

**DEVELOPMENT OF PEPTIDE NANOMATERIALS FOR NEURAL
REGENERATION**

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IN
MATERIALS SCIENCE AND NANOTECHNOLOGY**

By

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May, 2015

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REGENERATION**

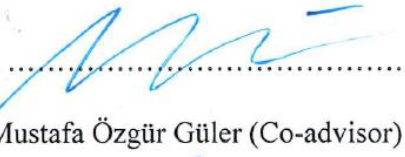
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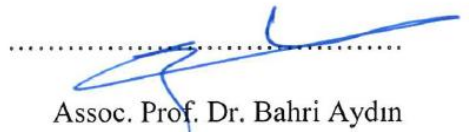
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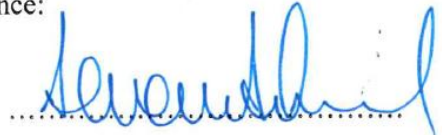


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ABSTRACT

DEVELOPMENT OF PEPTIDE NANOMATERIALS FOR NEURAL REGENERATION

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Nervous system consists of a dense network of cells and their connections and exhibits a high level of complexity. This complexity arises from the high variety of cell types with very specific functions, the high number of cells along with the abundance of connections between these cells. When combined with the nonproliferative nature of neural cells and inhibitory nature of the pathological extracellular matrix (ECM), this complexity leads to a very limited regenerative potential. Thus neurodegenerative disorders and traumatic injuries of neural tissues lead to lifelong disabilities due to the poor success of current therapies. Novel therapeutic approaches which can overcome barriers that impede neural regeneration are therefore required to be developed. Smartly designed nanomaterials that can direct cells towards desired functions can improve the regeneration of neural tissues.

Herein, I have described my work on development of peptide nanofibers for neuroregeneration and biological applications of these nanomaterials. To achieve the regeneration of the nervous system, the composition of the neural ECM under healthy conditions and during early development was mimicked through structural resemblance and bioactive epitope presentation using nanofibers. Laminin derived IKVAV peptide sequence and glycosaminoglycan mimicking, growth factor-binding sulfonated peptide sequence were presented on peptide nanofiber scaffolds. Differentiation of PC-12 cells, a model cell system for neuroregenerative studies, was found to be improved on these nanofiber scaffolds when compared to the cells on epitope free control scaffolds. Cells could even extend neurites on these scaffolds in the presence of inhibitory chondroitin sulfate proteoglycans. These nanofibers also proved to be efficient in sciatic nerve regeneration after injury. When injected into the lumen of polymeric nerve guidance channels, this bioactive nanofiber system provided guidance to the elongating axons and resulted in better axonal regeneration that was evident both from histological analysis and electromyography results. Results of *in vitro* and *in vivo* experiments were correlated and indicated the neuroregenerative potential of these peptide nanofibers. In addition, semiconductive oligothiophene was encapsulated in peptide nanofibers without compromising the biocompatibility. These hybrid nanofiber scaffolds can potentially be used for electrical stimulation of neurons that can further boost regeneration.

Keywords: peptide nanofibers, neural regeneration, scaffolds, sciatic nerve.

ÖZET

SİNİRSEL REJENERASYON AMAÇLI PEPTİT NANOMALZEMELERİN GELİŞTİRİLMESİ

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Sinir sistemi hücrelerin birbiriyle olan bağlantılarından oluşan yoğun ve kompleks bir ağa sahiptir. Sinir sisteminde farklı fonksiyonlara sahip çeşitli hücrelerin varlığı, hücre sayısının ve hücrelerin yaptığı bağlantıların fazla oluşu bu sistemi oldukça karmaşık kılmaktadır. Bu kompleksiteye ek olarak sinir hücrelerinin çoğalmaması ve patolojik durumlarda oluşan hücreler arası matriksin inhibe edici doğası hasar sonrası rejenerasyon imkanını sınırlamaktadır. Güncel tedavi seçeneklerinin düşük başarı oranı nörodejeneratif hastalıklar ve travmatik hasarlar nedeni ile birçok hastayı ömür boyu bakıma muhtaç bırakmaktadır. Bu nedenle yeni tedavi yaklaşımlarına ihtiyaç duyulmaktadır. Hücrelerin kaybedilen fonksiyonlarını yerine getirmeye yönlendirmek üzere tasarlanmış nanomalzemenin geliştirilmesi sinir rejenerasyonunda umut verici olabilir.

Bu çalışmada sinirsel rejenerasyon amaçlı geliştirilmiş peptit nanofiberler ve bu malzemelerin biyolojik uygulamaları anlatılmıştır. Sinir sisteminde rejenerasyon sağlayabilmek amacıyla sağlıklı doku ve embriyonik dokulardaki hücreler arası matriksi yapı ve biyolojik sinyaller yönünden taklit eden nanofiberler üretilmiştir. Bu nanofiberler laminin proteinine has bir sekans olan 'IKVAV' ve büyüme faktörlerine bağlanma özelliği olan glikozaminoglikan taklidi sülfonatlı bir diziden oluşmaktadır. Biyoaktif peptit nanofiber iskeleler üzerinde sinirsel yönde başkalaştırılan PC-12 hücrelerinin kontrol nanofiberler üzerindeki hücrelere göre daha iyi başkalaştığı görülmüştür. Aksonal büyümeyi inhibe edici kondroitin sülfat proteoglikanların varlığında dahi nörit uzatımı etkin bir şekilde devam etmiştir. Hücreler arası matriks taklidi bu nanofiberlerin siyatik sinir rejenerasyonunda da etkili olduğu görülmüştür. Polimerik sinir tüpünün içine enjekte edilen nanofiberlerle tedavi edilen hayvanlarda aksonal rejenerasyonun daha iyi olduğu histolojik analizler ve elektromiyografi sonuçları ile gösterilmiştir. Birbirini destekleyen *in vitro* ve *in vivo* sonuçlar, bu çalışmada kullanılan peptit nanofiberlerin sinir rejenerasyonu için etkili bir malzeme olduğunu göstermektedir. Ayrıca bu nanofiberlerin içine yarı iletken oligotiyofen enkapsüle edilmiş ve bu modifikasyonun hücre canlılığını etkilemediği gözlemlenmiştir. Yeni geliştirilen bu multifonksiyonel peptit nanofiberler daha iyi bir rejenerasyon için sinir hücrelerinin elektriksel uyarımına imkan verecektir.

Anahtar sözcükler: peptit nanofiberler, sinir rejenerasyonu, siyatik sinir

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LIST OF ABBREVIATIONS

ALS : amyotrophic lateral sclerosis

SCI : spinal cord injury

CNS : central nervous system

PNS : peripheral nervous system

ECM : extracellular matrix

PG : proteoglycan

CSPG : chondroitin sulfate proteoglycan

HSPG : heparan sulfate proteoglycan

DSPG : dermatan sulfate proteoglycan

KSPG : keratan sulfate proteoglycan

GAG : glycosaminoglycan

HA : hyaluronic acid

MAG : myelin-associated glycoprotein

OMgP : oligodendrocyte myelin glycoprotein

cAMP : Cyclic adenosine monophosphate

CREB : cAMP response element binding protein

NGF : nerve growth factor

BDNF : brain derived neurotrophic factor

GDNF : glial derived neurotrophic factor

IGF : insulin like growth factor

CNTF : ciliary neurotrophic factor

FGF-2 : fibroblast growth factor 2

TNF- α : tumor necrosis factor alpha

LIF : leukocyte inhibitory factor

IL : interleukin

tPA : tissue plasminogen activator

NT-3 : neurotrophin-3

RA : retinoic acid

PA : peptide amphiphile

SEM : scanning electron microscope

TEM : transmission electron microscope

AFM : atomic force microscopy

SHH : sonic hedgehog

CN : cavernous nerve

PDL : poly-D-Lysine

CD : circular dichroism

DMEM : Dulbecco's Modified Eagles Medium

RPMI : Roswell Park Memorial Institute Medium

MEM : Minimal Essential Medium

EthD-1 : ethidium homodimer I

SYN1 : synaptophysin 1

BSA : bovine serum albumin

PBS : phosphate buffered saline

HPLC : high performance liquid chromatography

LC-MS : liquid chromatography-mass spectroscopy

ANOVA : analysis of variance

PNI : peripheral nerve injury

NGC : nerve guidance conduit

HRP : horseradish peroxidase

SFI : sciatic functional index

EMG : electromyography

HE : hematoxylin-eosin

CaMK : calcium/calmodulin dependent protein kinase

MEK : mitogen activated protein kinase

ERK : extracellular signal regulated protein kinase

DRG : dorsal root ganglion

DH4T : 3,3'''-Dihexyl-2,2':5',2'':5'',2'''-quaterthiophene

THF : tetrahydrofuran

CHAPTER 1

INTRODUCTION

1.1. Neurodegenerative disorders and neural injuries

Neurodegeneration is the common term for defining progressive loss of structure and function of nerve cells. It generally occurs due to the accumulation of misfolded aggregated proteins in some parts of the aging brain, which results in cell death and inflammatory damage in those regions¹. Clinically, there are different types of neurodegeneration in distinct neurodegenerative diseases. Therefore, there are some variances in the proximal triggers and pathological markers of neurological diseases such as Lewy bodies in Parkinson's disease, plaques and tangles in Alzheimer's disease, demyelination in multiple sclerosis and motor-neuron death in ALS. Although initiated by different initial triggering factors, these diseases share some overlapping downstream and secondary pathways such as neuroinflammation². Prevalence of neurodegenerative disorders is on the rise due to the increase in the number of aged population. In a report published in 2012, it was stated that there exists 5.2 million people living with Alzheimer's in US. Prevalence above age 65 is quite high, with one in eight people suffering from the disease³. The number of patients suffering worldwide was reported to be 35.6 million in 2010 and this number is expected to double every 20 years due to the increase in life expectancy⁴. Approximately 50000 patients are diagnosed with Parkinson's disease each year⁵. Neurodegenerative disorders are not the only reason of neural cell loss; neuronal loss from traumatic injuries of brain and spinal cord also result in destructive effects on motor, sensory and autonomic functions. Spinal cord injury (SCI), for example, generally causes permanent loss of sensation and motor function below the site of injury. Mainly caused by motor vehicle and sports accidents, spinal cord injuries lead

to lifelong disabilities if not fatal. According to the 2014 report of National Spinal Cord Injury Statistical Center, approximately 12500 new injuries occur each year and there exists approximately 276000 patients suffering from SCI living in US. Less than 1% of patients could recover completely after injury⁶.

Peripheral nervous system (PNS) is even more open to injuries due to the absence of the cranium and spine which protect central nervous system (CNS) from external damages. PNS injuries result in a high number of patients suffering from neuropathic pain which brings an economic burden resulting from health care costs. 20 million patients suffer from peripheral neuropathy and approximately \$150 billion is spent annually for health care due to nerve injuries in United States⁷. Approximately a hundred thousand patients undergo peripheral nerve surgeries per year in United States and Europe⁸.

The number of patients suffering from neurological disorders and traumatic injuries demand the development of successful therapies for the restoration of lost neurological functions. Although the demand is high mostly due to age-related neurodegenerative disorders and injuries resulting from motor vehicle accidents, such efficient therapies are not present, yet. In order to develop effective therapies for the induction of neural regeneration, one must first understand the reasons behind the poor regenerative capacity of neural tissues, especially through the intrinsic properties of extracellular matrix (ECM) in health and disease state. Different properties of the ECM in CNS and PNS should also be considered when designing therapies, since such differences lead to differing regenerative capacity in these tissues.

1.2. Role of ECM in poor regenerative capacity of central nervous system

Fully restoring functional capacities of neurons after damage due to traumatic injuries or neurodegenerative disorders is still not possible. Although conventional therapies provide neural regeneration up to a certain level, they are not as efficient as desired. The term “neural regeneration” refers to several different mechanisms for the restoration of the functions of degenerated neural tissue. It either occurs by the generation of new neurons from the progenitor cells residing near the damaged area, generation of new synapses by the neurons that survive after the damage or by repair of the damaged axons/myelin sheaths to prevent secondary cell loss after injury. These strategies for regeneration of the nervous system are ideally combined to achieve a functional outcome. However, due to some intrinsic properties such as the unproliferative nature of neurons, presence of progenitors in localized areas and in low numbers together with the upregulation of inhibitory elements upon injury leading to glial scar formation, regeneration capacity of the central nervous system is quite limited.

Central nervous system consists of a dense network of cells leaving a smaller volume for the extracellular matrix components, 10-20% for the brain unlike most other tissues⁹. Before 1971, brain was thought to be totally free of ECM and consisting of only a very dense network of cells¹⁰. Yet, this small amount of ECM is sufficient to modulate the survival, neurite outgrowth and synaptic plasticity of neurons. Neural ECM undergoes prominent changes during the maturation of the nervous system.

Embryonic and postnatal ECM is rather a loose network, while some condensation occurs through adulthood starting from the postnatal period, according to specific needs of neural cells¹¹⁻¹². In fact, the balance among ECM components is so important that any imbalance leads to massive degeneration in the CNS.

Being quite different from that of other tissues, ECM content also differs dramatically in between specific locations in the CNS. Three different ECM types are deposited in basement membrane, perineuronal nets (PNN) and the gap filling interstitial matrix. Basement membrane (basal lamina) is more similar to other tissues in terms of the ECM components it contains. Collagen, entactin (laminin–nidogen complexes), fibronectin, dystroglycan and perlecan are the predominant proteins and proteoglycans of basement membrane¹³. This structure is associated with blood vessels and is important in the stabilization of the blood-brain barrier¹⁴.

Perineuronal nets are the dense network of matrix around cell bodies and mainly responsible for the maintenance of synaptic plasticity. Main components of PNN are hyaluronan, chondroitin sulfate proteoglycans (CSPGs) and tenascins^{13, 15}. Being absent in embryonic tissue, these structures form a barrier around the cells in mature tissue and prevent the formation of new synaptic connections while preserving the ones that are already formed. Mammalian nervous system has a considerable capacity for plasticity that continues through postnatal development until maturation of the CNS. During this period, named as critical period, nervous system continues to develop and produces new neural connections that are influenced by experience. Plasticity declines upon the closure of the critical period, the time of which coincides

with the formation of perineuronal nets indicating these structures as barriers to the establishment of new connections¹⁵.

Being absent from perineuronal nets, small amounts of fiber forming collagens and elastins along with fibronectin and laminin are present in the interstitial matrix. Sulfated proteoglycans and hyaluronan along with tenascins are the main components of this ECM¹³.

Proteoglycans (PGs) are the predominant components of the central nervous system ECM. Proteoglycans are classified into four main groups depending on the glycosaminoglycan (GAG) chain; chondroitin sulfate proteoglycans (CSPGs), heparan sulfate proteoglycans (HSPGs), dermatan sulfate proteoglycans (DSPGs) and keratan sulfate proteoglycans (KSPGs). CSPGs and HSPGs are the main PGs of the CNS, CSPGs being the most abundant¹⁵. Each group is also composed of a variety of PGs due to the different protein cores carrying GAG chains. Lecticans are the most abundant CSPG type in the brain and are classified into four subgroups. Neurocan and brevican are neural tissue specific lecticans while aggrecan and versican are common to the ECM of other tissues¹⁶. Besides sulfated GAGs that are found attached to protein cores, hyaluronic acid (HA) is also abundant in the CNS tissues. HA is a large polymeric carbohydrate chain that is nonsulfated and does not contain a protein core. It binds to other proteoglycans and forms a dense mesh-like ECM structure in perineuronal nets¹⁷.

Depending on the protein core and GAG chain, proteoglycans in the CNS are permissive or non-permissive to neurite extension. GAGs can exert their effects on

cells either by inducing direct signaling through cell surface receptors (like HA achieves through CD44 receptor) or by their affinity to growth activating molecules. Affinity of HSPGs to growth factors is quite important in the modulation of neuronal function, especially through FGF signaling (improved upon syndecan binding to FGF¹⁸). CSPGs can also bind a range of molecules including semaphorin 5A, binding to which is found to change permissive nature to inhibitory¹⁹. Binding of CSPGs to other ECM proteins, such as tenascin and laminin blocks the axon growth promoting ability of these proteins and thus inhibits axon growth²⁰. While binding of CSPG to laminin is inhibitory, binding by HSPG improves axonal elongation²¹. Binding of HSPGs to the same receptor in a competitive manner (as in the case of semaphorin 5A) abolishes the inhibitory effect of CSPG on neurotrophin responsiveness¹⁹. CSPGs can also inhibit neural regeneration by limiting calcium availability either by directly binding to extracellular calcium or binding to calcium channels, thereby preventing its uptake by neurons²². Binding of CSPGs to PTPsigma receptor on neurons leads to the phosphorylation of neurotrophin receptors TrkB and TrkC, possibly leading to a downregulation of responsiveness to neurotrophins²³. Inhibitory effects of CSPGs are either due to the protein core or sulfated GAG chain, depending on the CSPG type. For versican, neurocan and phosphacan, the protein core is indispensable for axonal inhibition, while the GAG chain is responsible for inhibition in aggrecan and brevican. Chondroitinase treatment, which degrades the GAG chains, eliminates the inhibitory effect of aggrecan and brevican while being ineffective in interfering with the function of the other CSPGs²⁴⁻²⁹.

Although they limit the regenerative capacity of CNS with their inherent inhibitory properties, CSPGs have indispensable roles in neural development, both for cell migration, and formation and stabilization of neural connections. Neural crest cells avoid migrating through local environments that are abundant in CSPG. Presence of CSPGs along their path therefore limits their movements and creates a trajectory for them to migrate to their defined positions³⁰. Repulsive nature of CSPGs for growth cones is also quite important in axonal path finding and the correct formation of neural networks during embryonic development as well as postnatal development in critical period. Treating developing nervous system with chondroitinase ABC leads to various axon path finding errors, verifying the importance of CSPGs in the process³¹. CSPGs are also important in adult CNS for proper stabilization of synaptic connections and prevention of improper axonal sprouting and formation of disorganized neural networks³².

