0.383	0.874	18.046	0.724	0.009	0.866
1% Alginate	22.111	0.337	0.924	15.185	0.691	0.005	0.852

BSA release is used as a model for release of the enzyme chondroitinase ABC that would act on chondroitin sulfate proteoglycans present in the scar tissue formed upon injury to the peripheral nerve. The amount of BSA loaded into the alginate hydrogels correspond to 250 U/mL of chABC (50 U in 200 μ L of hydrogel). The high number of units is to compensate for the limited penetration of the enzyme into the core of nerve bundles. Chondroitin sulfate proteoglycans generally accumulate in the injury site at least 14 days after induction of injury (Lee et al., 2010). Korsmeyer-Peppas model predicts that 100% of the drug will be released from 1% alginate hydrogel in ~3.2 days, which falls significantly short from the time point the enzyme should exert its activity. A way to overcome this problem is increasing the alginate concentration in order to create a denser polymer network, impeding water uptake and consequently lengthening the duration of the release.

3.3.1.1. Live/Dead Cell Viability Assay

In order to assess the performance of the NGs on supporting cell viability and alignment, NGs were seeded with rat pheochromocytoma cell line PC12. PC12 cell line has been commonly used as an *in vitro* neural cell model for neuron survival and neurite outgrowth studies (Foley et al., 2005; Grau and Greene, 2012). Upon exposure to nerve growth factor (NGF), they assume several neuronal characteristics in a dose dependent manner such as extension of neurite like processes (neuritogenesis),

electrical excitability, and synthesis, release and storage of catecholamines (i.e. dopamine) (Das et al., 2004).

PC12 cells were cultured in F12 (HAM's) media supplemented with 15% donor horse serum, 5% FBS, 50 ng/mL NGF, and 5% penicillin/streptomycin solution until confluency. Cells were passaged and seeded into the channels of vertical or horizontal NG setups as 2 μ L pellets or with a cell density of 50×10^3 cells/channel. TCPS controls were done in 24-well plates with a cell density that corresponds to the cell number in a whole NG. For example, if 3 channels are seeded, TCPS control would be seeded with 15×10^4 cells/well. Cell numbers of pellet seeded constructs were determined by suspending the same amount of pellet seeded in PC12 complete media and calculating the cell number with an automated mammalian cell counter (NucleoCounter[®] NC-100[™], Chemometec, Denmark).

Figure 3.12 shows Live/Dead staining of pellet and suspension seeded constructs with calcein-AM (live) and Ethidium Homodimer-1 (dead). A large fraction of cells seeded inside GelMA microchannels showed good attachment and an elongated morphology on day 1, similar to that observed on TCPS controls. Cell to cell contact via extended processes was observed 24 h after incubation using CLSM and Live/Dead staining where calcein-AM stains the cytosol elements including the filopodia (Figure 3.13), and cell viability was more than 95% (Figure 3.14).

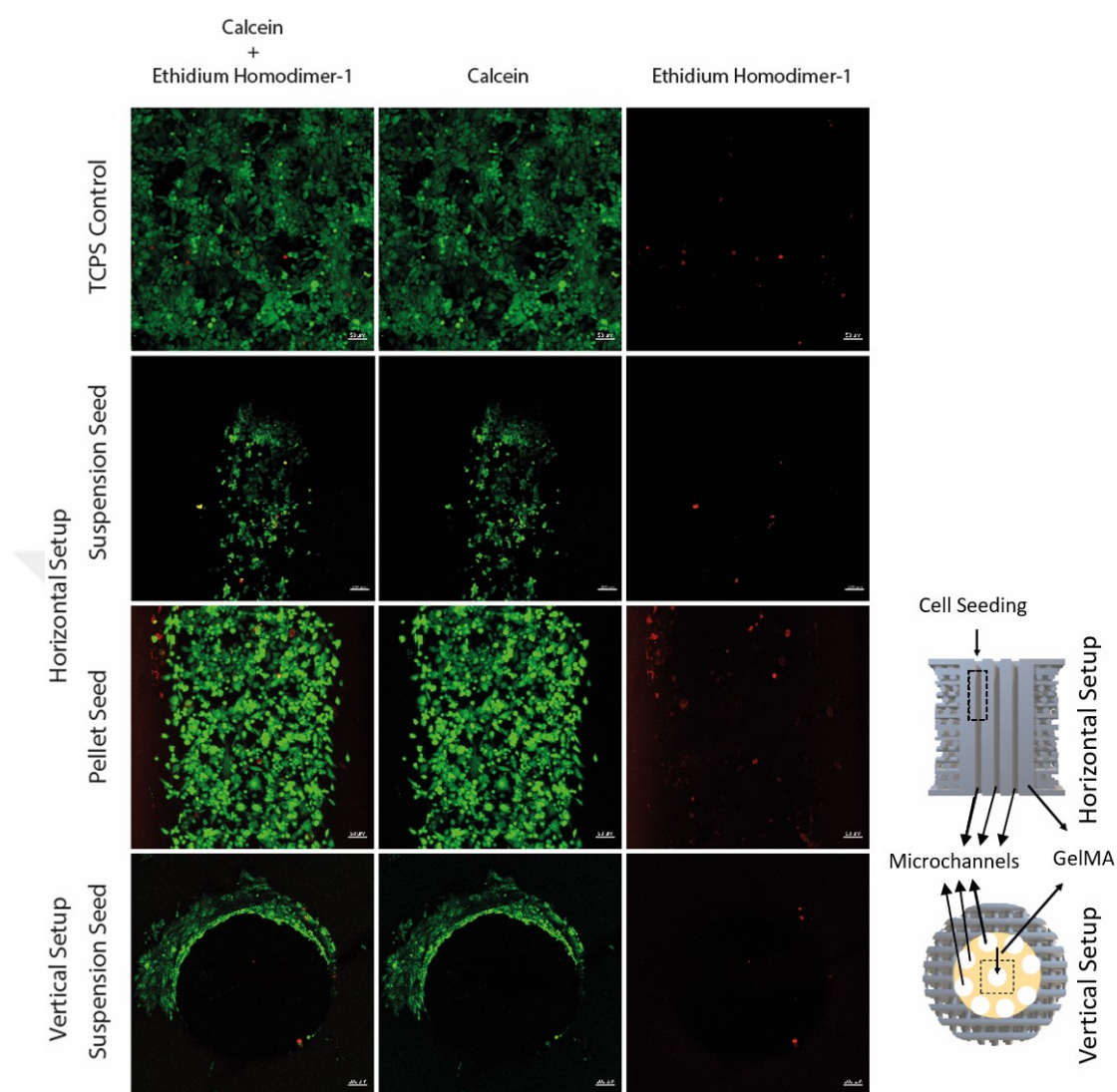


Figure 3.12. CLSM micrographs of Live/Dead staining of pellet and suspension seeded NG setups on Day 1. Pellet seeds have a cell density of 50×10^5 cells/channel. Horizontal and Vertical setup schemes shown on the right. Scale bars: 50 μm for TCPS control and pellet seed, 100 μm for suspension seed. Calcein-AM shows live cells, Ethidium Homodimer-1 shows dead cells.