Having a regulatory function on neural network formation in development and keeping connections stable, CSPGs are quite important in healthy CNS. However they should be kept in balance in order not to over-inhibit neural functions. This balance becomes disorganized upon a trauma leading to production of reactive astrocytes. Astrocytes start to proliferate and deposit dense ECM in the injury site and fill the gap caused by the injury. This preserves the overall structure of the tissue, stabilize blood brain barrier and limits the damage to injury site, thereby preventing secondary damage³³. Although important for the overall health of CNS, this hastily-deposited ECM is responsible for the absence of regeneration in CNS injuries. This *de novo* produced tissue is called as glial scar, and it is impermeable to axonal

sprouting due to the abundance of CSPGs. Hence, nearby healthy cells that have the potential to make new synaptic connections to tolerate the neural cell loss in the injury site cannot enter into the area. Besides, synaptic connections cannot be established by undamaged cells in the injury site due to the inhibitory effect of CSPGs on plasticity.

Besides CSPGs, myelin components are also inhibitory for neural regeneration after CNS injury. The major inhibitory myelin components can be listed as myelin-associated glycoprotein (MAG), Nogo-A, oligodendrocyte myelin glycoprotein (OMgP), and myelin lipid sulfatide³⁴⁻³⁵. Myelin inhibitors MAG, Nogo-A and OMgP are found to exert their inhibitory effect through binding to Nogo 66 receptor 1 (NgR1) on neurons³⁶⁻³⁹. Presence of NgR1 is essential for growth cone collapse upon encountering myelin inhibitors⁴⁰⁻⁴¹. Paired immunoglobulin-like receptor B (PirB), and its human homolog (LILRB2) are recently identified as a receptor for these three myelin inhibitors and blocking this receptor along with NgR1 largely eliminates myelin inhibition of neurite outgrowth⁴².

A third type of CNS inhibitory molecules includes the axon guidance molecules semaphorin and ephrin. Most semaphorins are inhibitory for neurons and play a role in network stabilization by limiting neurite outgrowth. In the glial scar, class 3 semaphorins (Sema3s) contribute to the inhibitory environment in a CSPG dependent manner. Interfering with the CSPG-Sema3s interaction eliminates the inhibitory nature of this molecule *in vitro*⁴³. Besides Sema3s, membrane associated semaphorins like Sema4D are also upregulated in the glial scar and inhibit neurite outgrowth⁴⁴. Ephrins exert their inhibitory effect by binding to EphA and EphB

receptor tyrosine kinase on neural cell surface. EphrinB3 is expressed in CNS myelin, ephrin B2 is upregulated upon injury by reactive astrocytes and ephrinA5 expression is found to be increased in cortical lesion and they all inhibit neurite outgrowth⁴⁵⁻⁴⁷.

1.3. Role of ECM in regenerative capacity of the peripheral nervous system

Reaction of the CNS to any injury leading to scar tissue formation acts as a barrier for tissue regeneration, while it is the opposite in PNS. Different types of glial cells in CNS and PNS, astrocytes and Schwann cells, lead to formation of completely different environments for axons struggling to heal after an injury. While astrocytes produce a growth inhibitory environment consisting of chemorepulsive guidance molecules, Schwann cells produce a growth promoting environment by the secretion of supportive ECM molecules, neurotrophins, as well as dealing with the clearance of inhibitory myelin products after injury. A chain of events occurring after injury in the peripheral nerve leads to formation of a permissive environment for axonal regeneration. Wallerian degeneration in the distal stump of the injured nerve leads to fragmentation of the axon and associated myelin products. Fragmented products are then cleared by macrophages that are recruited to the injury site⁴⁸. Myelin associated proteins are known to inhibit axonal elongation and their clearance in PNS after injury forms a permissive environment for regeneration as opposed to that in CNS. While the distal stump undergoes Wallerian degeneration, axonal regeneration starts from the proximal end. Schwann cells dedifferentiate and start to proliferate. They

myelinate newly elongating axon thus providing an electrical insulation to the regenerating axon⁴⁹.

Permissive environment, axon guidance cues and intrinsic growth capacity of peripheral nerves are crucial for the successful regeneration of the PNS. Unlike their CNS counterparts, peripheral nerves have an intrinsic growth capacity that is activated upon injury. Cyclic adenosine monophosphate (cAMP) level is upregulated upon peripheral nerve injury which activates protein kinase A⁵⁰⁻⁵¹. Protein kinase A is thought to take role in cytoskeletal reorganization by inhibiting RhoA. RhoA is normally activated by the presence of myelin debris and its activation halts the cytoskeletal reorganization required for axonal elongation. Hence, its inhibition by protein kinase A makes axon's cytoskeletal dynamics favorable for regeneration⁵². In addition, cAMP has a transcriptional role by activating cAMP response element binding protein (CREB) which in turn enables cell to overcome myelin associated inhibition of neurite outgrowth⁵³. Upregulation of intracellular cAMP levels also leads to an increase in cell-surface localization of TrkB receptors, which in turn increases the axon's responsiveness to neurotrophins⁵⁴⁻⁵⁵.

Unlike the CNS, PNS is abundant in basal lamina. Secreted by Schwann cells, the basal lamina of peripheral nerve tissue contains laminin, type IV collagen, and heparan sulfate proteoglycans⁵⁶. Being present in healthy peripheral nerve, basal lamina production is upregulated in the injured nerve⁴⁸. In addition to their importance in the myelination of nerves by Schwann cells, basal lamina components are also important for axonal elongation. Laminin is a positive regulator of neurite outgrowth and has been found to be over expressed after peripheral nerve injury.

Abolishing the expression of laminin by Schwann cells severely impairs axon regeneration. Besides their role in neurite outgrowth, laminins are also important for the proper differentiation of Schwann cells and myelination of growing axons⁵⁷. In addition to laminin, NGF, BDNF, GDNF, IGF, CNTF and FGF-2 are also upregulated in the injured peripheral nerve. Increased amount of these growth factors in the injury site provide a better axonal elongation along with proper myelination by Schwann cells⁵⁸.

Myelin inhibition, which severely constraints the regeneration of the CNS, is not a major issue in the PNS. There are several routes by which peripheral nerves deal with this problem. Responsiveness of the axon to myelin products is thought to be different in the PNS when compared to the CNS, which is actually related to the intrinsic growth capacity of peripheral nerves. Intracellular cAMP levels, for example, determine whether MAG will act as growth inhibitory or not. Besides, lack of some myelin proteins in PNS myelin is thought to make PNS myelin less inhibitory though major components such as MAG still exist in its structure⁴⁸. Clearance of myelin from the injury site, which does not take place in CNS, is another important concern. Myelin is rapidly cleared away from the injured peripheral nerve by the coordinated action of Schwann cells and macrophages during Wallerian degeneration. In the early stages after injury, Schwann cells are the main phagocytic cells in the injured nerve and they start myelin clearance as early as 5 hours after injury. They secrete phospholipase A2 which degrades phosphatidylcholines that are abundant in myelin. Lysophosphatidylcholine is produced by the hydrolysis of phosphatidylcholine by phospholipase A2 and it has a

myelinolytic activity⁵⁹. Schwann cells also recruit macrophages to the injured peripheral nerve by secreting several chemokines including TNF- α , LIF, IL-1 α and IL-1 β , IL-6⁶⁰. Infiltration of macrophages to the nerve is essential in clearing myelin debris from the distal stump during Wallerian degeneration. Inhibitory myelin proteins are not cleared in CNS due to the lack of macrophages in the lesion site, infiltration of which is restricted by the blood-brain barrier.

Following peripheral nerve injury, fibrinogen from blood starts to infiltrate into the nerve where it is converted to fibrin. Accumulated fibrin prevents Schwann cell migration to the injury site and prevents remyelination⁶¹. Schwann cells upregulate production of tissue plasminogen activator (tPA) and thereby activate fibrinolytic pathway. Lysis of fibrin enables Schwann cell to enter the injured nerve and start myelination of the growing axon⁶².

1.4. Mimicking healthy ECM with synthetic scaffolds

Limited capacity of regeneration in neural tissues is aimed to be improved by materials designed for stem cell culture, differentiation and transplantation, which are called ‘scaffolds’. Besides being used as a vehicle for transplantation of cells, scaffolds can also be designed to present biological signals specific to the tissue of interest or to provide slow release of encapsulated therapeutical small molecules.

Stem cells are promising candidates for treatment of neurological disorders since they can differentiate into neural cells when induced appropriately. Their differentiation can be enhanced by using bioactive materials, which can also be used

as vehicles for cell transplantation to damage site. Various synthetic materials have been studied as scaffolds for their *in vitro* and *in vivo* potential in neural differentiation and nerve regeneration⁶³⁻⁶⁶.

Natural cues for directing cell fate consist of different chemical, physical and biological signals in the extracellular matrix, neighboring cell interactions, soluble factors such as chemokines, growth factors and hormones, and the inherited potency of the cell⁶⁷. Combination of these factors and their interactions affects ultimate cellular differentiation mechanisms (Figure 1.1). Understanding this complex environment and mimicking it as efficiently as possible will enable directing cell differentiation in a desired manner. One way of mimicking the natural environment of cells *in vitro* is to supply soluble factors in the culture media. This approach is relatively simple but costly since it requires high concentrations of soluble factors. Also it is not very efficient since it does not mimic the mechanical and structural properties of the cells' natural environment. Cells are embedded in tissues in a three-dimensional (3D) manner and they can migrate or grow extensions such as axons and dendrites of neural cells. Physical properties of the 3D network including stiffness, roughness and pore size are also important and should be considered while designing a synthetic system that mimics the native tissue. As conventional *in vitro* cell culture surfaces cannot provide an appropriate environment to cells, 3D scaffolds combined with soluble signals or bioactive signals tethered to the surface are used to mimic the natural neural niche.

Natural extracellular matrix proteins or synthetic scaffolds have been used for developing 3D scaffolds to mimic biological, chemical and mechanical properties of

natural matrices of the cells. Although natural proteins are biocompatible, it is not easy to functionalize these scaffolds with desired bioactive groups, or to manipulate their mechanical properties such as stiffness. Thus, these scaffolds might not be sufficient to direct the differentiation of cells especially into complex cells like neurons⁶⁸⁻⁷². On the other hand, synthetic materials can be synthesized with desired functionalities such as specific bioactive groups, stiffness and roughness in order to mimic the natural environment of cells.

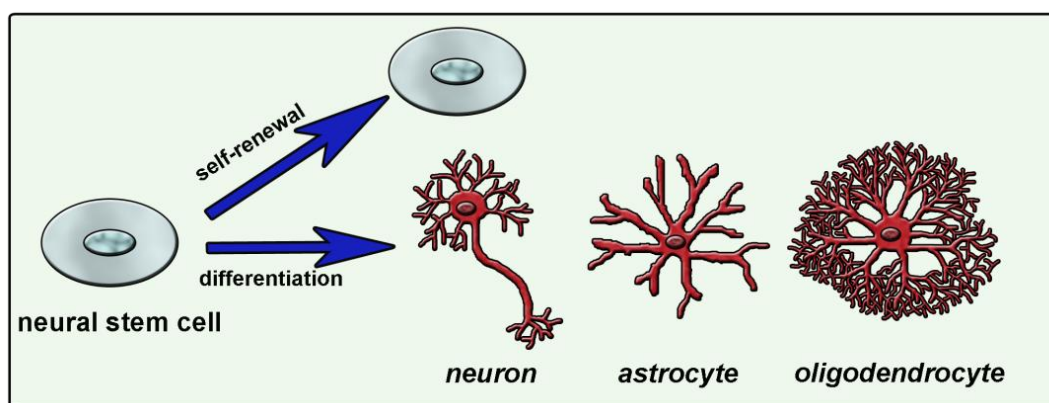


Figure 1.1 . Neural stem cell fate determination is mainly guided by extracellular matrix molecules, soluble factors and cell-to-cell interactions. Reproduced by permission of The Royal Society of Chemistry⁷³.

1.4.1. Different approaches in the design of synthetic materials for neural differentiation

A variety of different methods can be used to develop scaffold materials with desired properties including support of cell survival and induction of differentiation

into desired cell type. Besides changing material type, nerve regeneration studies have also focused on designing scaffolds with incorporated bioactivity for induction of neurogenesis. One approach to induce differentiation is adding soluble inducers while culturing cells on non-bioactive scaffolds. In this approach, scaffold is a better environment for cell survival compared to the tissue culture plate. However, as the scaffold itself does not include any signals for differentiation, differentiation is induced solely by the soluble inducers added to the growth media. Soluble inducers commonly used for neural differentiation include neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and retinoic acid (RA).

Another approach is embedding soluble inducers mentioned above into the scaffold in order to provide gradual release or tethering these inducers to the surface of the scaffold through functional groups in order to provide spatial organization to cells. In the case of gradual release, scaffold is not bioactive but has a role in differentiation by releasing inducers at desired concentrations in a longer time period. When tethering the inducers to the surface, the scaffold is bioactive; however, its bioactivity is not directly related to differentiation process. Bioactive groups are presented on the materials' surfaces so that they bind to the soluble inducers and present them similarly to the extracellular matrix components (such as heparan sulfate proteoglycans) presenting growth factors to the cells. Soluble factors can also be immobilized on materials' surfaces directly by covalent attachment without the use of growth factor affine proteoglycans⁷⁴. Loss of biological function

due to the hindering of bioactive part might pose a problem in this approach and it should be carefully analyzed to be sure to detect any loss of function.

Functionalization of the surface with bioactive signals is a method used for differentiation induction directed by scaffold. Bioactive groups can be either chemical groups inspired from the natural environment of cells in a specific tissue, like brain, or functional groups of natural inducers such as peptides found in neural differentiation inducing proteins. Besides addition of bioactive signals, scaffolds can also be functionalized by tuning their mechanical properties and physical morphology in order to support neural cell survival and differentiation. Since they present bioactive groups, these scaffolds can be used alone for induction of differentiation, depending on the complexity of the final cell type and potency of the starting cell for differentiation into the desired cell phenotype (Figure 1.2). Such a scaffold can induce differentiation into different neuronal subtypes. However, it might not be sufficient for maturation of the induced cells into functional cells. Additional inducers can be added to the culture medium to promote maturation or the scaffold can be functionalized with multiple bioactive groups to overcome maturation problem.

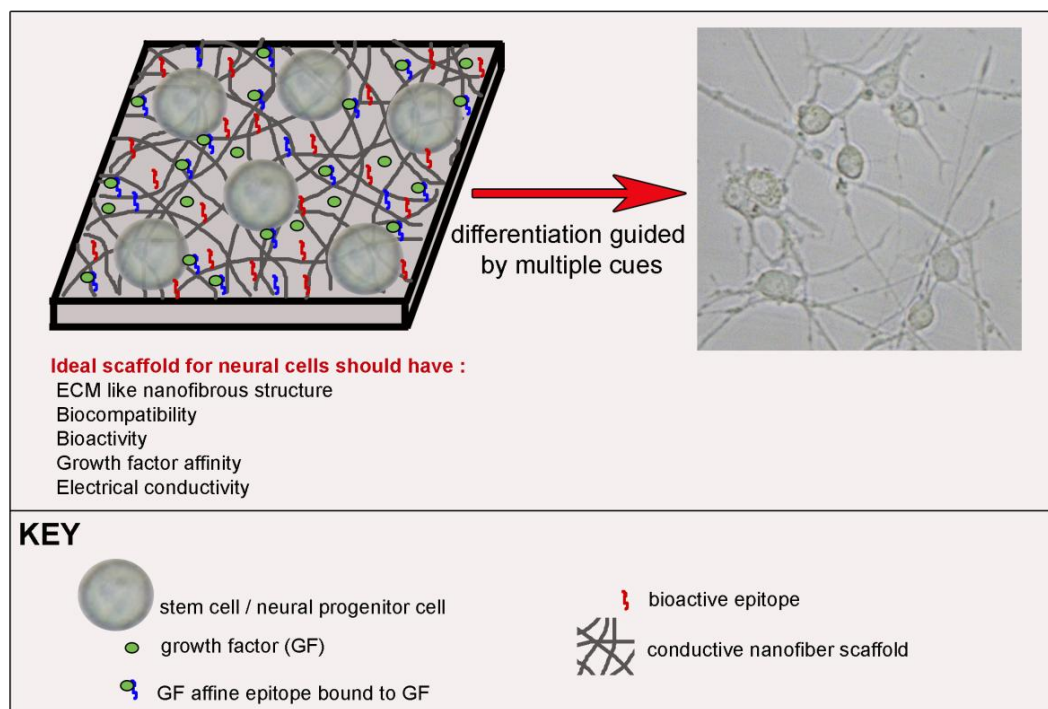


Figure 1.2. Bioactive scaffold design for neural differentiation of stem cells. Reproduced by permission of The Royal Society of Chemistry⁷³.

1.4.2. Peptide nanofibers as scaffolds for neural regeneration

Self-assembled peptide nanofiber scaffolds are powerful tools for producing bioactive scaffolds that are tailored according to the demands of specific cells. Peptide amphiphiles (PA), which are molecules consisting of a hydrophobic alkyl chain conjugated to a peptide sequence, can be used as building blocks of these scaffolds (Figure 1.3). Peptide part of the PA molecules usually contains an amino acid sequence containing β -sheet forming amino acids such as valine or alanine along with some charged amino acids and an epitope sequence for bioactivity⁷⁵. Charged amino acids are important both for solubility of the peptide and for

nanofiber formation. PA molecules of opposite charges come together by electrostatic interactions in aqueous environment along with the hydrophobic interactions of alkyl groups. Through these interactions, peptide nanofibers, that are 8-10 nm in diameter, are produced. These nanofibers are actually cylindrical micelles where hydrophobic tails are embedded in the nanofibers and hydrophilic peptide sequences containing the epitope remains on the periphery of the nanofibers. During formation of the nanofibers, water is trapped in the peptide nanofiber networks leading to formation of a hydrogel system. Physical structures of these nanofiber networks are quite similar to the structure of fibrous extracellular matrix⁷⁵⁻⁷⁶. Besides having a similar physical structure to ECM, these scaffolds can be produced with similar bioactivity by incorporating short peptide sequences in the epitope region, which are derived from the cell surface receptor interacting domains of ECM proteins. RGDS⁷⁷, KRSR⁷⁸, YIGSR⁷⁹, DGEA⁷⁸, REDV⁸⁰ and IKVAV⁸¹⁻⁸² are some of these sequences that have been previously derived from ECM proteins and used to obtain bioactive peptide nanofiber scaffolds.