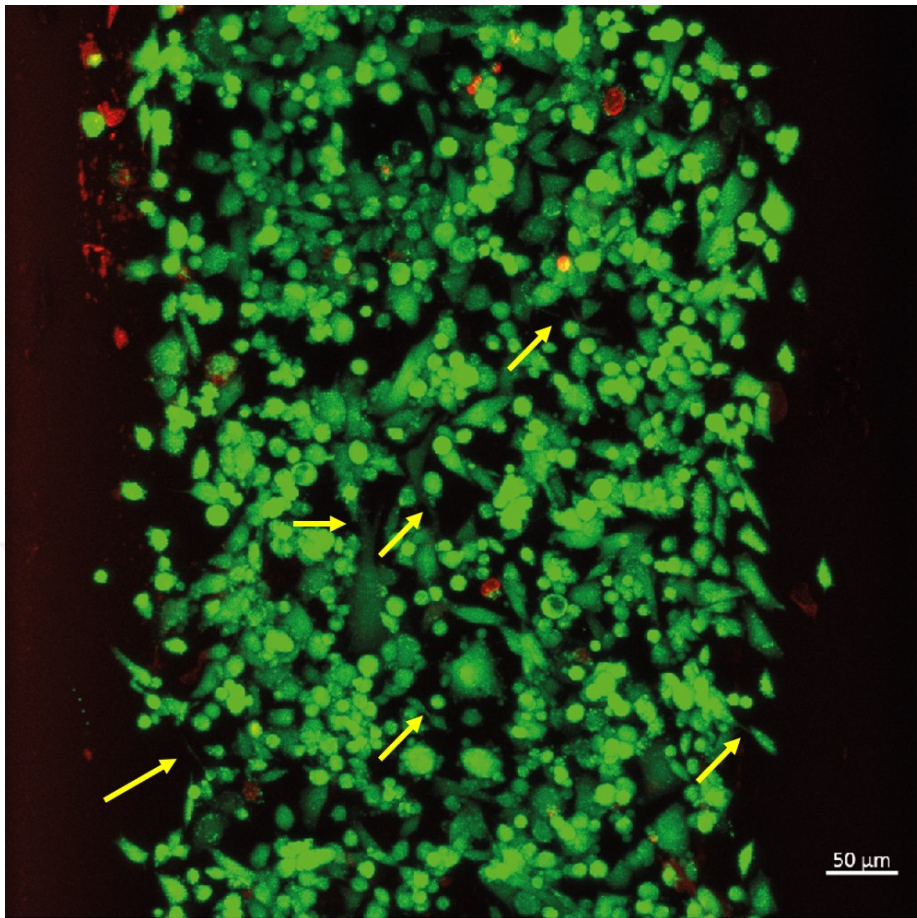


Figure 3.13. Cell to cell contact observed on Day 1 of incubation. Arrows indicate the processes PC12 cells protrude to contact. Green: Calcein-AM, live. Red: Ethidium Homodimer-1, dead. Scale bar: 50 μm .

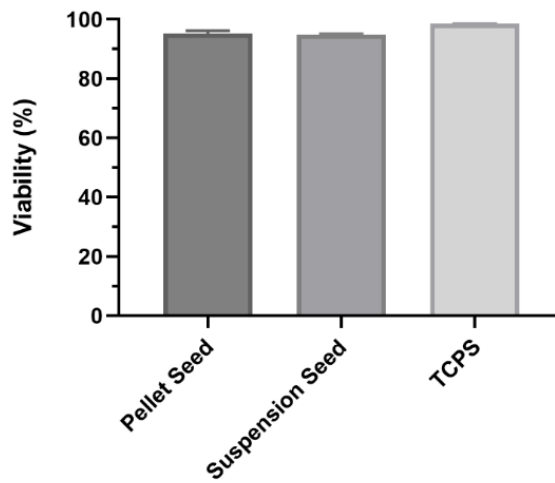


Figure 3.14. Quantitative analysis representing percent cell viability on day 1 of incubation.

Z-stack and tiles modules in CLSM were used to collect data from the proximal and distal portions of the NGs on day 1 (Figure 3.15, Figure 3.16 and Figure 3.17). Cells showed high levels of viability down ~2mm into the channels in the vertical setup (Figure 3.15A). Similarly, in horizontal setup, a large number of viable cells were dispersed along the channel length and showed good attachment to the channel walls (Figure 3.17).