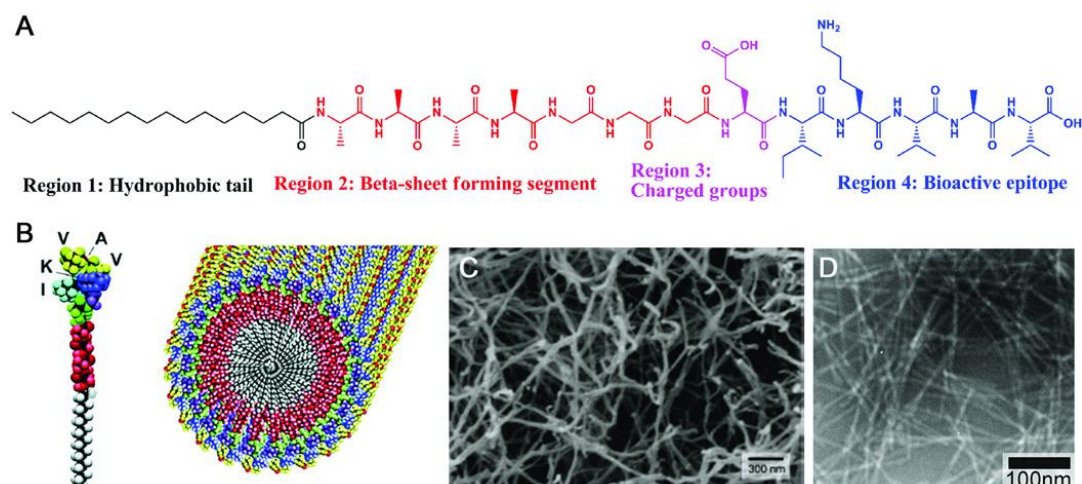


Figure 1. 3. Chemical structure, SEM and TEM images of a peptide amphiphile molecule. Functional units of a representative peptide amphiphile and an illustration of self assembly are given A and B. SEM image of peptide nanofiber networks and TEM image of individual nanofibers are given in C and D. From Silva G.A et al, Science 2004, 303(5662): 1352-1355. Reprinted with permission from AAAS⁸².

Peptide amphiphile nanofibers have been widely used for tissue engineering applications in different tissues. Using the same core structure while changing the epitope sequence enables direction of different cell types into desired functions. Use of Tenascin-C derived epitopes for example directed osteogenic differentiation of mesenchymal stem cells while use of glycosaminoglycan mimicking sequences lead angiogenesis by HUVECs *in vitro* and when applied *in vivo* by boosting the effect of VEGF⁸³⁻⁸⁴. Pre-chondrogenic cells cultured on these GAG mimetic PA nanofibers were found to better differentiate into chondrocytes when compared to those on epitope free scaffolds⁸⁵. Peptide amphiphile nanofibers can also be used as a template

for formation of mineralized tissue. Dopa containing PA nanofibers were found to initiate hydroxyapatite formation which derived differentiation of mesenchymal stem cells into mature osteoblasts⁸⁶.

Extracellular matrix derived PA nanofibers are also used for engineering neural tissues. PA nanofibers with laminin derived epitope (IKVAV) were found to induce selective differentiation of neural progenitor cells into neurons while suppressing astrocyte differentiation (Figure 1.4)⁸⁷. IKVAV presenting PA nanofibers were also used in spinal cord injury (SCI) model in mice. IKVAV-PA injection led to a reduction in astrogliosis and cell death while increasing the number of oligodendroglia at the site of injury. IKVAV peptide alone was not able to promote recovery, which showed that both nanofiber structure of the PA and the IKVAV sequence were required⁸⁸. Anatomical basis of the behavioral recovery coming from the injection of IKVAV-PA was analyzed in a separate study and the major factor for this improvement was found to be the increased density of serotonergic fibers close to the lesion⁸⁹.

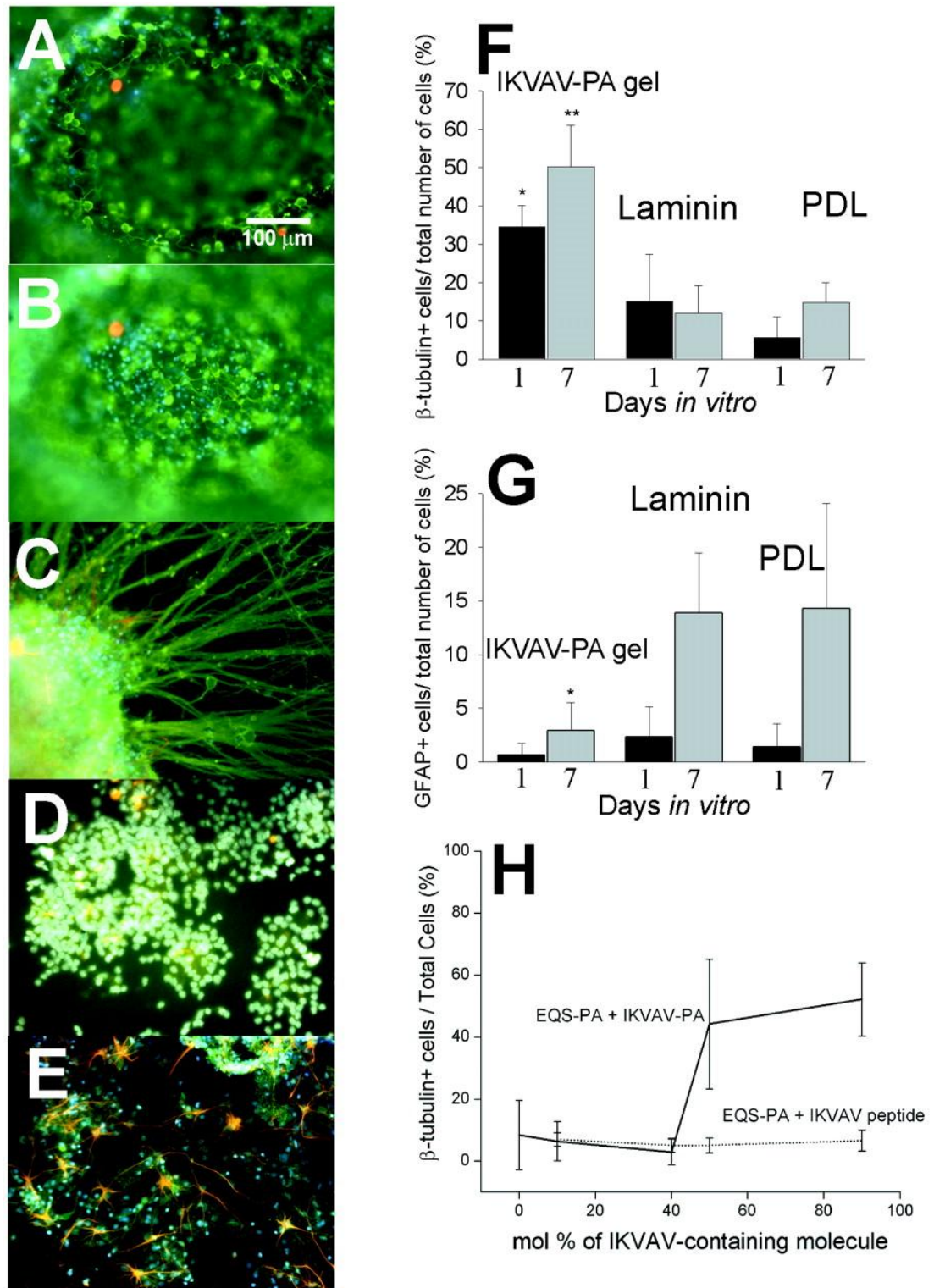


Figure 1. 4. Immunohistochemical analysis of neural progenitor cell differentiation on IKVAV-PA gels. Cells are stained against neural marker β -III-tubulin (green) and

astrocyte marker GFAP (red). Cells in PA gels apparently undergo differentiation towards neural lineage at day 7 (C) when compared to those on laminin (E). Images of cells at day 1 in PA gels (A, B) and on laminin (D) are also given. Quantifications given in F, G and H clearly demonstrate preference of neural lineage over glial lineage in IKVAV-PA gel when compared to cells on laminin and poly-D-lysine (PDL). EQS-PA is epitope free control peptide amphiphile. From Silva G.A et al, Science 2004, 303(5662): 1352-1355. Reprinted with permission from AAAS⁸².

Mechanical properties of PA nanofiber scaffolds can also be modified by using different proportions of signal incorporated PAs and non-bioactive but mechanically more stable molecules. Such an approach leads to formation of more stable and stiffer gels⁹⁰. In addition, their gelation kinetics can be modified by changing amino acid sequences that are important for structural properties of the fibers. By including more hydrophilic and bulky amino acids, gelation can be slowed down without disrupting bioactivity, which can be important for *in vivo* applications such as injections⁹¹.

The PA gels can also be used for efficient delivery of biological molecules to the treatment site. One such example is the delivery of Sonic hedgehog (SHH) via monodomain gels containing aligned PA nanofibers. SHH protein has important roles in peripheral nerve regeneration. In this animal model, SHH signaling was inhibited in rats and their cavernous nerve (CN) was crushed to form peripheral nerve damage. SHH was then delivered to crushed CN from PA gel and promoted

CN regeneration, suppressed penile apoptosis and improved erectile function. Such a treatment might be crucial in regeneration of the CN in prostatectomy and diabetic patients where SHH levels are decreased⁹².

Peptide amphiphiles can be mixed with biological materials also. A hybrid matrix approach with the combination of type I collagen and IKVAV containing peptide amphiphile nanofibers was shown to support neuronal survival, morphogenesis, and fine control over dendrite and axon growth of Purkinje cells. While collagen provided favorable mechanical support, laminin epitope concentration was adjusted to control matrix bioactivity by using PAs. Therefore, this system enabled the adjustment of laminin epitope density to control bioactivity without affecting its structural integrity⁷⁹.

CHAPTER 2

ECM MIMETIC PEPTIDE NANOFIBERS FOR NEURAL DIFFERENTIATION

This work is partially described in the following publication:

Mammadov B, Mammadov R, Guler MO, & Tekinay AB (2012) Cooperative effect of heparan sulfate and laminin mimetic peptide nanofibers on the promotion of neurite outgrowth. *Acta Biomater* 8(6):2077-2086.

2.1. INTRODUCTION

Traumatic nerve injuries including the peripheral nerve injuries and spinal cord injuries lead to disability of more than 17,000 people in the United States every year⁹³⁻⁹⁴. Although some of the peripheral injuries can partially recover spontaneously, some injuries do not recover even after painful surgeries. Poor regenerative capacity of neural tissue results in loss of motor abilities in patients leading to serious economical loss due to patient care expenses. For a successful therapeutic approach, the natural environment of cells that contains inducers of neural differentiation and neurite outgrowth should be considered. Neural extracellular matrix is composed of a variety of proteins and proteoglycans that are either permissive, some being instructive in fact, or restrictive for neurite outgrowth. Balance between the abundance of permissive and restrictive components is essential for the central nervous system to function accurately in terms of neurite outgrowth and synaptic activity. This balance becomes chaotic after CNS injury by upregulation of chondroitin sulfate proteoglycans, the main inhibitory ECM component, in glial scar which leads to poor regeneration⁹⁵⁻⁹⁷. Hence, any attempt aiming neural regeneration after injury should consider this unbalanced environment in which neurons that still survive reside in. In order for these remaining neurons to regain functionality, a properly designed extracellular niche with neurite outgrowth promoting clues should be provided. This designed niche should also aid neurons to overcome the pathologically abundant inhibitory components and make new synaptic connections to tolerate the loss of neurons caused by the injury⁹⁸.

Laminin is the major ECM element in CNS and PNS basement membranes and it is essential for axonal growth as well as for myelination of axons by Schwann cells⁹⁹. Laminin works as an effective neurite outgrowth promoting molecule *in vitro* as well¹⁰⁰⁻¹⁰⁴. Besides interacting with neurons through integrin receptors, laminin also interacts with other ECM components like heparan sulfate proteoglycans¹⁰⁵⁻¹⁰⁹. Laminin-HSPG complex formation upon their noncovalent interaction was observed to enhance neurite promoting activity *in vitro*^{21, 110}. Heparan sulfate and heparin are highly sulfated glycosaminoglycans that are known by their high affinity to growth factors. Growth factor-GAG interactions stabilize growth factors, increase their local concentration in the ECM and provide proper conformation for receptor interaction²¹. Hence, laminin-HSPG and laminin-heparin complexes have potential of being a dual inducer of neurite outgrowth by inducing cells through integrin receptors and HSPG stabilized growth factors simultaneously. Although affinity of HSPG and heparin to neurotrophins such as nerve growth factor (NGF) is moderate, their affinity to heparin enables their slow release in the abundance of heparin¹¹¹. Binding of NGF to heparin also enhances bioactivity of NGF¹¹¹. In addition, heparin stabilizes laminin networks and enhances their functions leading to improved neuritogenesis by heparin-laminin complexes¹¹²⁻¹¹³.

Bioactive epitopes from neurite inducer proteins have previously been used for decoration of scaffold surfaces instead of their full-length versions in order to obtain biofunctional materials. These synthetic materials can mimic the natural environment of neural cells and enhance neurite outgrowth as well as neural differentiation of stem cells. Peptide amphiphile (PA) nanofibers are among the most versatile

scaffolds because of their ease of modification by using simple chemistry. These nanofiber scaffolds can be designed according to the needs of a variety of tissues just by changing the biofunctional peptide sequence. PA molecules form nanofibers by internalization of hydrophobic alkyl tails in aqueous environment where epitope forming amino acids are presented on the surface of the nanofibers. Laminin epitope (IKVAV) carrying peptide amphiphiles were shown to efficiently induce neural differentiation of stem cells⁸². These laminin-derived PAs were also tested *in vivo* and found to promote axonal growth, serotonergic fiber plasticity and inhibit glial scar formation after spinal cord injury⁸⁸⁻⁸⁹.

In this study, heparan sulfate mimicking PA (PA-GAG) molecules were used for the first time for promoting neurite outgrowth. Heparan sulfate interacts with a variety of ECM molecules as well as growth factors in microenvironment of cells. Herein, a self-assembling peptide scaffold was formed by heparan sulfate mimicking peptide nanofibers along with laminin derived peptide nanofibers. Heparan sulfate mimicking functional groups on this scaffold provide a close similarity to permissive elements of neural ECM where laminin interacts with HSPG. Full-length laminin-HSPG complexes were shown to be more effective in neurite outgrowth when compared to laminin alone. In accordance with this finding, heparan sulfate mimicking moieties and laminin derived epitopes presented on PA nanofibers were analyzed to find any cooperative work in neurite outgrowth process resulting in a more effective neural induction when compared to these epitopes alone. In addition, cells cultured on these PA scaffolds were analyzed under inhibitory conditions provided by chondroitin sulfate proteoglycans in order to find any effect of PA

nanofibers in overcoming inhibition of axonal growth. Inhibition of axonal regeneration by extracellular environmental elements in CNS is the main problem leading poor regeneration after any damage to CNS and overcoming this barrier is a promising approach for therapy if it can be achieved.

2.2. MATERIALS and METHODS

2.2.1. Materials

9-fluorenylmethoxycarbonyl (Fmoc) and tert-butoxycarbonyl (Boc) protected amino acids, [4-[a-(20,40-dimethoxyphenyl)Fmocaminomethyl]enoxy]acetamidonorleucyl-MBHA resin(Rink amide MBHA resin), Fmoc-Asp(OtBu)-Wang resin and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from NovaBiochem and ABCR. The other chemicals were purchased from Fisher, Merck, Alfa Aesar or Aldrich. NGF was purchased from Thermo Scientific (RP-86969). Poly-D-Lysine (PDL) and collagen type I (C7661) were purchased from Sigma. Chondroitin sulfate proteoglycan mixture (CSPG) was purchased from Millipore (CC117). Live/Dead Assay (L3224) and other cell culture materials were purchased from Invitrogen. Proliferation assay was purchased from Roche (11647229001). Antibodies and reagents for ELISA assay were purchased from R&D.

2.2.2. Synthesis and purification of peptide amphiphiles

PAs were constructed on either Rink Amide MBHA resin or Fmoc- Glu (OtBu)-Wang resin. Amino acid couplings were performed with two equivalents of Fmoc protected amino acid, 1.95 equivalents HBTU and three equivalents of N,N-diisopropylethylamine (DIEA) for 2 h. Fmoc removal was performed with 20% piperidine/dimethylformamide solution (DMF) for 20 min. 10% acetic anhydride solution in DMF was used to block remaining free amine groups after amino acid coupling. After each step, resin was washed with 3 times DMF, 3 times DCM and 3 times DMF again, respectively. Sulfobenzoic acid was added to the side chain of lysine to synthesize sulfonated PA (PA-GAG). A lysine residue with 4-methytrityl (Mtt) side chain protection was used for selective deprotection of amine groups. Mtt removal was performed by shaking resins for 5 min with TFA:TIS:H₂O:DCM in the ratio of 5:2.5:2.5:90. Cleavage of the PAs from the resin was carried out with a mixture of TFA:TIS:H₂O in the ratio of 95:2.5:2.5 for 2 h. Excess TFA was removed by rotary evaporation. The remaining viscous PA solution was triturated with ice-cold ether and the resulting white precipitate was dried under vacuum. PAs were characterized by liquid chromatography–mass spectrometry (LC–MS). Mass spectrum was obtained with Agilent LC-MS equipped with Agilent 6530 Q-TOF with an ESI source and Zorbax Extend-C18 2.1 x 50 mm column for basic conditions and Zorbax SB-C8 4.6 mm x 100 mm column for acidic conditions. A gradient of (a) water (0.1% formic acid or 0.1% NH₄OH) and (b) acetonitrile (0.1% formic acid or 0.1% NH₄OH) was used. In order to purify the peptides, Agilent preparative reverse-phase HPLC system equipped with Zorbax Extend-C18 21.2 x 150 mm column was

used for basic conditions and Zorbax SB-C8 21.2 x 150 mm column was used for acidic conditions. A gradient of (a) water (0.1% TFA or 0.1% NH₄OH) and (b) acetonitrile (0.1% TFA or 0.1% NH₄OH) was used.