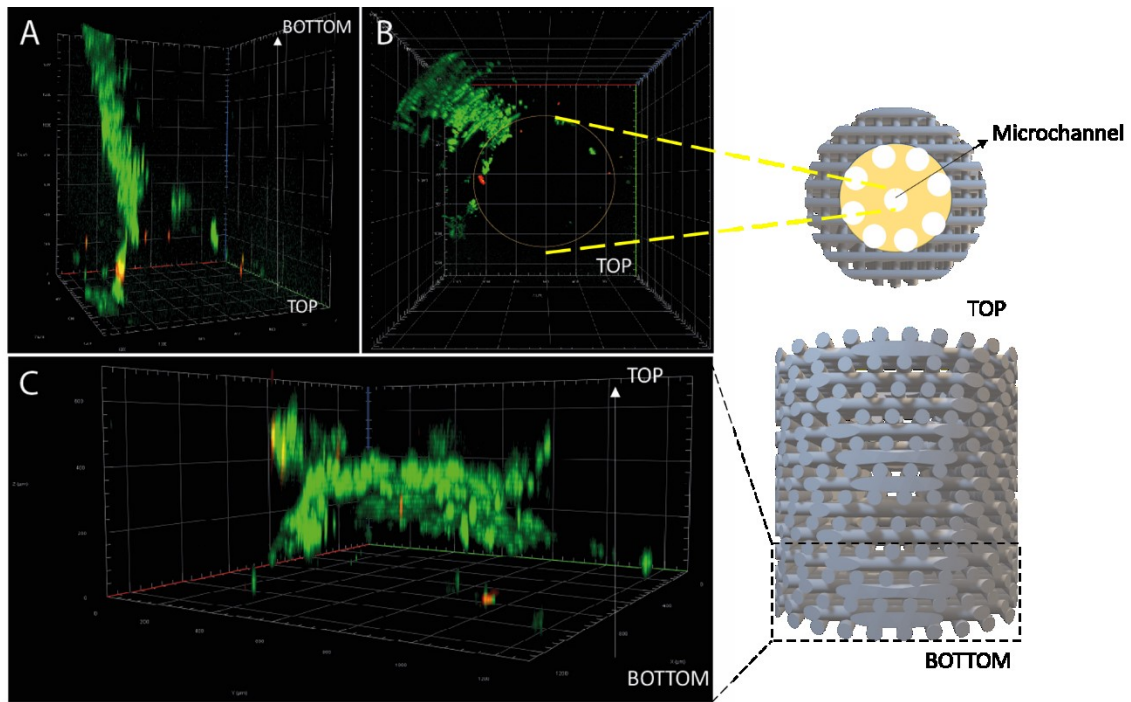


Figure 3.15. CLSM micrographs of PC12 cells attached to the walls of the channels of a vertical setup on Day 1. A) Z-stack image captured starting from the top of the construct scanning $\sim 2\text{mm}$ along the channel. B) Top view of z-stack image in A. C) Z-stack image captured starting from the bottom of the construct scanning $\sim 500\ \mu\text{m}$ along the channel. Green: Calcein-AM, live. Red: Ethidium Homodimer-1, dead.

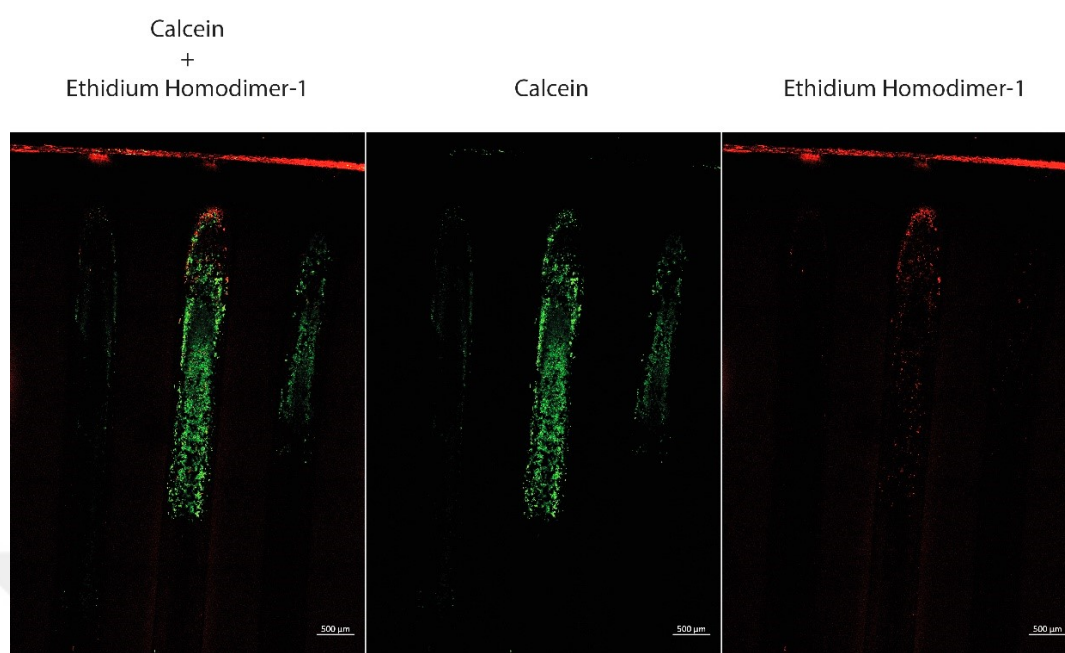


Figure 3.16. CLSM micrograph of suspension seeded PC12 cells in the microchannels of a horizontal setup on Day 1. Calcein-AM dye shows live cells, Ethidium Homodimer-1 shows dead cells. Scale bars: 500 μm .

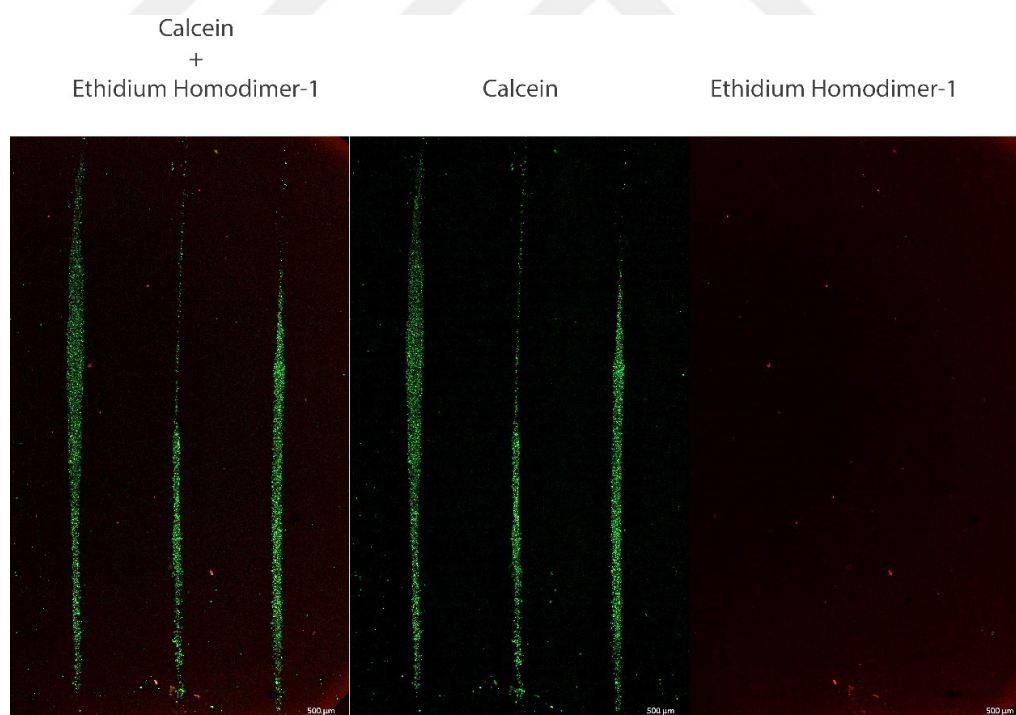


Figure 3.17. CLSM micrograph of pellet seeded PC12 cells in the microchannels of a horizontal setup on Day1. Calcein-AM dye shows live cells, Ethidium Homodimer-1 shows dead cells. Scale bars: 500 μm .