2.2.3. SEM imaging of PA nanofiber matrices

PA nanofiber networks were observed by imaging with a scanning electron microscope. Oppositely charged PA solutions (1 wt %) were mixed in appropriate volume ratio (final volume is 50 μ L) to produce gels with neutral charge. Gels were dehydrated by incubating sample in 20%, 40%, 60%, 80% and 100% v/v ethanol/water sequentially. They were critical point-dried by using Autosamdri®-815B equipment from Tousimis. Dried PA gels were coated with 9 nm Au/Pd and SEM (FEI Quanta 200 FEG) images were taken by using an Everhart–Thornley Detector (ETD) at high vacuum mode with 15 keV beam energy.

2.2.4. AFM imaging of PA nanofiber matrices

PA nanofibers were also observed by imaging with atomic force microscopy (AFM). Oppositely charged PA solutions (0.04 wt%) were mixed in appropriate volume ratios (final volume being 100 μ L) to produce nanofibers with neutral charge. PA solutions were drop-cast onto a 1 cm² silicon wafer. After 1 min, solvent on the wafer was removed by using dust-free tissue paper, and the rest was air-dried. Non-contact mode AFM was performed by using model MFP-30 from Asylum Research. Resonance frequency and spring constant of AFM tips used in this study were 150 kHz and 5 N m⁻¹, respectively. All images were taken with a 0.5–1 Hz scan rate.

2.2.5. Circular dichroism

A JASCO J815 CD spectrometer was used at room temperature. Oppositely charged 2.5×10^{-4} M PA solutions were mixed in appropriate volume ratios (final volume being 500 μ L) to produce nanofibers with net neutral charge. Measurements were carried out from 300 nm to 190 nm; data interval and data pitch being 0.1 nm, and scanning speed being 100 nm min⁻¹, all measurements with three accumulations. Digital Integration Time (DIT) was selected as 1 s, band width as 1 nm, and the sensitivity was standard.

2.2.6. Oscillatory rheology

Oscillatory rheology measurements were performed with Anton Paar Physica RM301 Rheometer operating with a 25 mm parallel plate configuration at 25 °C. Each sample of 250 μ L total volumes with a final PA concentration of 1 wt% and equimolar concentrations of each PA component was carefully loaded onto the center of the lower plate and incubated for 10 min before measurement. After equilibration, the upper plate was lowered to a gap distance of 0.5 mm. Storage moduli (G') and loss moduli (G'') values were scanned from 100 rad s⁻¹ to 0.1 rad s⁻¹ of angular frequency, with a 0.5% shear strain. Three samples were measured for each PA gel.

2.2.7. NGF release assay by ELISA

500 ng NGF was encapsulated into PA-LN+PA-E (n = 5) or PA-LN+PA-GAG (n=4) gels. Molar ratios of PAs were adjusted to neutralize the final charge, similar to cell culture experiments. 25 μ L of positively charged PA (PA-LN) was mixed with 1 μ L

NGF (500 ng) and then 25 μ L from either of PA-GAG or PA-E was added to induce gel formation. After 30 min incubation at 37 °C for a stable gel formation, 250 μ L PBS was added carefully without disturbing the gel at the bottom of tubes. For measurement of NGF release, 200 μ L PBS over gels was collected at different time points (3 h, 2.5 days, 5 days and 10 days) while the same volume of fresh buffer was added carefully. NGF ELISA (R&D Systems) was performed according to the manufacturer's instructions to detect NGF concentration in these samples.

2.2.8. PA charge optimization for PC-12 differentiation

Charge of the PA nanofiber scaffolds was optimized by using scaffolds with -1 charge and those with no net charge. Equal volumes of oppositely charged PA solutions were used in both cases. Concentration of each PA solution was adjusted according to the desired net charge. As an example, to obtain a -1 charged PA-LN+PA-GAG gel, 1.5 mM PA-GAG was mixed with 1 mM PA-LN at equal volumes while both PAs of 1.5 mM was used to obtain a gel with no net charge. Charge and molecular weight of all PAs used in the study are provided in Table 2.1.

Table 2.1. Charge, sequence and molecular weight of PA molecules used in the study.

PA molecule	Sequence	Molecular weight (g/mole)	Net charge at pH7
PA-LN	C ₁₂ -VVAGKKIKVAV	1292.74	+3
PA-GAG	C ₁₂ -VVAGEGD(K-pbs)S	1226	-3
PA-K	C ₁₂ -VVAGK	654	+1
PA-E	C ₁₂ -VVAGE	655.82	-2

After formation of the gels, plates were incubated at 37 °C for 1 h after which they were dried overnight in laminar flow hood. They were sterilized under UV for 1 h before seeding cells.

5x10³cells/well were seeded in maintenance medium and the day after seeding, media over cells was replaced with the induction media. Induction medium is composed of RPMI, 2% horse serum, 1% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 100 ng/mL NGF. Media was changed every 3 days during experiment. Images were taken at day 7 and neurites were quantified from these images by using Image J software.

2.2.9. PA concentration optimization for PC-12 differentiation

PA gels with no net charge were prepared by mixing 50 µL of negatively charged component with equal volume of the positively charged component in 96 well-plates.

Bioactive PA components (PA-LN and PA-GAG) were used in three different concentrations: 0.5, 1.5, 3 mM; and the concentration of the other component (PA-E or PA-K) was adjusted so that the gel will have neutral charge. PA-LN is combined with either PA-GAG or PA-E and PA-GAG is combined with either PA-LN or PA-K. As PC-12 cells are suspension cells and do not attach to tissue culture plate, uncoated surface was not included in the experiment. Collagen I coated surfaces (35 μ L/well, 0.01%) were used as positive control.

Plate preparation, cell seeding and induction was carried out by following the protocol in the section 2.2.8. Images were taken at day 7 and neurites were quantified from these images by using Image J software.

2.2.10. Optimization of neural induction medium

Three different induction media were tried to find the optimal medium for neural induction. 5×10^3 cells/well was seeded to PA coated (bioactive PA concentration being 1.5 mM in each combination and net charge being 0) or Collagen I (0.01%) coated surfaces. Coating was done as previously mentioned. The day after seeding, neural induction was carried out by changing media with either DMEM, RPMI or MEM containing 2% horse serum, 1% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 100 ng/mL NGF. Media was changed every 3 days during experiment. Images were taken at day 7 and neurites were quantified from these images by using Image J software.

2.2.11. Optimization of cell density

To find out the optimal cell density for neural differentiation and neurite outgrowth by PC-12 cells, cells were seeded at three different densities. PA and collagen I coating was done as previously mentioned and 2.5×10^3 , 5×10^3 and 1×10^4 cells/well were seeded to coated 96-well plates. The day after seeding, cells were induced for neural differentiation by changing the media with induction medium (MEM, 2% horse serum, 1% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 100 ng/mL NGF). Media was changed every 3 days during experiment. Images were taken at day 7 and neurites were quantified from these images by using Image J software.

2.2.12. Optimization of NGF amount

In order to determine the optimal concentration of NGF used for neural induction, cells were induced with 25, 50, or 100 ng/mL NGF. PA and collagen I coating was done as previously mentioned and 5×10^3 cells/well were seeded to coated 96-well plate wells. The day after seeding, cells were induced for neural differentiation by changing the media with induction medium (MEM, 2% horse serum, 1% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and NGF). Induction media without NGF was used as negative control and no neurite outgrowth was observed in the absence of NGF. Media was changed every 3 days during experiment. Images were taken at day 7 and neurites were quantified from these images by using Image J software.

2.2.13. Optimization of PA coating

As there was some inhomogeneity in coated surfaces when 100 μL /well PA volume was used for coating 96 well-plates, a lower volume is tried to get rid of those defects. 80 μL /well total PA volume was used for coating. It consisted of 40 μL of positively charged PA component along with the 40 μL of negatively charged PA component. The rest of the coating procedure was same with the above mentioned protocols. 5×10^3 cells/well were seeded to PA coated surfaces and the day after seeding neural induction was carried out by changing the media with induction medium (MEM, 2% horse serum, 1% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 20 ng/mL NGF). Media was changed every 3 days during experiment. Images were taken at day 7 and neurites were quantified from these images by using Image J software.

2.2.14. Analysis of cell viability on PA nanofiber coated surfaces

96-well plates were coated with PA nanofibers or 0.1% PDL as described above. 5×10^3 cells/well were seeded and cultured in culture media as described above for three days. Live/Dead assay (Invitrogen) was carried out at day 3 by treating cells with 2 μM Calcein AM and 2 μM ethidium homodimer I (EthD-1) for 30 min at RT. Images were taken at four random points per well with a fluorescent microscope at 200x magnification. All samples were studied in triplicate. Live (stained green by Calcein AM metabolization) and dead (stained red by EthD-1 internalization) cells were counted by using Image J and live cell percentage was calculated for each sample. Results were evaluated by one-way ANOVA for statistical analysis.

2.2.15. SEM imaging of PC-12 cells on PA substrates

13 mm glass coverslips placed in 24 well plates were coated with PA gels similar to other experiments except that gel volume was 300 μL /well (150 μL /each PA solution). 3×10^4 cells/well were seeded and samples were prepared for SEM imaging at day 3 and day 6 after neural induction ($n = 2$ /experimental group). Cells were first fixed with 2% glutaraldehyde (diluted in PBS, 400 μL /well) for 1 h and then with 1% OsO_4 for 1 h at RT. After washing with PBS, dehydration steps were carried out by dipping coverslips to 20%, 40%, 60%, 80% and 100% ethanol (v/v) for 30 sec - 1 min/each. Samples were then dried by critical point drying and coated with Au/Pd, 3 nm twice before imaging. SEM imaging was carried out at 30 kV with ETD detector at high vacuum mode.

2.2.16. BrdU assay for analysis of cell proliferation

3×10^3 cells were seeded (96 well-plate) on coated surfaces and BrdU assay was carried out at day 7 after neural induction. NGF induced cells were compared to cells cultured in the absence of NGF ($n = 3$ /each experimental group). BrdU (10 μM final concentration) was added to media and 12 h after adding BrdU, cells were fixed and cells were incubated with peroxidase conjugated anti-BrdU antibody (1:100 dilution) for 1.5 h at RT. After washing with PBS three times, substrate is added. 5 min after adding substrate absorbances at 370 nm were measured.

2.2.17. Immunofluorescent staining against SYN1

13 mm glass coverslips placed in 24 well plates were coated with PA gels similar to other experiments except that gel volume was 300 μL /well (150 μL /each PA solution). 3×10^4 cells/well were seeded and immunofluorescence against synaptophysin 1 (SYN1) was carried out at day 7 after neural induction. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT and permeabilized with permeabilization buffer (0.5 wt% NP40, 10 mM Tris-HCl pH 7.5 and 0.2 mM ZnCl_2) for 15 min at RT. After washing with PBS, blocking was carried out with 10% goat serum and 1% bovine serum albumin (BSA) in PBS with 0.3% Triton-X for 30 min at RT. Cells were then treated with primary antibodies against Synaptophysin I (mouse monoclonal, Millipore) at 1:400 dilution and incubated overnight at 4 °C. After washing with PBS several times, cells were incubated with Cy2 conjugated goat anti-mouse secondary antibody (Millipore, 1:200) for 1 h at RT. To counter-stain nuclei, cells were incubated with 1 μM TO-PRO-3 (Invitrogen) in PBS for 15 min at RT after which they were washed with PBS several times. Coverslips were then mounted with Prolong Gold Antifade Reagent (Invitrogen) and sealed with nail polish. Samples were imaged with confocal microscopy (Zeiss LSM510).

2.2.18. Neurite outgrowth inhibition assay

To analyze whether PA nanofiber scaffolds can overcome inhibitory effects of chondroitin sulfate proteoglycans (CSPGs), PC-12 cells were differentiated on these surfaces in the presence of CSPGs. To optimize the method by which cells will

encounter CSPGs, three different methods along with 5 different concentrations of CSPGs was tried on collagen I. In the first method, CSPG was mixed with 0.01% Collagen I and wells were coated with this mixture. In the second method, Collagen I precoated wells were coated with CSPGs. Third method was treating cells cultured on Collagen I coated wells with soluble CSPG. CSPG concentrations used in this experiment were 0.5, 1, 2.5 and 10 $\mu\text{g/mL}$. 5×10^3 cells were seeded to each well and $n = 3$ for each experimental group. Neural induction conditions were same with above mentioned previous experiments. Cells were observed under microscope at predetermined time points for inhibition of neurite outgrowth. 10 $\mu\text{g/mL}$ CSPG with the first method led to most effective inhibition. Hence, further experiments were carried out by using this method.

Neurite inhibition assay on PA coated surfaces was carried out similarly as neural induction assays that are above mentioned except that 10 $\mu\text{g/mL}$ CSPG (1 $\mu\text{g/cm}^2$) was mixed with negatively charged PA solution before gel formation. Images were taken at day 2 after neural induction and neurites were quantified from these images by using Image J software.

2.3. RESULTS

2.3.1. Peptide amphiphile design and characterization

In order to study the neurite outgrowth supporting ability of a novel peptide amphiphile scaffold containing heparan sulfate and laminin derived epitopes, four different peptide amphiphile molecules were designed and synthesized. All PA molecules had a hydrophobic alkyl tail consisting of lauric acid and a β -sheet

forming peptide sequence, VVAG. This sequence consists of four nonpolar amino acid residues among which valine has a very high beta sheet forming propensity provided by its hydrophobic side chain while glycine is used as a spacer between the hydrophobic part of the sequence and hydrophilic epitope region^{75, 114}. Lauryl-VVAGKKIKVAV-Am (PA-LN) was designed as laminin mimetic PA molecule, since IKVAV epitope was previously shown to induce differentiation of neural progenitor cells into neurons⁸². Lauryl-VVAGEGDK(pbs)S-Ac (PA-GAG) was developed to mimic heparan sulfates with its sulfonate, hydroxyl and carboxylic acid groups incorporated as amino acid side chains. This molecule was shown to have affinity for heparin binding growth factors⁸⁴. PA-LN+PA-GAG scaffolds consisted of the two bioactive PAs, laminin derived PA-LN and heparan sulfate mimetic PA-GAG. The other two PA molecules that we have used were Lauryl-VVAGE-Ac (PA-E) and Lauryl-VVAGK-Am (PA-K). These two PAs did not bear a bioactive epitope sequence and they were used for gel formation with oppositely charged PAs to obtain PA-K+PA-GAG and PA-LN+PA-E nanofibers. PA-K+PA-E nanofibers were used as non-bioactive gel control having the nanofibrous structure without any bioactive functional groups. Chemical structures of PA molecules used in this study are given in Figure 2.1. The PA molecules were synthesized by solid phase peptide synthesis and purified with HPLC. Molecular mass and purity of PA molecules were characterized by LC-MS. Purity of HPLC purified products were found to be above 95% with MS verifying the molecular weight of molecules synthesized for all PAs used in here (Figure 2.2-2.9).

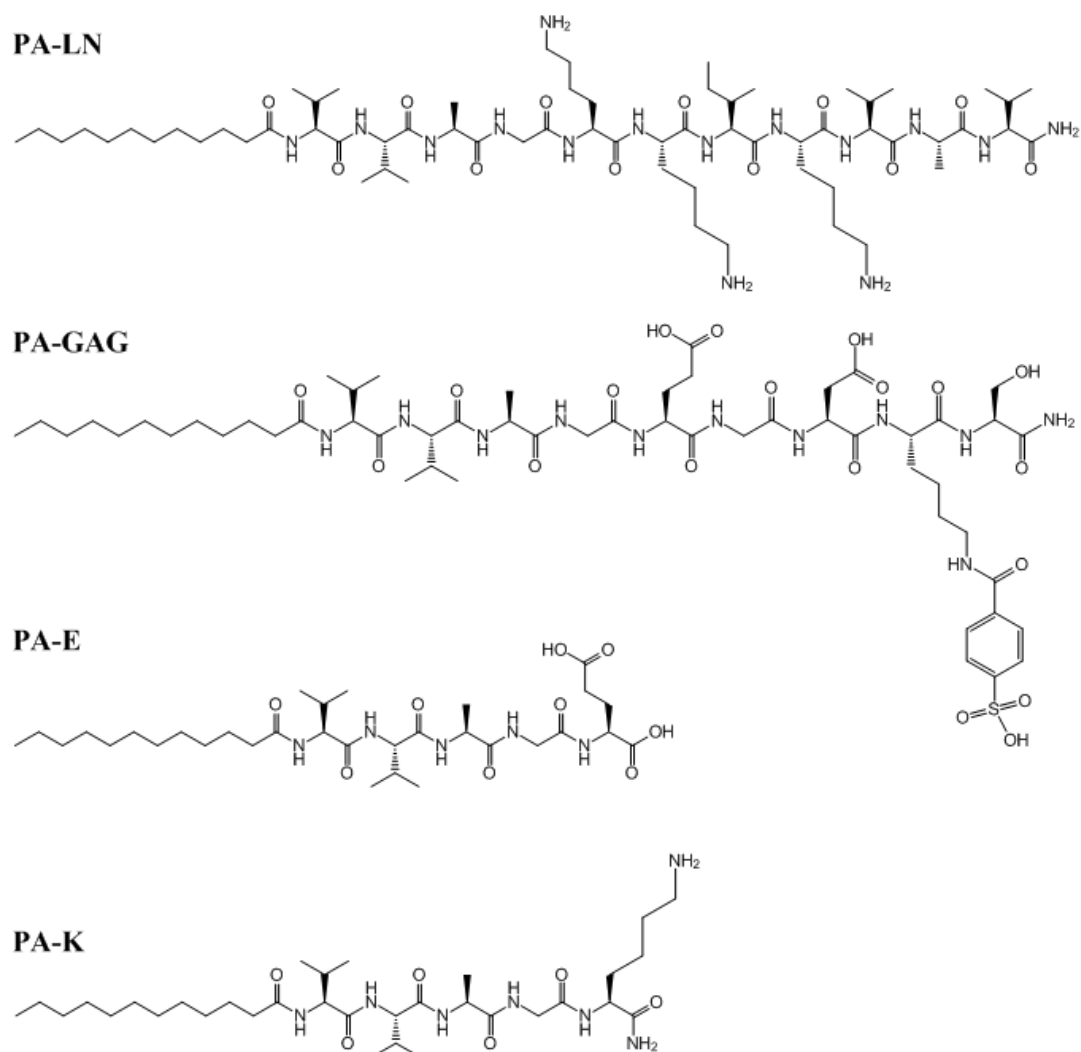


Figure 2. 1. Chemical structures of the PA molecules used in this study.