The high level of viability of cells on day 1 indicates that the medium flow inside the microchannels is sufficient for the nutrient exchange the cells require for viability. It was observed during CLSM imaging that some of the cells, while attached, did not take the spindle morphology of PC1. Axonal growth cones continuously monitor the substrate stiffness through their actin cytoskeleton, to form focal adhesions. The stiffness of the environment affects the focal adhesion, and subsequently attachment and neurite elongation. Wu et al. (2019) reported that the GelMA concentration significantly affects the adhesion and spreading of PC12 cells on the hydrogel substrate. Their findings are that PC12 cells do attach stiffer hydrogels (20% and 30% GelMA concentration) after 24h, but it takes 3 to 5 days for cells to elongate their neurites and assume their natural spindle-like morphology. Furthermore, the longest neurite lengths were observed on hydrogels produced from intermediate gel concentrations (10%-20% range). Their findings are complementary to the observations we made on PC12 attachment to GelMA15. PC12 adhesion to GelMA15 was investigated by coating the base of a 24-well plate with GelMA15 and then seeding PC12 on the gels with a density of 10×10^4 cells/well. Plain TCPS' were seeded with the same density for positive control. They were all incubated at 37 °C in 5% CO₂ in an incubator. Cell attachment was studied under a phase contrast microscope after 4 h (the average duration for cell attachment to a substrate) and 24 h of incubation. Majority of cells seeded on TCPS showed attachment and elongation after 4h of incubation, whereas PC12 cells were still floating over the GelMA15 and had a circular morphology (Figure 3.18). After 24 h, PC12 cells seeded on TCPS had projected axon-like processes while cells seeded on GelMA15 had just attached to the surface and assumed spindle morphology (Figure 3.18).

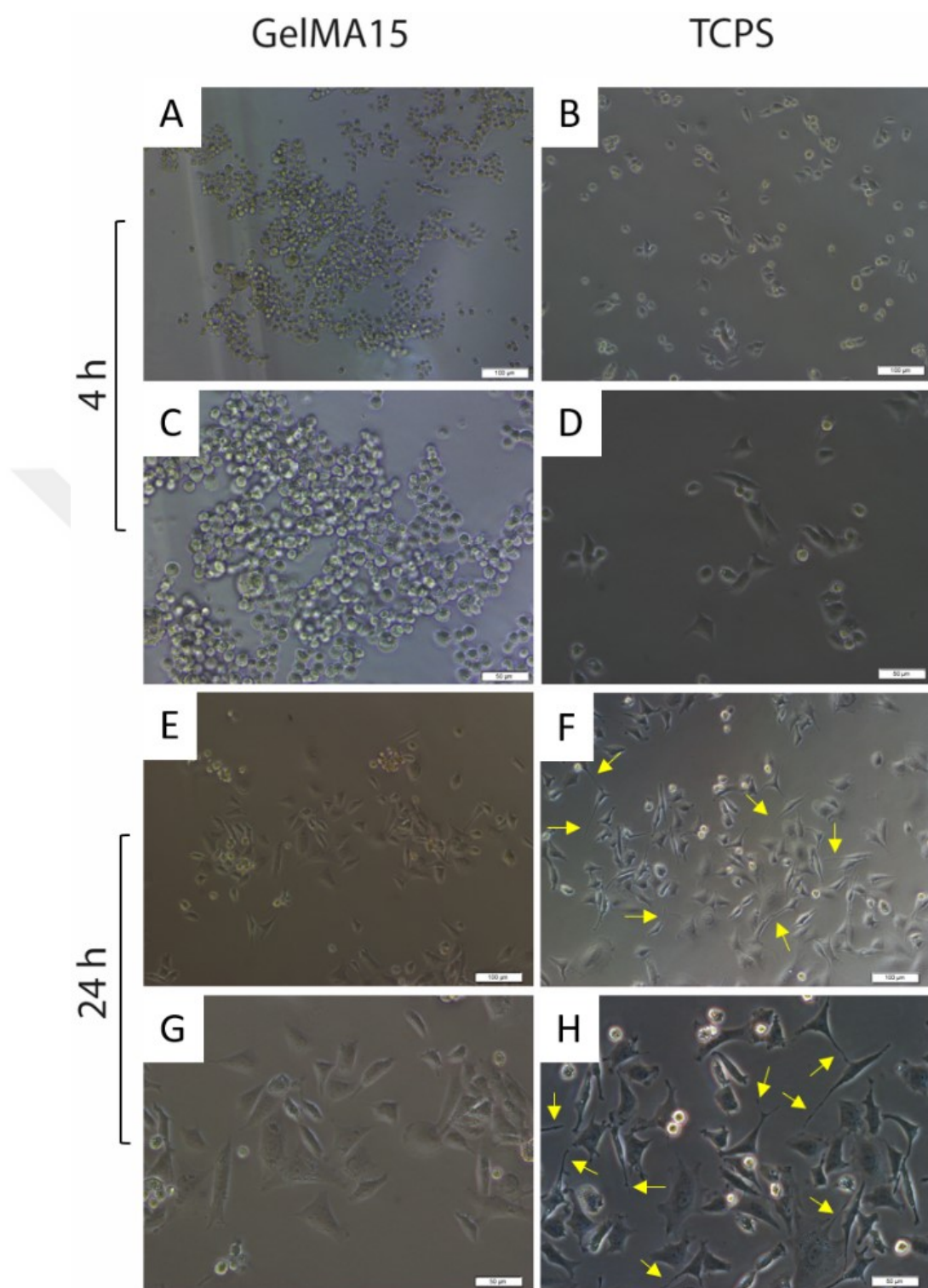


Figure 3.18. Phase contrast micrographs of PC12 seeded GelMA15 (A, C, E, G) and TCPS (B, D, F, H). Cell attachment on the two substrates in 4 h (A, B, C, D) and 24 h (E, F, G, H) is shown. Arrows indicate axon-like processes of PC12 cells. Scale bars: 50 μm (C, D, G, H) and 100 μm (A, B, E, F).

Live dead staining on Day 3 showed less cells and the majority of PC12 cells beginning to adopt morphological deformations (Figure 3.19). Even though still alive, the cells presented a circular shape and the cytoplasms were fragmented. Calcein-AM stains live cells by passive transport into the cytosol and metabolism by intracellular esterases located in the cytosol or endoplasmic reticulum. These morphological changes observed are somewhat similar to characteristic early morphological signs of necrosis and apoptosis. Cells which showed the normal spindle-like morphology were still attached and were aligned along the microchannel walls (Figure 3.20).