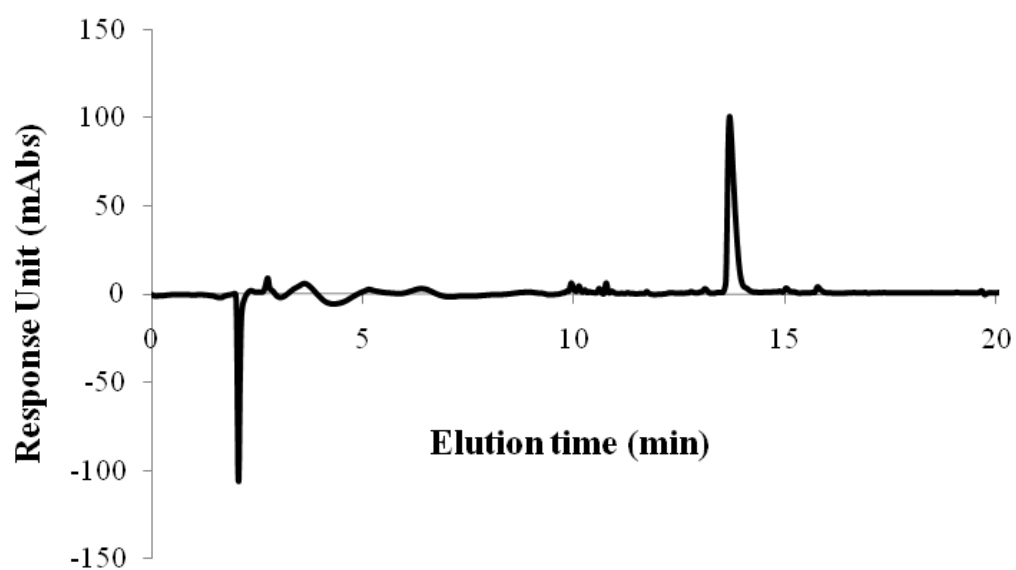


Figure 2. 2. Liquid chromatography analysis of PA-LN.

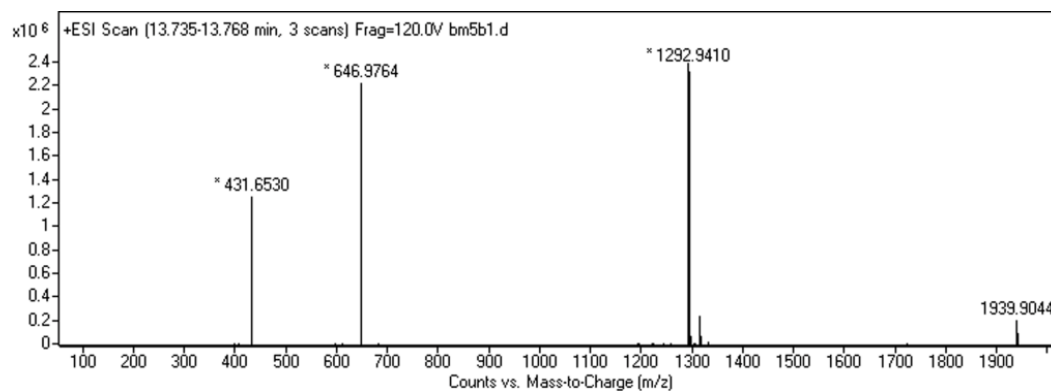


Figure 2. 3. Mass spectrometry analysis of PA-LN.

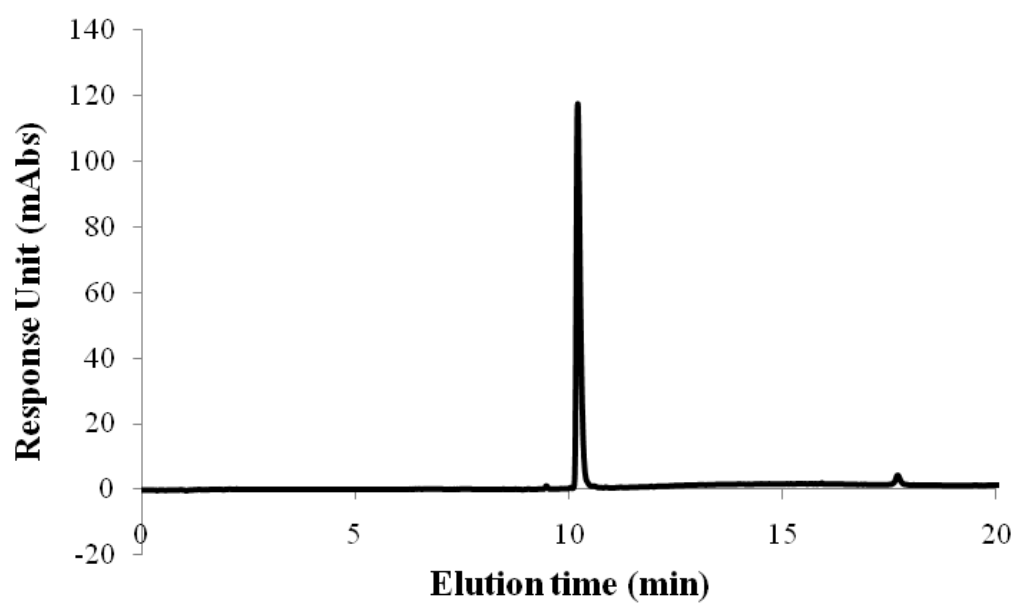


Figure 2. 4. Liquid chromatography analysis of PA-GAG.

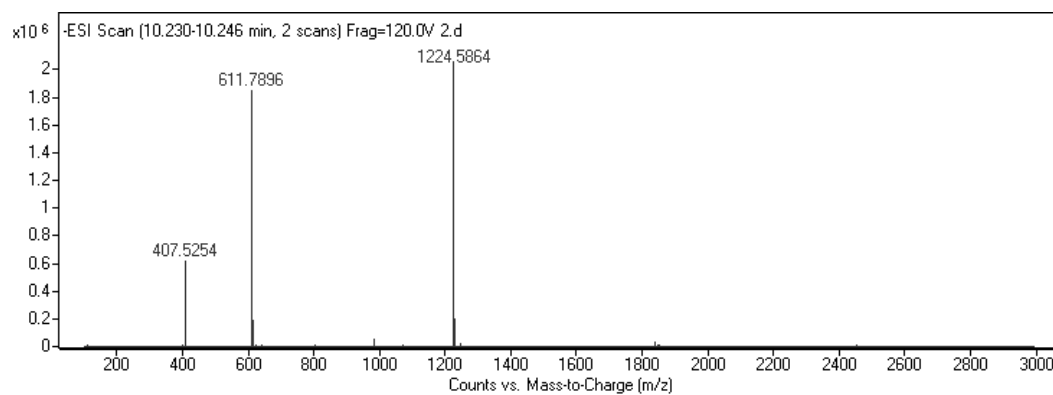


Figure 2. 5. Mass spectrometry analysis of PA-GAG.

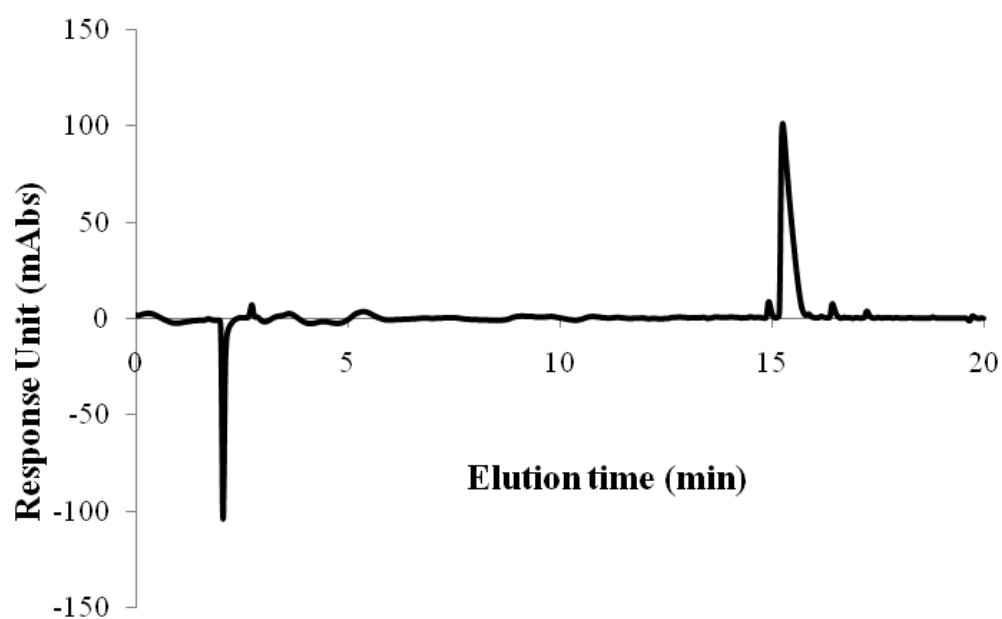


Figure 2. 6. Liquid chromatography analysis of PA-K.

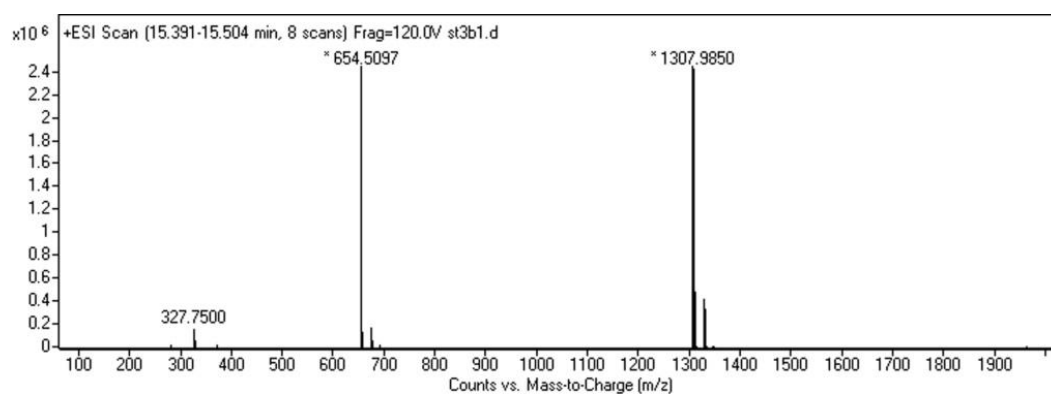


Figure 2. 7. Mass spectrometry analysis of PA-K.

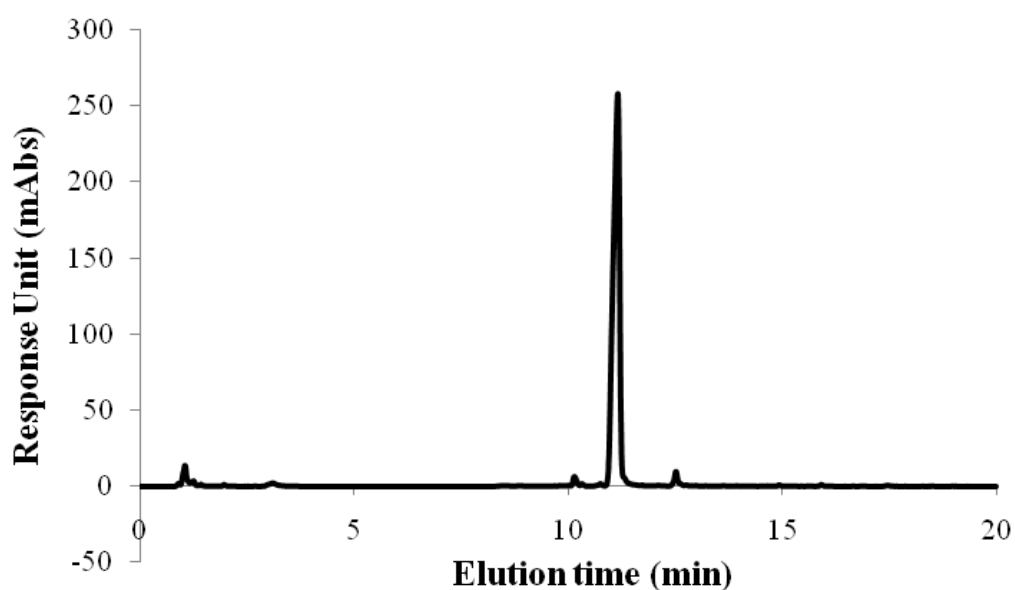


Figure 2. 8. Liquid chromatography analysis of PA-E.

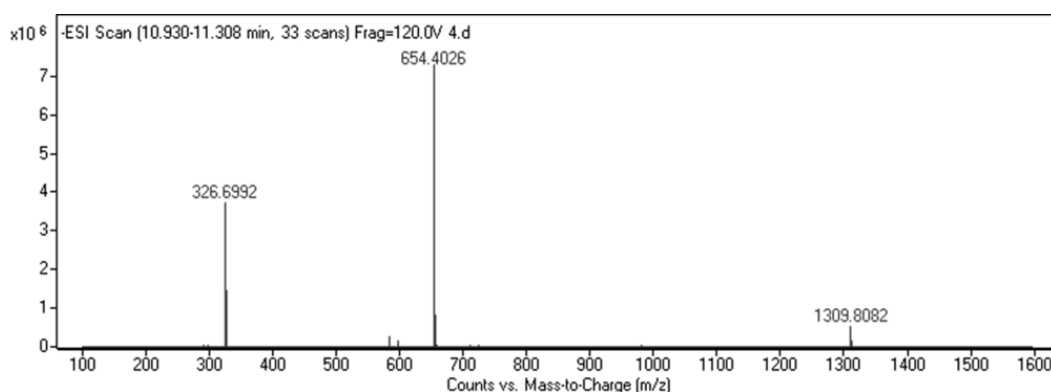


Figure 2. 9. Mass spectrometry analysis of PA-E.

Atomic force microscopy (AFM) was used for visualization of peptide amphiphile nanofibers. AFM images taken for each PA combination showed that all these combinations formed nanofibers whose lengths ranged from a few hundred nanometers to several microns length (Figure 2.10). Also bundling of individual

nanofibers can be observed for each combination. The SEM imaging of nanofiber networks in PA gels (Figure 2.11) revealed morphological similarity of nanofibrous PA scaffolds to natural ECM that surrounds the cells in tissues⁷⁶. Neural ECM is quite different in developing and mature CNS tissues in terms of the contents and physical structure. Fibrous proteins having important roles in nervous tissue development, especially as guidance molecules are down-regulated during maturation leading to an ECM with low content of fibrous structures⁸². PA scaffolds used in study resemble embryonic ECM of neural tissues in terms of physical appearance of fibrous networks. As neural regeneration involves new tissue formation as well as guidance of nerve terminals for appropriate formation of neural networks in regenerated tissue, similar to developmental processes such a physical similarity can be speculated to improve effectiveness of the PA nanofibers used in this study.