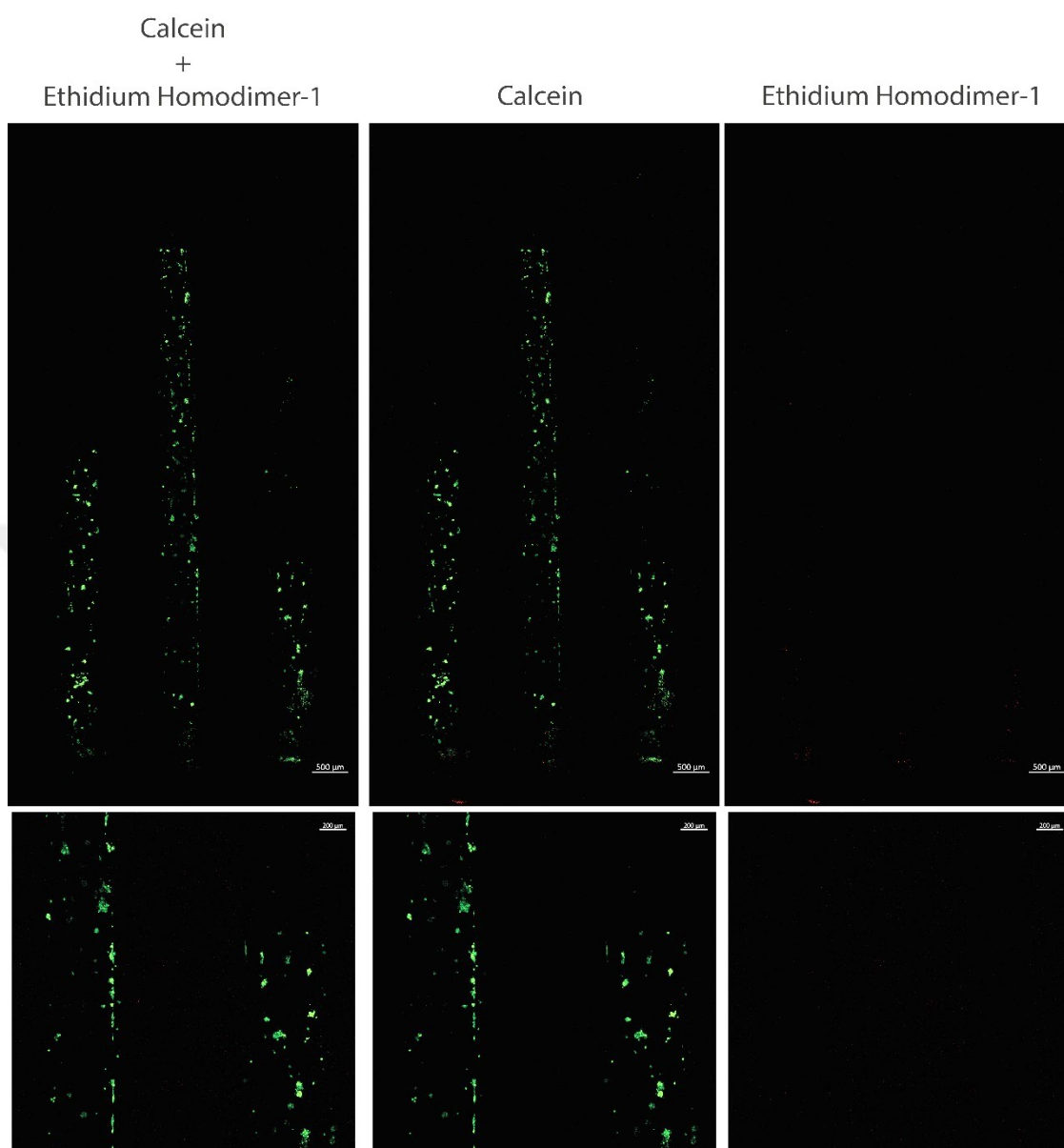


Figure 3.19. CLSM micrographs of PC12 cells on Day 3 of incubation. PC12 cells are viable but had shrunk and fragmented morphology along the whole length of the microchannels, with some cells showing alignment (top row). A higher magnification of the cells is presented in the bottom row. Calcein-AM dye shows live cells, Ethidium Homodimer-1 shows dead cells. Scale Bars: 500 μ m (top row), 20 μ m (bottom row).

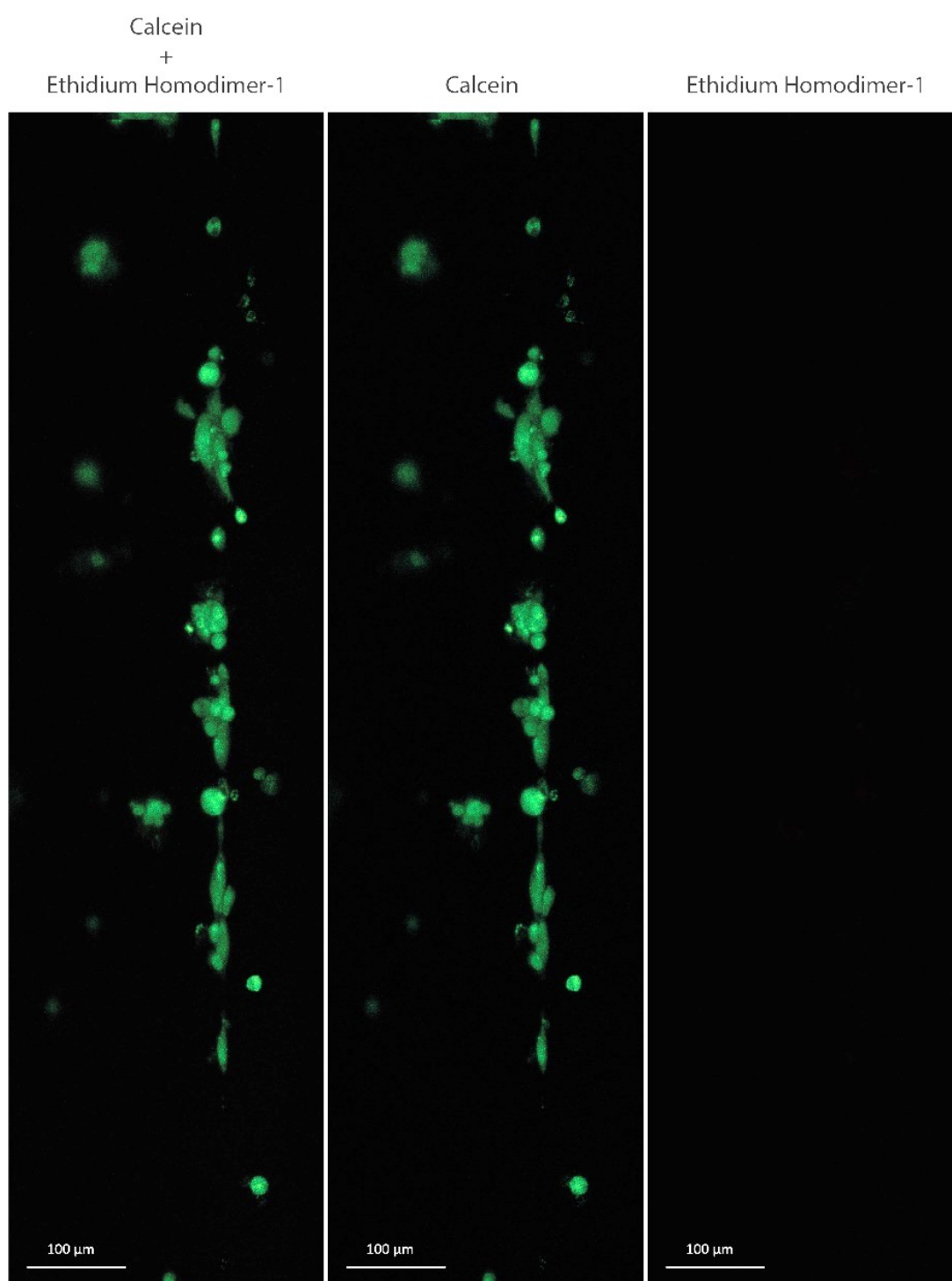


Figure 3.20. CLSM micrograph of PC12 cells aligned along the channel wall on Day 3 with higher magnification. Calcein-AM dye shows live cells, Ethidium Homodimer-1 shows dead cells. Scale bar: 100 μm .

Live/Dead staining on Day 7 showed that the pellet and suspension seeded PC12 cells did not survive in the NG setup (Figure 3.21). All cells showed the same fragmented cellular morphology with a loss of membrane integrity, pointing to necrosis. As cells seeded in pellet suspension form were lost, and the cells showed a high viability rate on day 1 for both, necrosis due to core hypoxia as a result of high density seeding in the microchannel was not considered to be the cause. Instead, Dental SG was reinvestigated for toxicity, as the initial structure used in the cytotoxicity testing did not have a bulk of the material in the incubation chamber as the horizontal setups did. The initial cytotoxicity testing was performed with a fibrous mat of Dental SG with a fiber diameter of 100 ± 9 , while the vertical setup had a increase of four-fold in fiber diameter and the horizontal setup had a bulk rectangular block that is used for stabilizing the halved tubular exterior.

Indirect cytotoxicity testing was performed with the horizontal setup structures on PC12 cells seeded onto 24-well plates with a density of 10×10^4 cells/well. Briefly, the Dental SG structures were incubated in complete media for PC12 cells for 1 and 3 days and these were used as the extracts for cytotoxicity testing. After a day of incubation in 1-day and 3-day extracts, changes in cell number were determined with Alamar Blue cell viability testing. The results show that cell proliferation was impeded with 1-day Dental SG extract incubation compared to the TCPS controls which presented a doubling in cell number (Figure 3.23). With 3-day extract, cell viability is significantly decreased (~25% of its original level) after a day of incubation (Figure 3.23). Apparently, Dental SG exerted a time dependent toxic effect on PC12 cells when in bulk form, which caused chemical necrosis. This toxicity may be explained by incomplete curing of the monomer due to low penetration of UV into the bulk material. As previously shown in in situ degradation studies in section 3.2.4, Dental SG degrades extensively in physiological conditions over the course of 2 weeks. Any unreacted resin monomers entrapped in the core of the product would be released into the media, increasing its toxic effect of chemically stressing the PC12 cells.