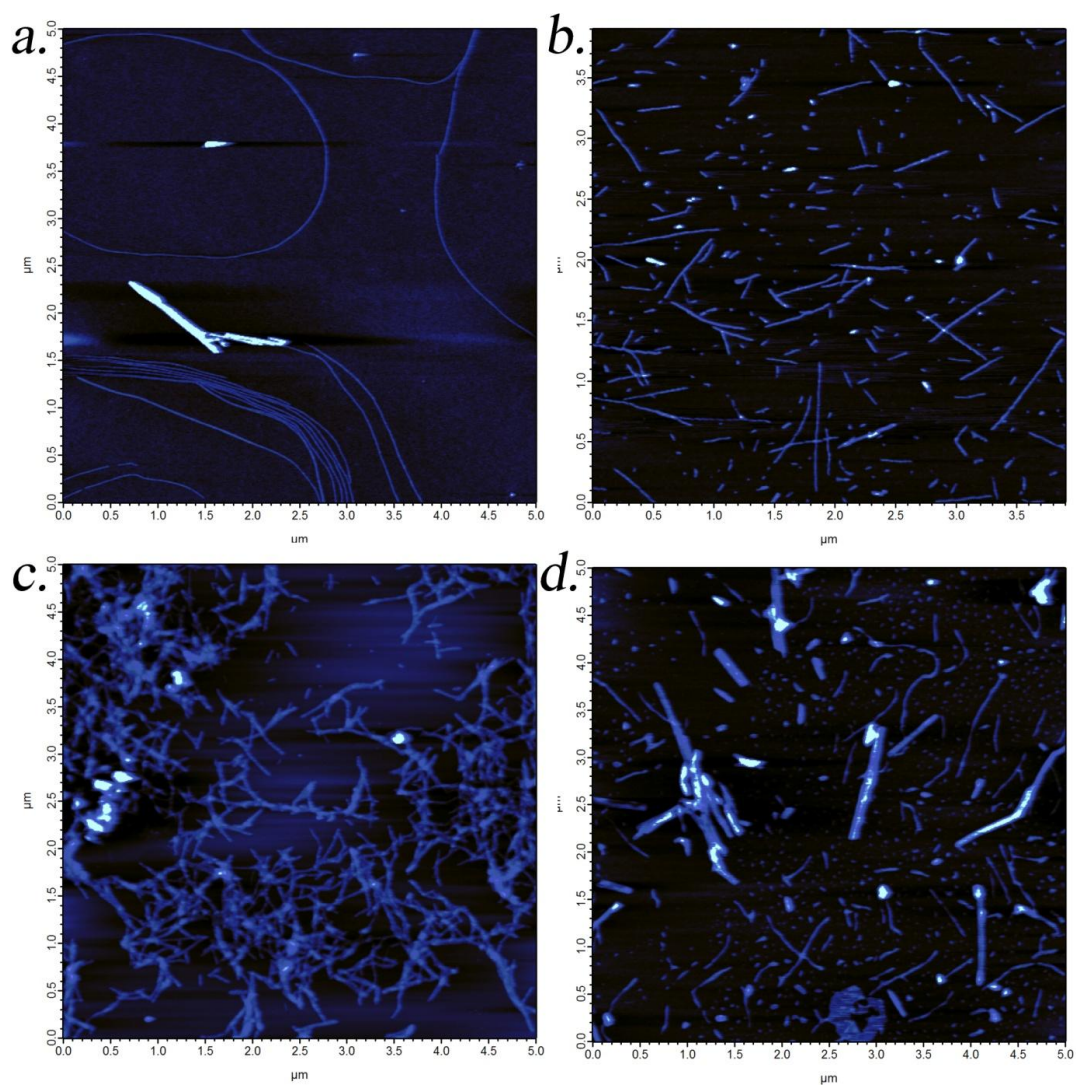


Figure 2. 10. AFM images of peptide amphiphile nanofibers. Nanofibers are observed in PA-K+PA-GAG (a), PA-LN+PA-E (b), PA-LN+PA-GAG (c) and PA-K+PA-E (d) combinations.

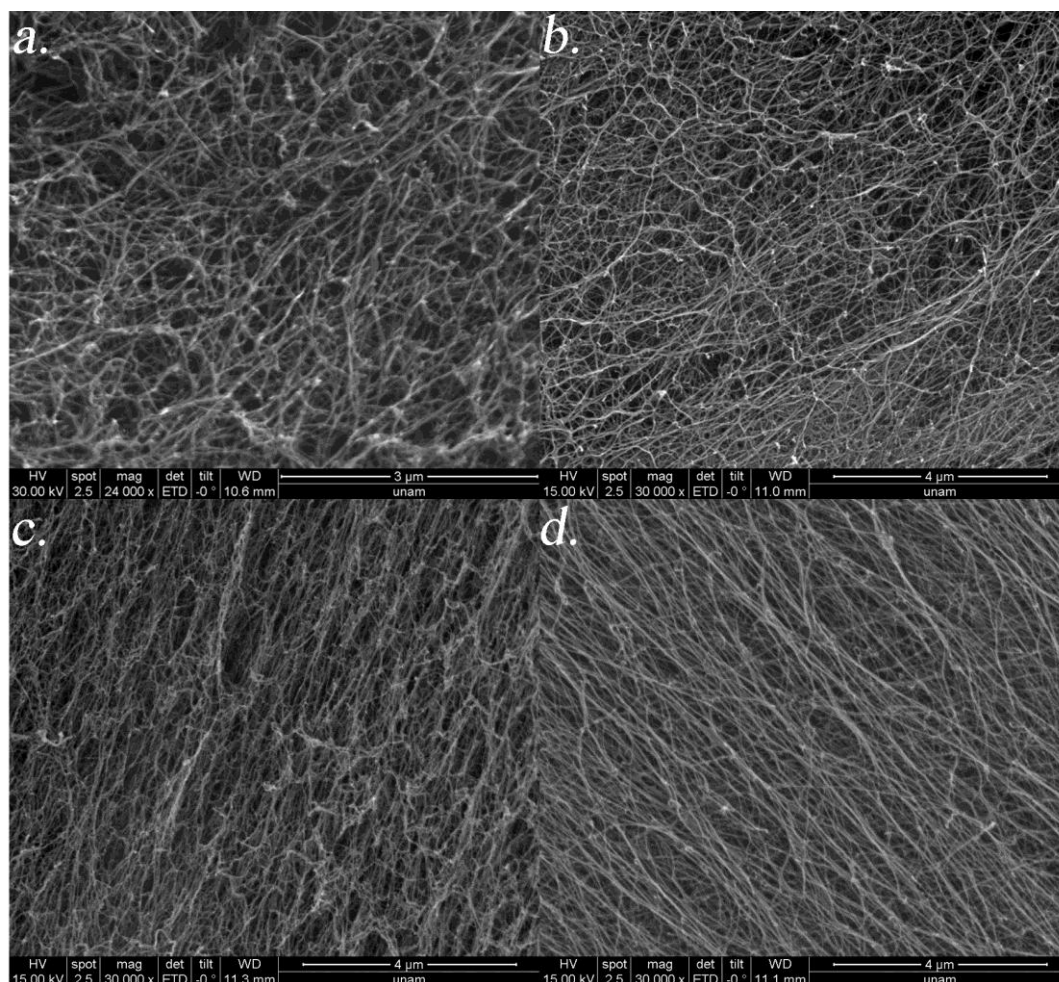


Figure 2. 11. SEM images of peptide amphiphile gels. PA-K+PA-GAG (a), PA-LN+PA-GAG (b), PA-LN+PA-E (c), PA-K+PA-E (d) scaffolds show similar morphology to natural ECM.

We employed circular dichroism (CD) spectrophotometer for analysis of secondary structure of the PA nanofibers. Secondary structures formed by hydrogen interactions are important in self-assembly of peptide nanostructures and different secondary structure motifs are found to derive formation of different supramolecular structures¹¹⁵. In order to find the initial assembly at molecular level that derives

formation of higher order structures, circular dichroism analysis was carried out. Protein secondary structures show characteristic peaks when analyzed with circular dichroism spectrophotometry. A positive peak around 195 nm along with a negative peak around 220 nm is the characteristic spectra of β -sheet structures. CD results of the PA combinations used in here revealed predominant β -sheet structures in peptide amphiphiles (Figure 2.12).

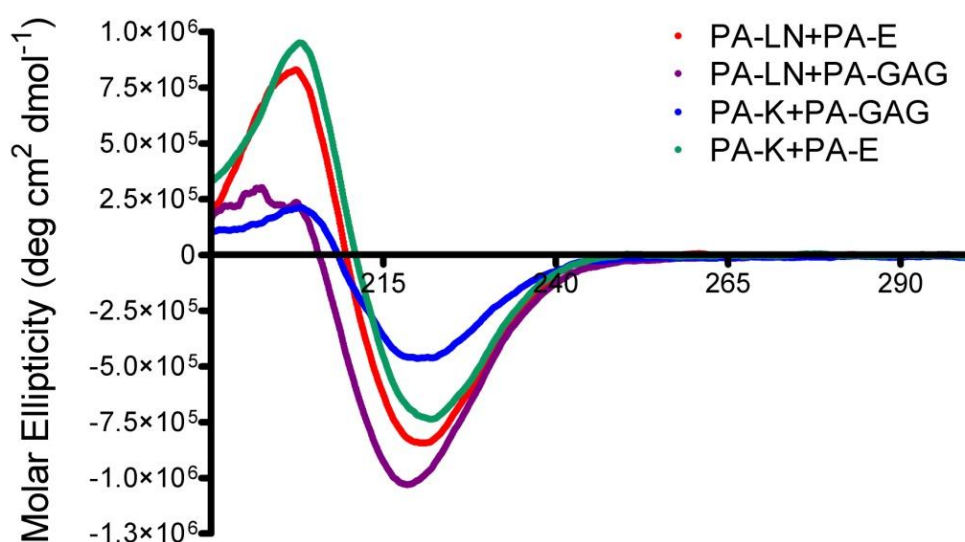


Figure 2. 12. Characterization of secondary structure of peptide amphiphiles by CD.

All nanofibers are found to have predominant β -sheet secondary structure.

Since mechanical properties of the ECM are crucial for neuritogenesis, we utilized oscillatory rheology to study mechanical properties of the PA gels. Due to technical limitations, gels measured with rheology are not prepared with the same method of

those used in cell culture experiments. PA gels used for cell culture experiments are quite thin after rehydration with cell culture media which limits measurement with oscillatory rheology. There might be some difference in the conditions tested with rheology and used in cell culture experiments due to these limitations but still rheological data given in Figure 2.13 provides valuable information about the suitability of the PA gels for soft tissue (nervous system in our case) applications beyond the extent of *in vitro* work. Rheology results confirmed that these materials formed gels as storage moduli of all PA combinations were higher than the loss moduli. In addition, storage moduli of PA gels used in this study, which refer to the stiffness of materials, indicated similar properties to natural neural ECM. Neural tissues such as the brain are soft tissues as indicated by measurement of rat brain's shear modulus as $330 \pm 100 \text{ Pa}$ ¹¹⁶. Though stiffness of PA gels measured here are higher (from 0.86 ± 0.08 to $2.72 \pm 0.7 \text{ kPa}$), they are still soft materials. Besides, gels used in these measurements are around four fold concentrated than used in cell culture. Hence, PA scaffolds used in this study are suitable for neural cell culture in terms of their mechanical properties.

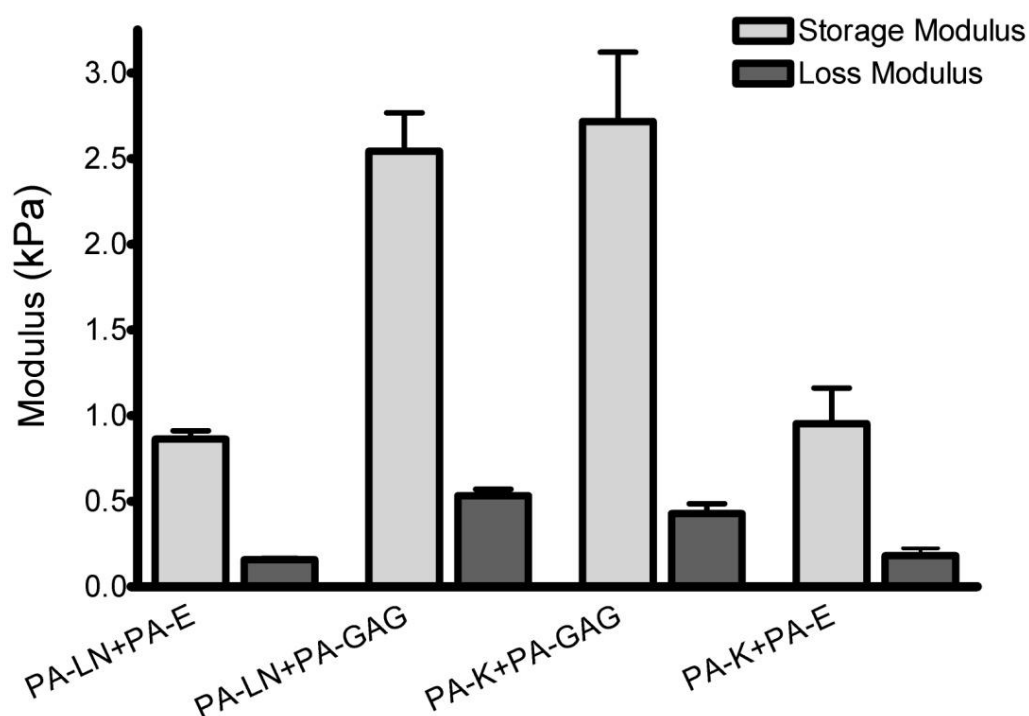


Figure 2. 13. Measurement of stiffness of PA gels by oscillatory rheology. Rheology measurements indicate that PA gels used in this study are soft materials suitable for neural tissue engineering.

2.3.2. Neural differentiation of PC-12 cells on PA scaffolds

In order to determine neurite outgrowth promoting effect of the PA nanofibers, PC-12 cells were used. PC-12 is a cell line derived from rat adrenal gland pheochromocytoma and it is a commonly used model system to determine effects of different molecules on neural differentiation and axonal growth as these cells can be induced to terminally differentiate into neural cells in the presence of NGF¹¹⁷⁻¹¹⁸.

Materials developed for neuroregenerative purposes should promote neurite outgrowth, since it is an essential step in making functional synapses, which is crucial in neuroregeneration. In nervous system, neurite outgrowth takes place through collaborative effort of a number of soluble factors and cell adhesion molecules as well as extracellular matrix elements. Herein, we combined mimics of two neural ECM components, laminin and heparan sulfate, which were previously shown to promote axonal growth and neurite outgrowth when used in combination^{21, 110, 119}.

Before analyzing the effect of bioactive epitopes presented by PA substrates on neural differentiation we optimized differentiation conditions. In order to optimize PA substrates, we optimized final charge of scaffolds and PA concentrations used. Scaffolds with a final charge of 0 and -1 were used and those with no net charge were found to induce extension of longer neurites (Figure 2.14). Neutral charged scaffolds are selected to be used in further experiments after this finding. For concentration optimization, cells were cultured on scaffolds containing 0.5 mM, 1.5 mM and 3 mM PA. Neurite lengths quantified on day 7 of NGF treatment indicated no significant difference between different PA concentrations at this range (Figure 2.15). Gel formation was more stable at 1.5 mM when compared to 0.5 mM PA gels and 1.5 mM PA gels were easier to observe under light microscope. 1.5 mM PA substrates were selected for further use due to the advantages mentioned for this concentration.

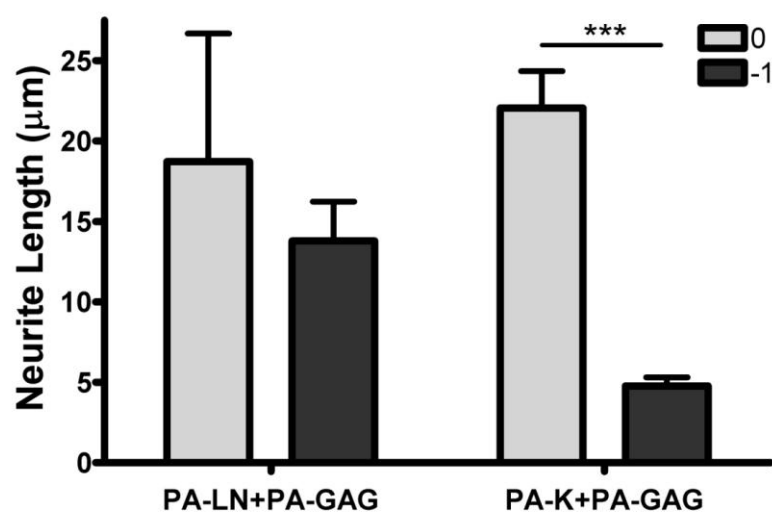


Figure 2. 14. Effect of final charge of PA substrate on neurite outgrowth by PC-12 cells. Scaffolds with neutral charge induced extension of longer neurites. Student's t-test is performed within each substrate group.

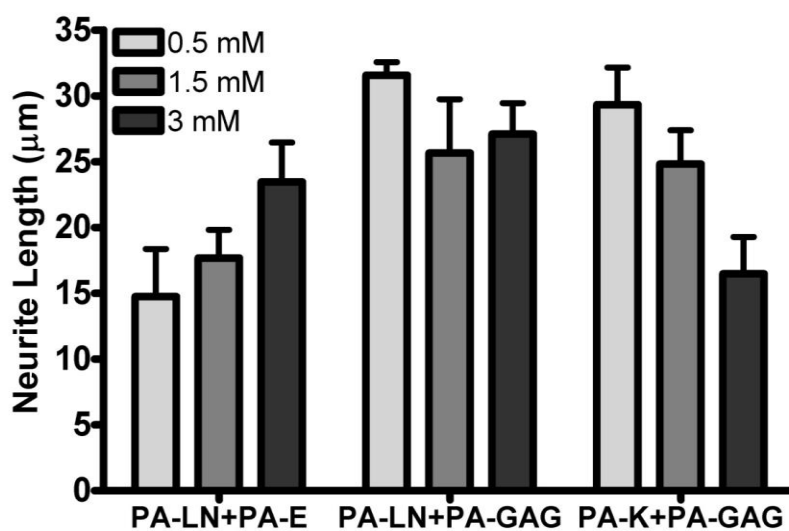


Figure 2. 15. Effect of PA concentration on neurite outgrowth by PC-12 cells. No significant difference in neurite length is observed on gels of different PA concentrations.

Media in which differentiation is carried out is also important as the nutrients and their concentrations provided in the media will affect the cell's decision to undergo proliferation or differentiation. DMEM, MEM and RPMI with 3% serum and 20 ng/mL NGF were used during differentiation. MEM was found to induce much longer neurites when compared to other media, which can be attributed to MEM being a basic medium that provides all the essential nutrients but at minimal amounts (Figure 2.16). Cells can be speculated to undergo proliferation in nutrient rich DMEM and RPMI while favoring differentiation in MEM. MEM was selected to be used for further experiments.