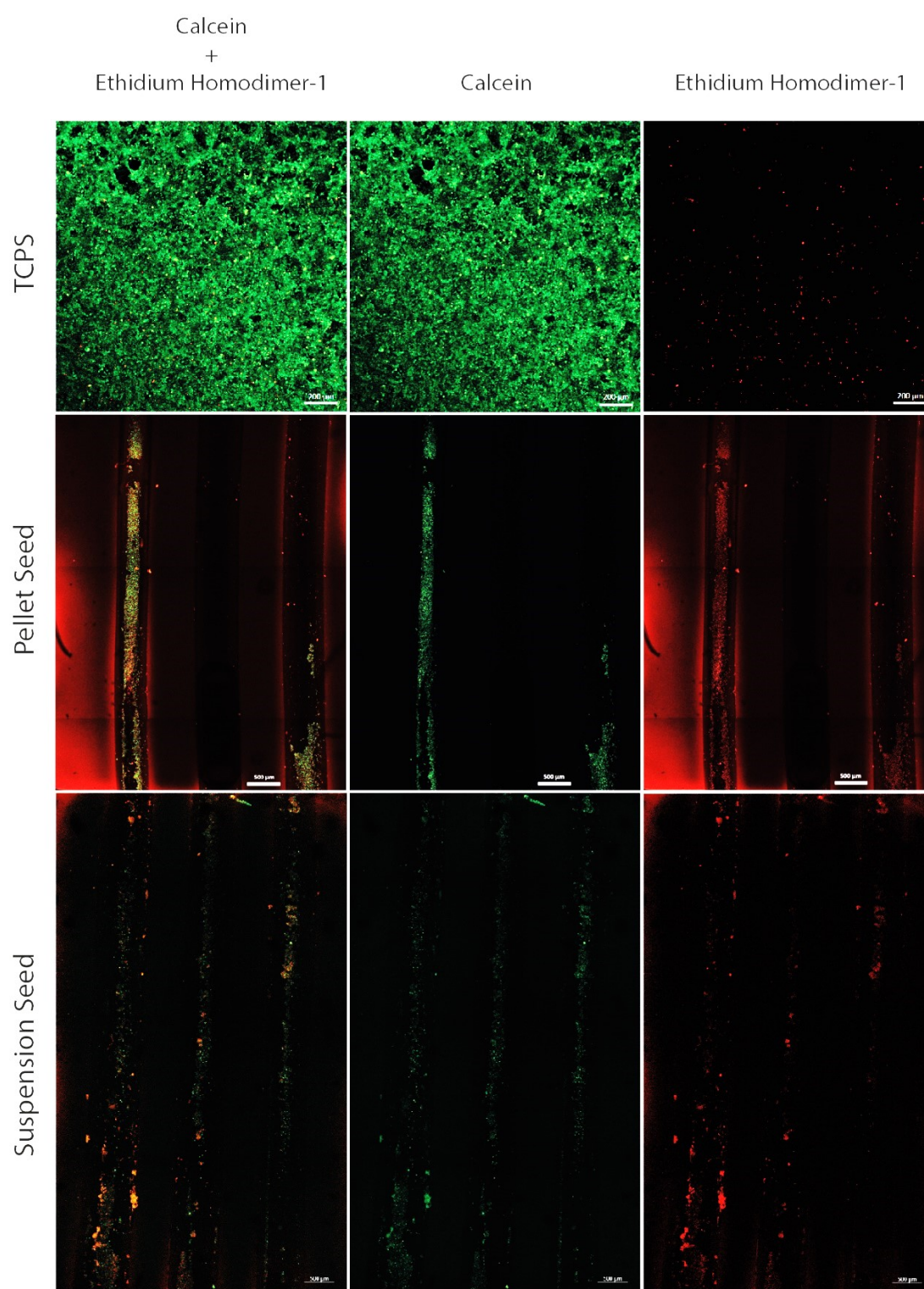


Figure 3.21. CLSM micrographs of PC12 cells on horizontal NG setups on Day 7 of incubation. Calcein-AM dye shows live cells, Ethidium Homodimer-1 shows dead cells. Scale bars: 200 μm (top row), 500 μm (middle and bottom rows).

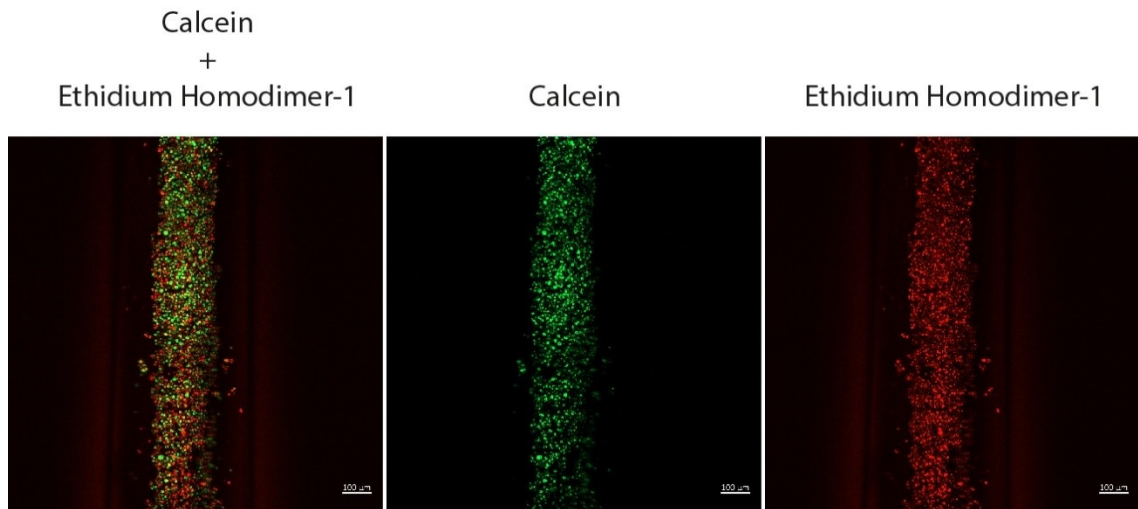


Figure 3.22. Higher magnification of CLSM micrographs of PC12 cells on horizontal NG setups on Day 7 of incubation. Calcein-AM dye shows live cells, Ethidium Homodimer-1 shows dead cells. Cells show a fragmented cytosol and disintegrated membrane indicated by Ethidium Homodimer-1 staining. Scale bars: 100 μm .

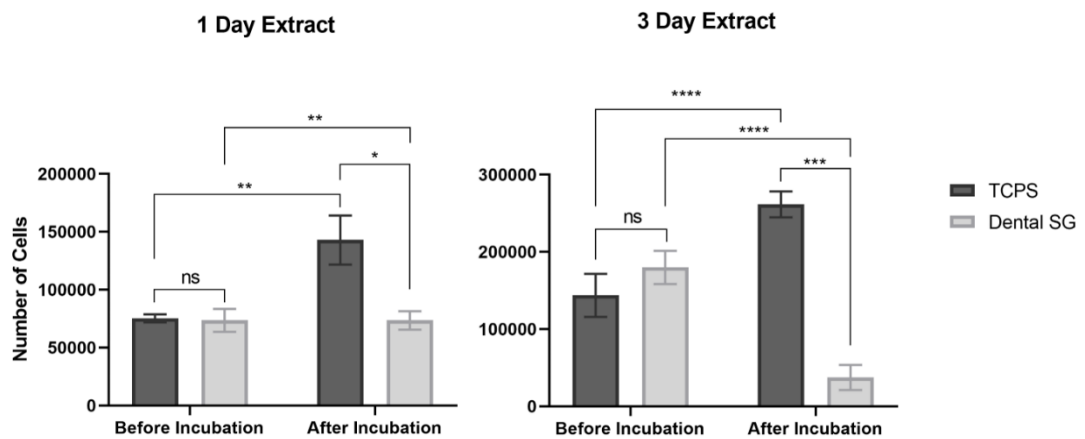


Figure 3.23. Indirect cytotoxicity test results performed using 1 and 3 day extracts of Dental SG in PC12 complete media and PC12 cells. TCPS controls were incubated with normal PC12 complete media. (n=3; ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$)

CHAPTER 4

CONCLUSION

Nerve guides are tubular structures designed to bridge together the proximal and distal ends of a transected nerve, provide physical support, and guide regenerating axons using topographical and chemical cues integrated in them.

In this study, a nerve guide of methacrylated gelatin (GelMA) hydrogel inner matrix with microchannels and chondroitinase ABC-laden alginate hydrogel exterior was designed. Stereolithography, an additive manufacturing method based on photopolymerization of resins was used to produce the NG exterior. BSA was used as a model of chondroitinase ABC in the release studies.

The study consisted of 4 phases: (i) Methacrylated gelatin synthesis, characterization, and optimization for hydrogel production, (ii) 3D printing of Dental SG nerve guide exterior tube, and its *in vitro* cytotoxicity testing, (iii) nerve guide assembly by filling lumen of tubular exterior with GelMA and creation of microchannels in GelMA matrix with capillary glass molds, and loading BSA embedded alginate hydrogel to the exterior, (iv) *in vitro* testing of nerve guide for cell viability and guidance.