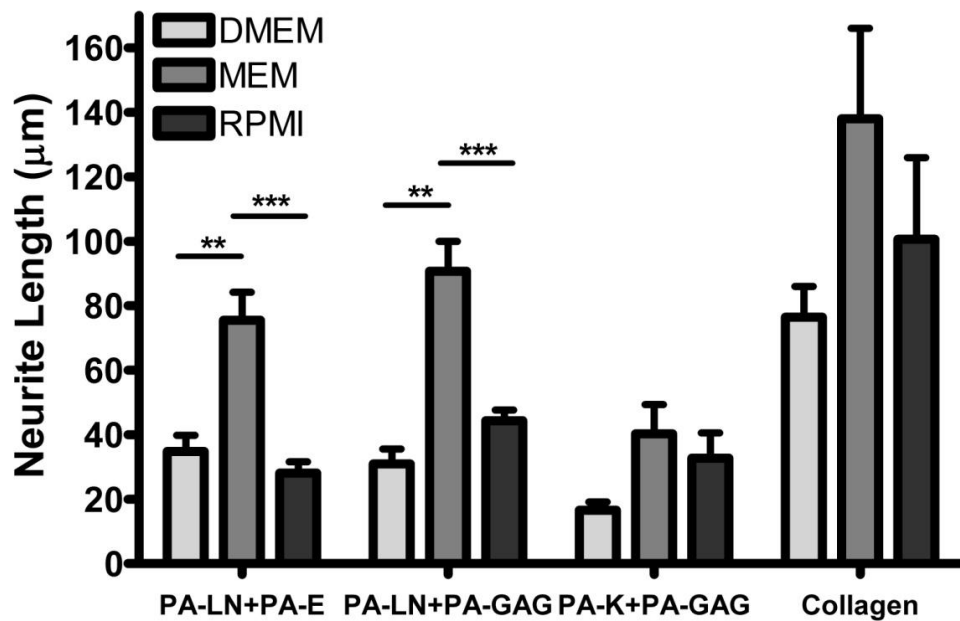


Figure 2. 16. Effect of induction medium on neurite outgrowth by PC-12 cells. MEM was found to induce much longer neurites when compared to DMEM and RPMI. One-way ANOVA is performed within each substrate group.

In order to find the minimal amount of NGF required for neural induction, cells were induced with 25, 50 and 100 ng/mL of NGF. Quantification of neurite lengths at day 7 after induction revealed no significant differences between different doses of NGF indicating that 25 ng/mL is enough for neural differentiation of PC-12 cells (Figure 2.17). It was further lowered to 20 ng/mL of NGF and used at this concentration in further experiments.

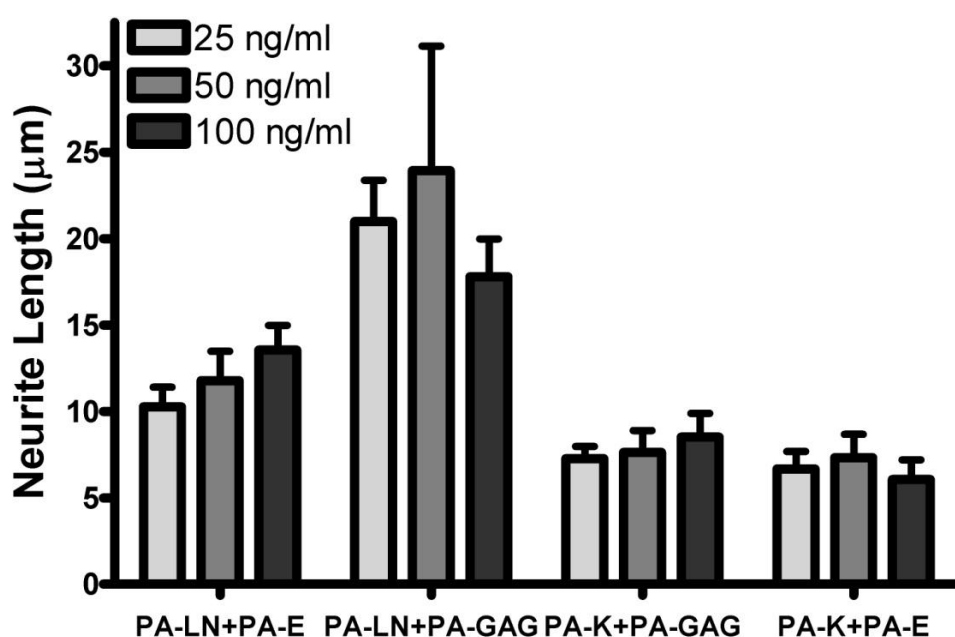


Figure 2. 17. Effect of NGF amount on neurite outgrowth by PC-12 cells. One-way ANOVA is performed within each substrate group. No significant difference in neurite length is observed when different amounts of NGF are used.

In order to analyze the biocompatibility of PA scaffolds, we assessed viability of the PC-12 cells cultured on PA-LN+PA-GAG, PA-LN+PA-E, PA-K+PA-GAG and PA-

K+PA-E scaffolds along with those cultured on poly-D-lysine (PDL). As these cells are suspension cells and do not adhere to uncoated tissue culture plate, bare surfaces could not be used in any of the experiments. Live-dead assay results indicated a quite high viability on all PA substrates as well as PDL and there was no sign of toxicity. It can be inferred that all PA combinations used in this study were biocompatible (Figure 2.18).

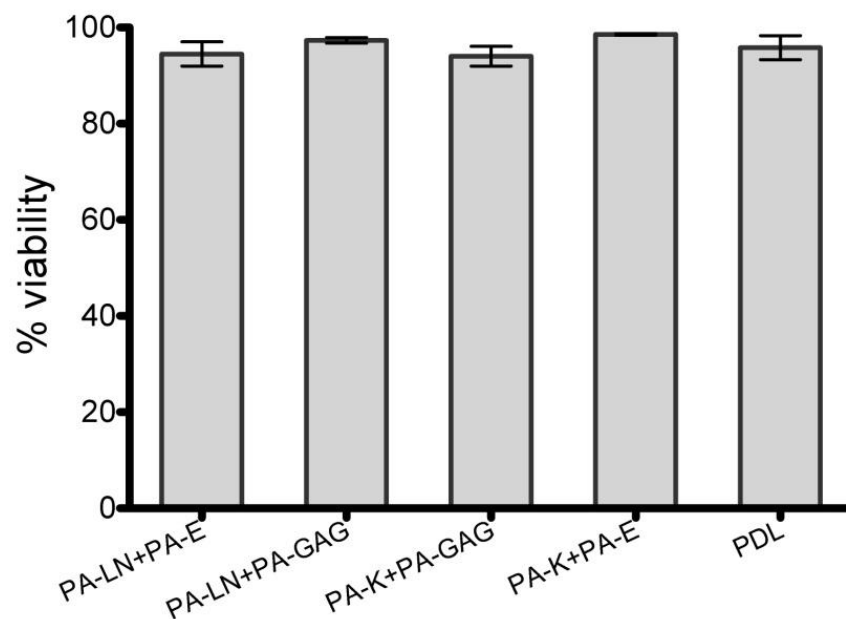


Figure 2. 18. Results of live-dead assay on PA substrates and PDL. Viability of PC-12 cells that are cultured on PA scaffolds is similar to the cells cultured on poly-D-lysine (PDL). There exists no significant difference between groups (one-way ANOVA is performed).

Detailed morphology of PC-12 cells on PA substrates was observed by SEM imaging. As these cells are suspension cells, whether they would adhere to PA substrates was a question mark. However, as can be seen from the SEM images they could adhere quite well, with high numbers of small extensions from the cell body. They could also extend neurites on these surfaces at day 3 after NGF addition. (Figure 2.19).

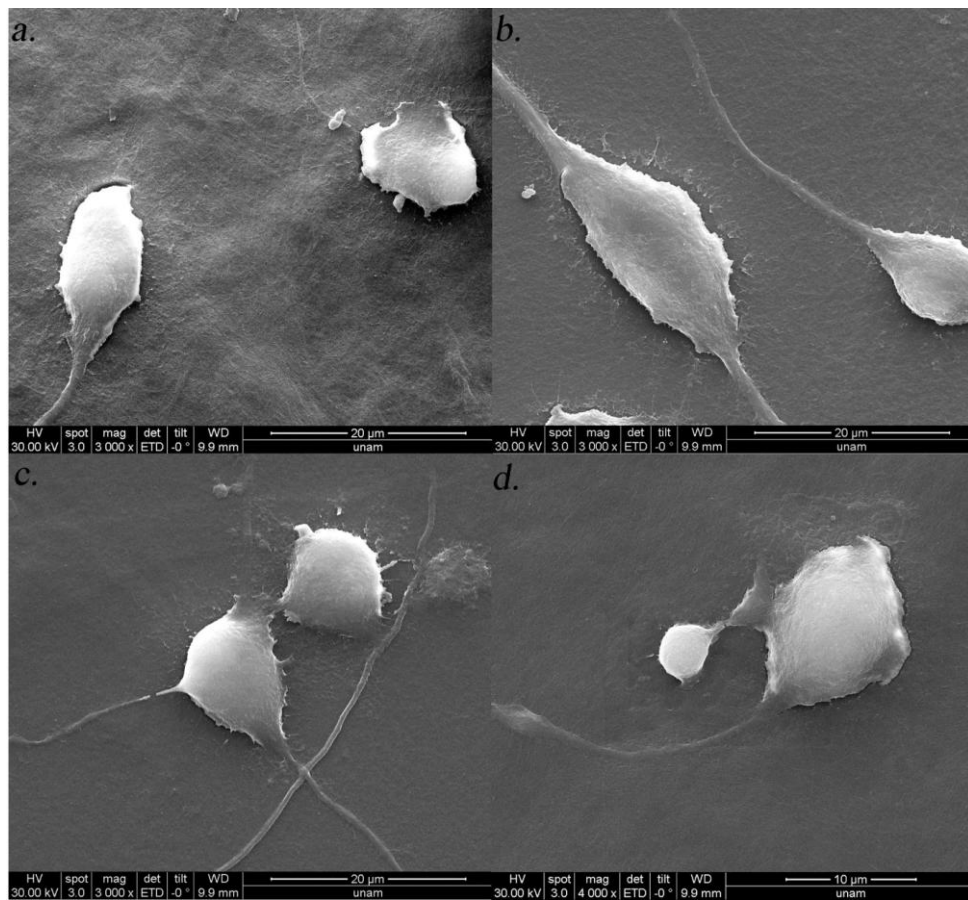


Figure 2. 19. SEM images of PC-12 cells cultured on PA gels. Cells are cultured on PA-K+PA-GAG (a), PA-LN+PA-GAG (b), PA-LN+PA-E (c), and PA-K+PA-E (d) gels for three days after neural induction with 20 ng/mL NGF. PC-12 cells can adhere to PA surfaces and extend neurites in the presence of NGF.

Neuroprogenitor cells in early developmental stages have mitotic activity, while during late stages of neural development they exit from cell cycle and cease proliferation as they start to differentiate¹²⁰. Hence, ceasing proliferation is an indication of differentiation for neural cells, as terminally differentiated neurons do not proliferate. In order to analyze proliferation profile of PC-12 cells on PA scaffolds, BrdU assay was carried out one week after neural induction with NGF. BrdU is a thymidine analog and when supplemented in cell culture media, it is incorporated into DNA during duplication. Hence, presence of BrdU in DNA indicates cell division. Results indicated that proliferation was diminished on all PA surfaces. This indicates neural differentiation on these surfaces, leading to formation of mature neurons that do not divide (Figure 2.20).

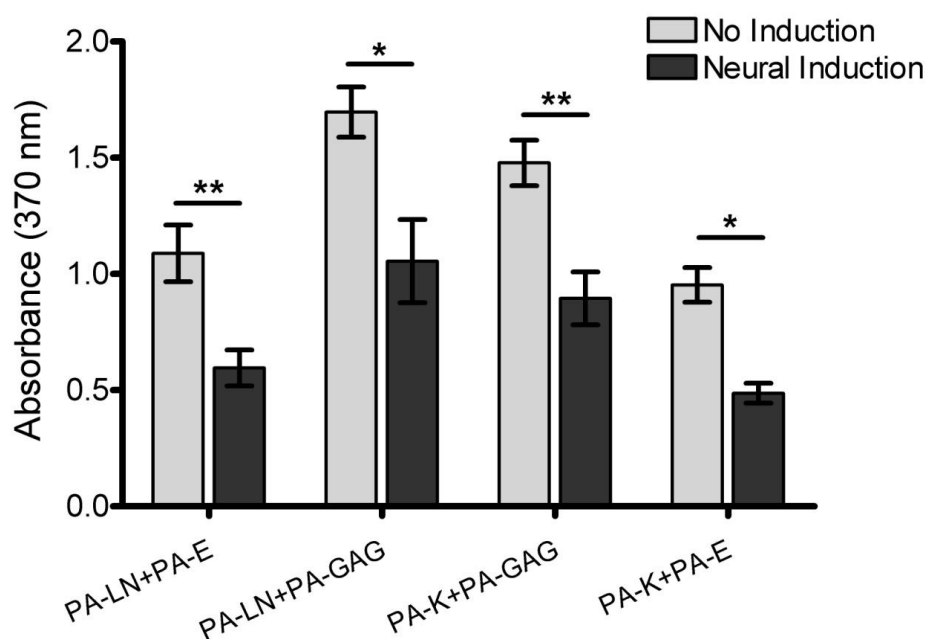


Figure 2. 20. Proliferation assay results of PC-12 cells on day 7 after neural induction. Results verified neural differentiation on PA scaffolds as proliferation was reduced in cells treated with NGF. Student's t-test is performed within each substrate group.

In order to analyze the effect of neural ECM mimetic peptide nanofibers on neural differentiation, we also measured lengths of neurites produced by PC-12 cells. Measurement of neurite length during NGF treatment provides quantitative information about neural differentiation¹²¹. Neurite lengths that were measured a week after neural induction pointed out PA-LN+PA-GAG nanofibers as the most potent neurite inducer scaffold (Figure 2.21). Neurites on this scaffold were found to be significantly longer when compared to those on PA-LN+PA-E and PA-K+PA-GAG nanofibers (Figure 2.22). Besides, significantly higher number of cells could extend neurites on PA-LN+PA-GAG nanofibers when compared to non-bioactive

PA-K+PA-E scaffold (Figure 2.23). PA-LN+PA-GAG scaffold was the only bioactive scaffold on which number of cells extending neurites was significantly different from the control PA scaffold. Although the number of cells with neurites cultured on PA-LN+PA-E and PA-K+PA-GAG scaffolds were higher than those on non-bioactive PA-K+PA-E scaffold, the differences were not statistically significant.

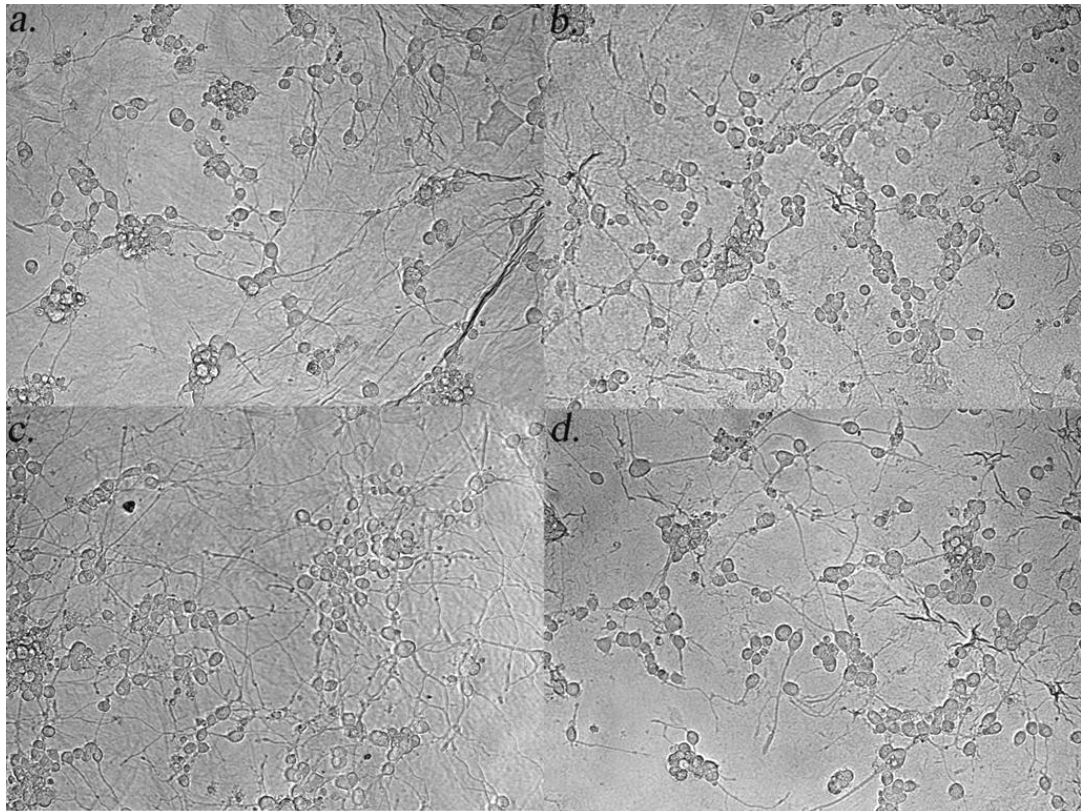


Figure 2. 21. Neurite outgrowth of PC-12 cells on PA scaffolds. Optical images of PC-12 cells on day 7 after neural induction with 20 ng/mL NGF indicates PA-LN+PA-GAG (c) as the most suitable scaffold for neurite outgrowth. Cells extended much longer neurites on this scaffold when compared to PA-K+PA-GAG (a), PA-LN+PA-E (b) and PA-K+PA-E (d) scaffolds. Images were taken at 200X magnification.