The optimum GelMA concentration was determined as 15% (w/v) as a result of *in situ* and enzymatic degradation testing in the first phase. Hydrogels of selected concentration were stable in physiological conditions. *In vitro* studies showed GelMA hydrogel provides cells with the optimal conditions for attachment and growth. The chondroitinase release from the alginate matrix of the nerve guide was studied. BSA was released from the alginate hydrogel matrix in accordance to Korsmeyer-Peppas release model with a 100% release achieved in ~3 days. Dental SG material tested for cytotoxicity in the second phase was shown to have no adverse effects on cells. However, the solid product was shown to be unstable under physiological conditions

with significant degradation observed in the presence of cells. In the following phase, the effectiveness of nerve guide in supporting cell viability and growth was tested *in vitro* with nerve cell model PC12. These cells showed good attachment to GelMA microchannel walls and were viable for 3 days of incubation, with visible alignment along the microchannels. However, due to possible toxic effect of Dental SG degradation products on PC12 cells, the cells did not survive until Day 7.

In the future studies, the nerve guide will be tested under physiological conditions with another material as an exterior tubular structure. Additionally, an axonal guidance cue MGF-Ct24E will be introduced to the nerve guide design to further the axonal migration towards the distal end. Improvements on sustained release duration will be investigated to achieve a total release period of around two weeks. *In vivo* studies will be conducted to evaluate the performance of the nerve guide in restoring motor function.

In conclusion, the inner microchannel structure of the designed nerve guide is capable of targeting axon elongation towards distal end via microchannels if suitable incubation conditions are available. It is also efficient in releasing scar-tissue dissolving agent in the first few days of incubation, indicating a successful sustained release.

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APPENDICES

A. Calibration Curves for Alamar Blue Viability Testing

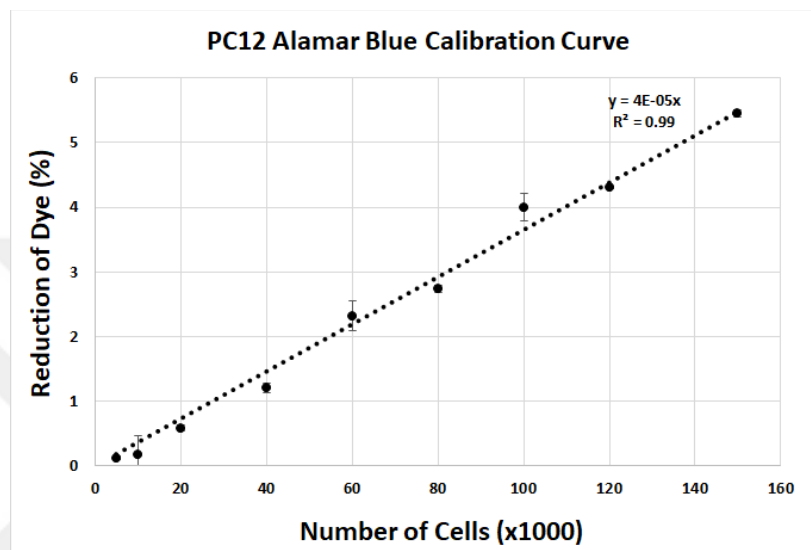


Figure A.1. Calibration curve for PC12 viability tested with Alamar Blue

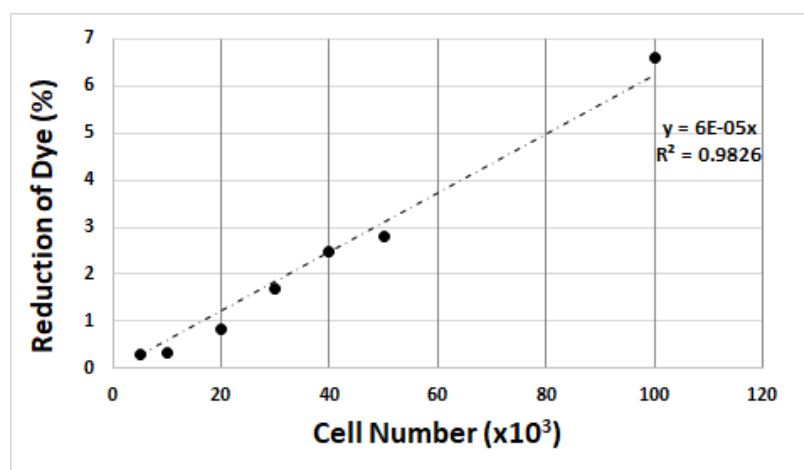


Figure A.2. Calibration curve for L929 viability tested with Alamar Blue

B. Calibration Curve for BSA Release in PBS

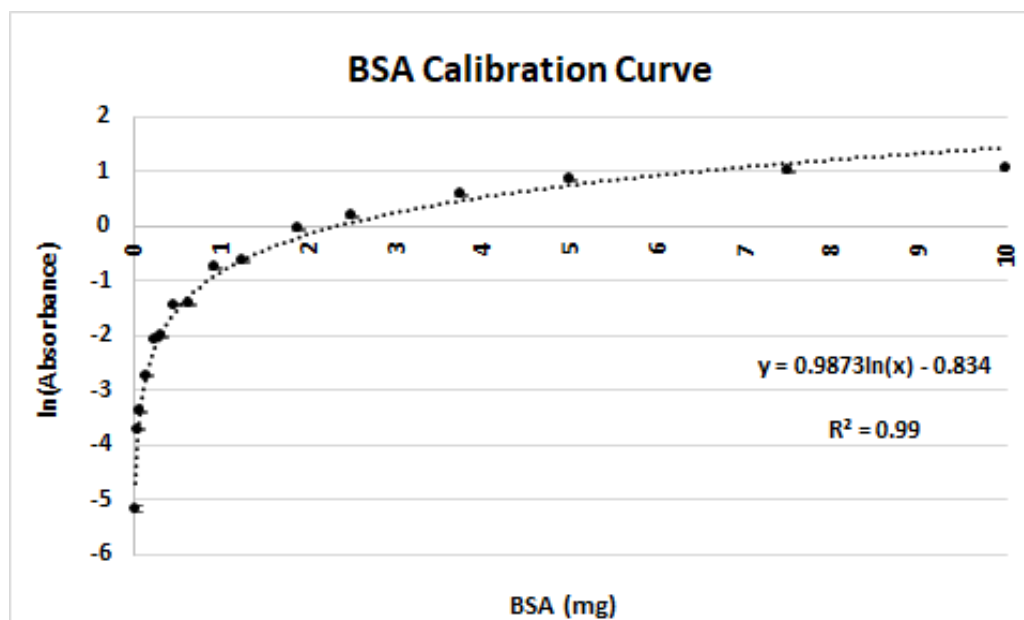


Figure B.1. Calibration curve for BSA release studies.