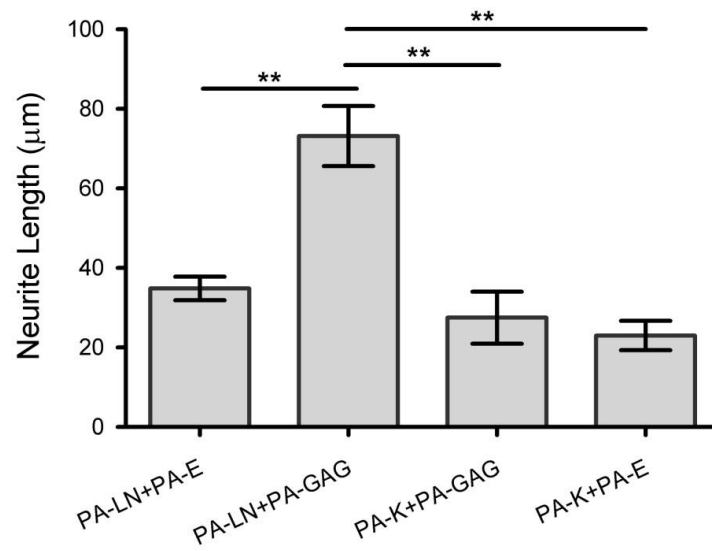


Figure 2. 22. Quantified results of neurite length on PA substrates at day 7. One-way ANOVA is performed for statistical analysis.

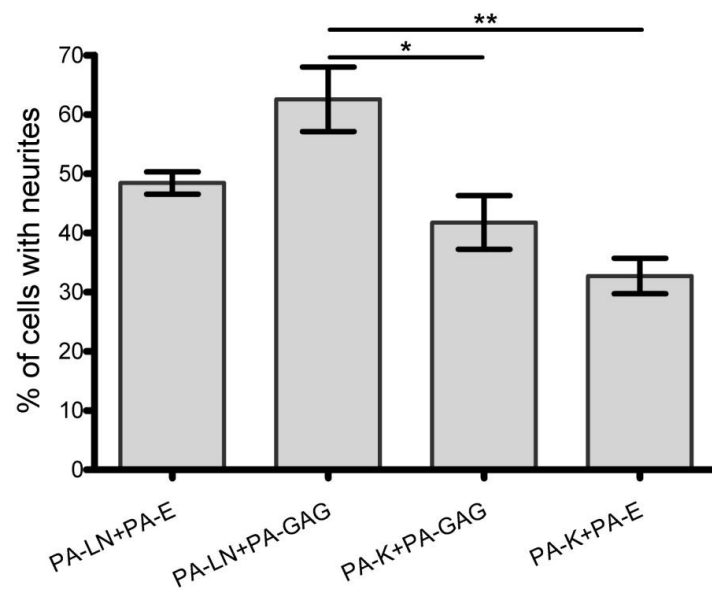


Figure 2. 23. Quantified results of percentage of cells extending neurites on PA substrates at day 7. One-way ANOVA is performed for statistical analysis.

As another evidence of neural differentiation and maturation, cells cultured on all PA combinations were found to express Synaptophysin I revealed by immunostaining (Figure 2.24). Synaptophysin I is the most abundant synaptic protein at presynaptic nerve terminals having a role in synaptic vesicle endocytosis¹²². Localization of synaptophysin in axonal protrusions by transportation from the cell body along the elongated axon is an indication of presynaptic nerve terminal development¹²³. Expression of synaptophysin was localized heavily on the nerve terminals of the differentiated PC-12 cells on PA substrates indicating the presynaptic nerve terminal development and synaptic potential of these cells.

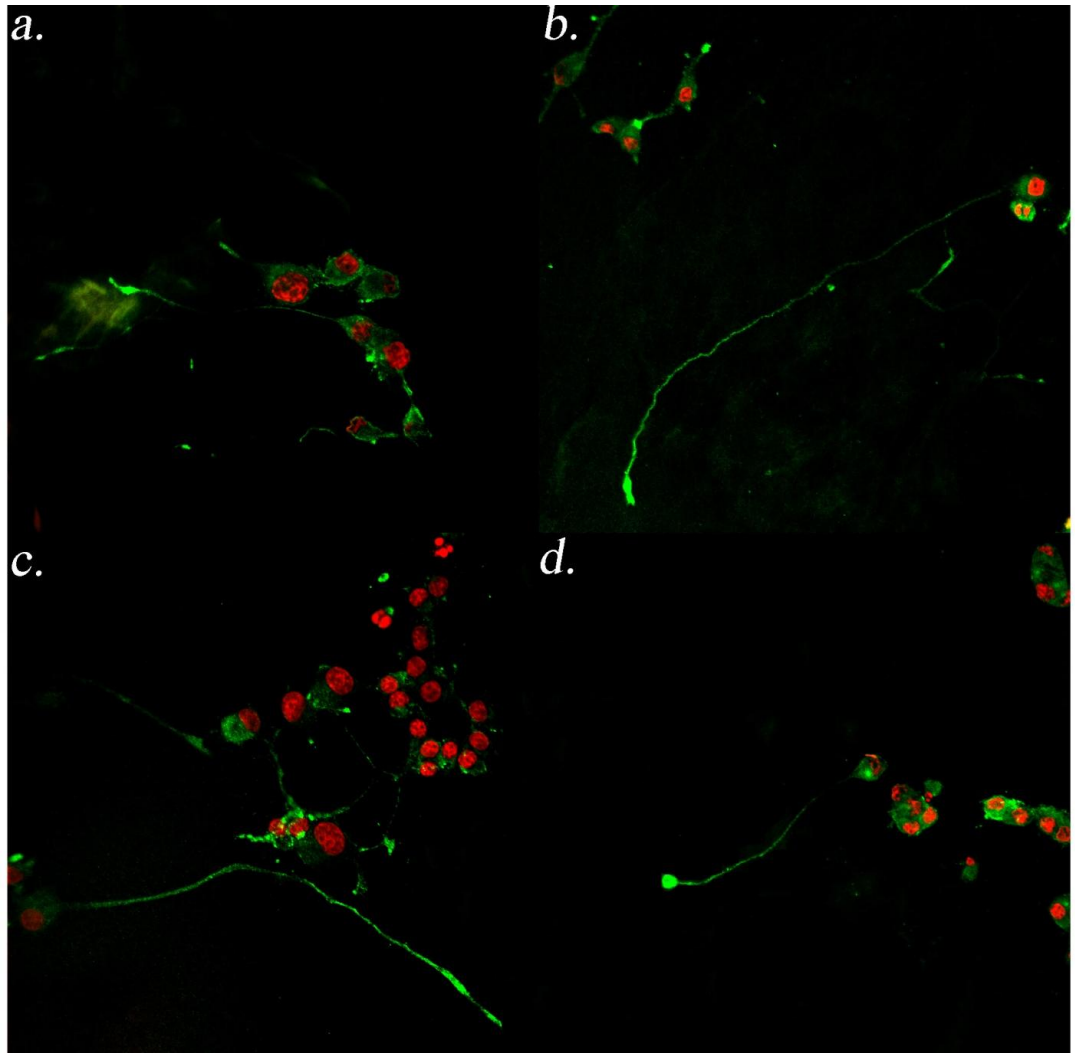


Figure 2. 24. Immunostaining of PC-12 cells cultured on PA nanofiber scaffolds against synaptophysin 1. Cells cultured on PA-K+PA-GAG (a), PA-LN+PA-GAG (b), PA-LN+PA-E (c) and PA-K+PA-E (d) scaffolds for 7 days in the presence of NGF express synaptophysin. Concentration of synaptophysin at nerve terminals indicates presynaptic nerve terminal development. Nuclei are stained with TO-PRO-3 (red). Images are taken at 400x magnification.

These results indicate that the presence of both laminin-mimetic and heparan-sulfate mimetic molecules enhance each other's abilities to induce neural differentiation and synthetic PA scaffolds with both laminin and heparan sulfate derived signals on the surface can successfully mimic the natural system in the neural ECM.

2.3.3. Investigation of NGF/PA-GAG interaction

Laminin derived epitope acts through interacting with cells via integrin receptors. Heparan sulfates induce differentiation of cells by interacting with growth factors and we have previously shown that PA-GAG interacts with vascular endothelial growth factor inducing differentiation of endothelial cell¹²³. Since our results showed that PA-LN+PA-GAG nanofibers act as a potential dual promoter of neurite growth, I investigated whether PA-GAG's role in this system was through its interaction with NGF, which is the main growth factor that is responsible for neural differentiation.

Effective binding of NGF to heparan sulfate or heparin was shown previously^{111, 124}. The nanofibers formed by PA-GAG molecules contain an abundant number of functional groups presented on the surface and can provide affinity to NGF. NGF release assay was performed to assess the release rate of the NGF from PA nanofibers, which indirectly gives information about NGF's affinity with this three dimensional nanofiber network comprising PA gels. As it is shown in Figure 2.25, NGF encapsulated within the PA gel system was released in the same rate from both PA-LN+PA-E and PA-LN+PA-GAG gels at earlier time points. Released amount of NGF was low for both gels which can be explained by NGF being wrapped up in nanofiber bundles during the process of nanofiber network formation, delaying

release of growth factors, even though they do not have strong affinity to individual nanofibers. Since we performed this assay in buffer system, no enzymatic degradation was possible. Dynamic nature of PA nanofiber system is thought to allow slow release of NGF physically in a time dependent manner. Time-dependent linear increase of NGF release from control PA gel (PA-LN+PA-E) supports this notion. Release from PA-LN+PA-GAG gel ceased after 2.5 days. It is reasonable to assume that growth factors bound to the nanofiber network were not released during this time period. Oppositely, release from PA-LN+PA-E gels proceeds linearly until 10 days, where experiment was ended. These data show that PA-LN+PA-GAG gel network interacts with NGF with higher affinity than PA-LN+PA-E gel, which can be attributed to the functional groups presented on PA-GAG nanofibers. As a result, we suggest that heparan sulfate mimicking chemical groups on the surface of the nanofibers effectively present NGF to cell surface receptors while IKVAV epitopes induce neurite outgrowth through integrin signaling simultaneously leading to a more effective neurite outgrowth.

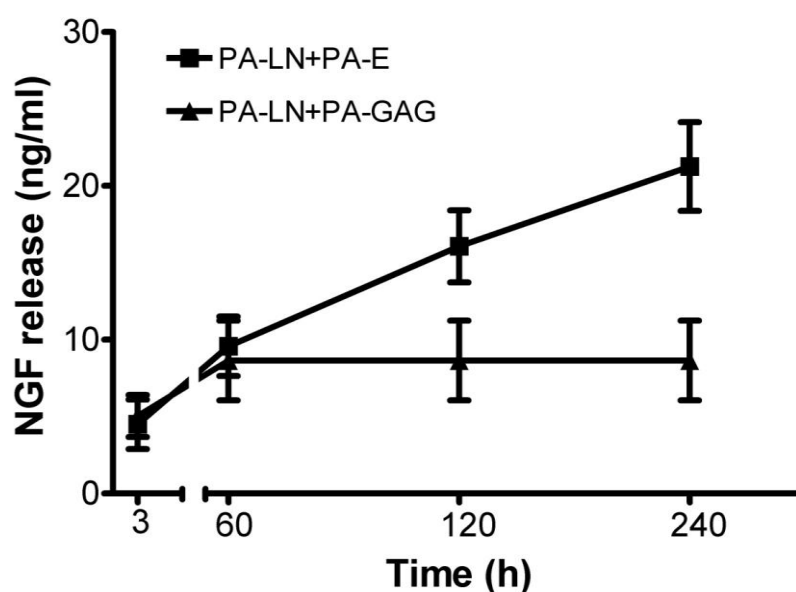


Figure 2. 25. Time-dependent NGF release from PA gels. PA-GAG confers advantage in terms of growth factor binding to the gel system it is included. Release from PA-LN+PA-GAG was much lower when compared to that from PA-LN+PA-E. NGF concentration released from gels was determined with ELISA method.

2.3.4. Neurite inhibition assay by CSPGs

After CNS injury, chondroitin sulfate proteoglycans (CSPG) are abundantly expressed in glial scar and exert inhibitory effect on axonal growth leading to poor regeneration⁹⁵⁻⁹⁷. Eliminating the restrictive effect of CSPGs and promoting neurite outgrowth in this inhibitory environment is an essential barrier to overcome for neural regeneration studies. In order to analyze the effect of CSPGs on the neurite outgrowth promoting activity of PA gels, we performed neurite outgrowth assay with PC-12 cells in the presence of CSPGs. Presence of CSPGs led to almost complete

inhibition of neurite growth of PC-12 cells cultured on PDL surfaces (Figure 2.26). Cells on collagen I coated surfaces experienced 92% reduction in neurite length and 95% reduction in the number of cells with neurites in the presence of CSPGs (Figure 2.27-2.28). These results were in agreement with previous reports on the inhibitory effect of CSPGs on neurite growth¹²⁵⁻¹²⁶. Strikingly, cells that were cultured on PA scaffolds, could overcome the inhibitory effect exerted by CSPGs. When cells were cultured on PA scaffolds, neurite length and number of cells extending neurites was not effected from presence of CSPGs as those on Collagen and PDL. None to 40% decrease in neurite length was observed on PA scaffolds while a dramatic reduction in neurite length (90%) was observed on Collagen (Figure 2.29). These data further indicate that the effect of laminin derived PA scaffolds, which were previously shown to enhance recovery after spinal cord injury⁸⁸⁻⁸⁹, could in part be attributed to CSPG inhibition overcoming effect of the PA nanofibers used in this study. The ability of PA nanofibers to help neural cells to overcome the inhibitory effect of CSPGs further strengthens the potential of these PA nanofibers for neuroregenerative therapies, which currently face with the CSPG inhibition problem.

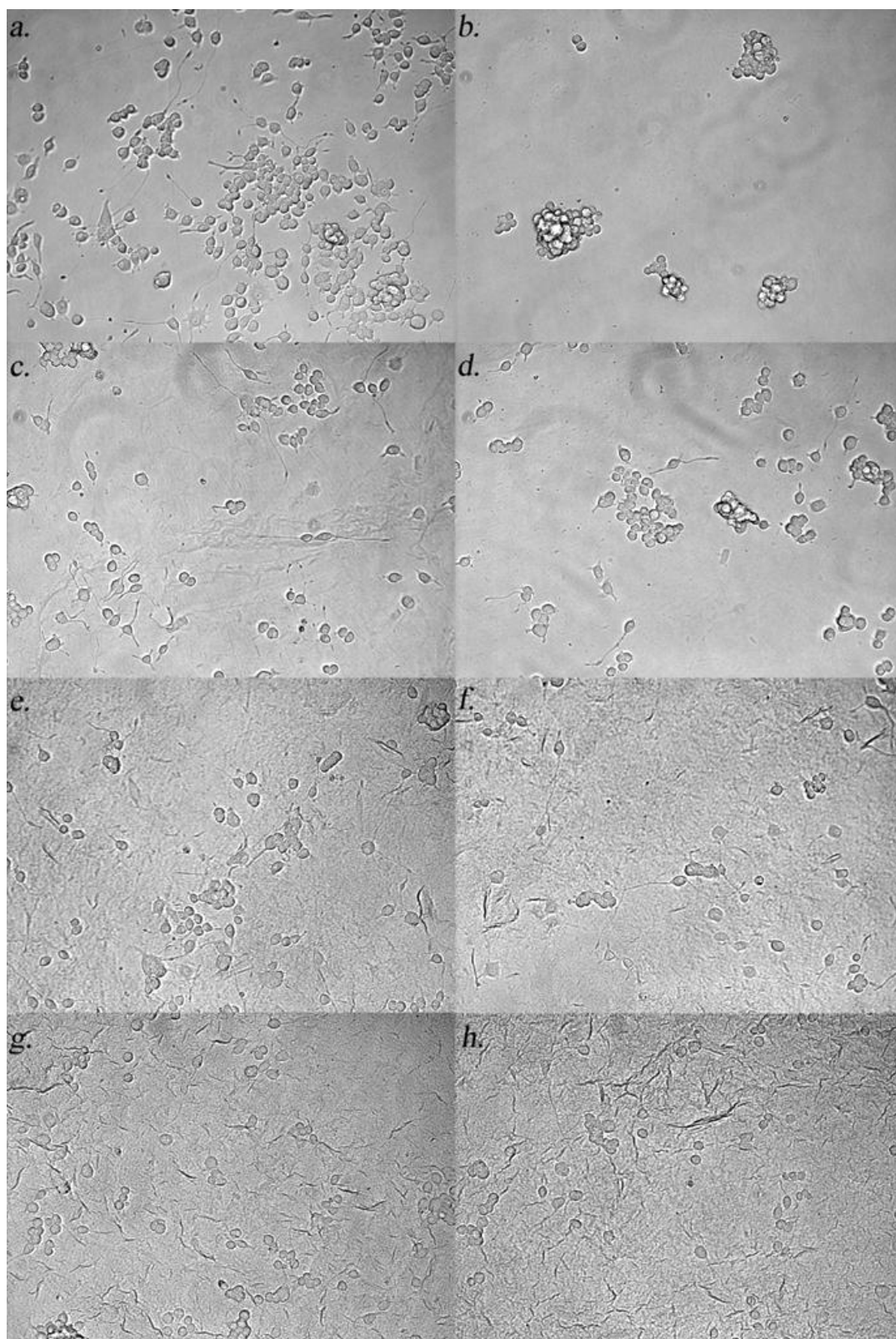


Figure 2. 26. Optical images of cells on substrates with CSPG. Images are obtained at day 2. When cultured on PDL with CSPG (b) neurite extension was almost completely inhibited compared to the PDL without CSPG (a). However, almost no inhibition was observed for PA scaffolds PA-K+PA-GAG (d), PA-LN+PA-E (f) and PA-K+PA-E (h) in the presence of CSPG when compared to those without CSPG (c, e, and g). Images were taken at 200x magnification.

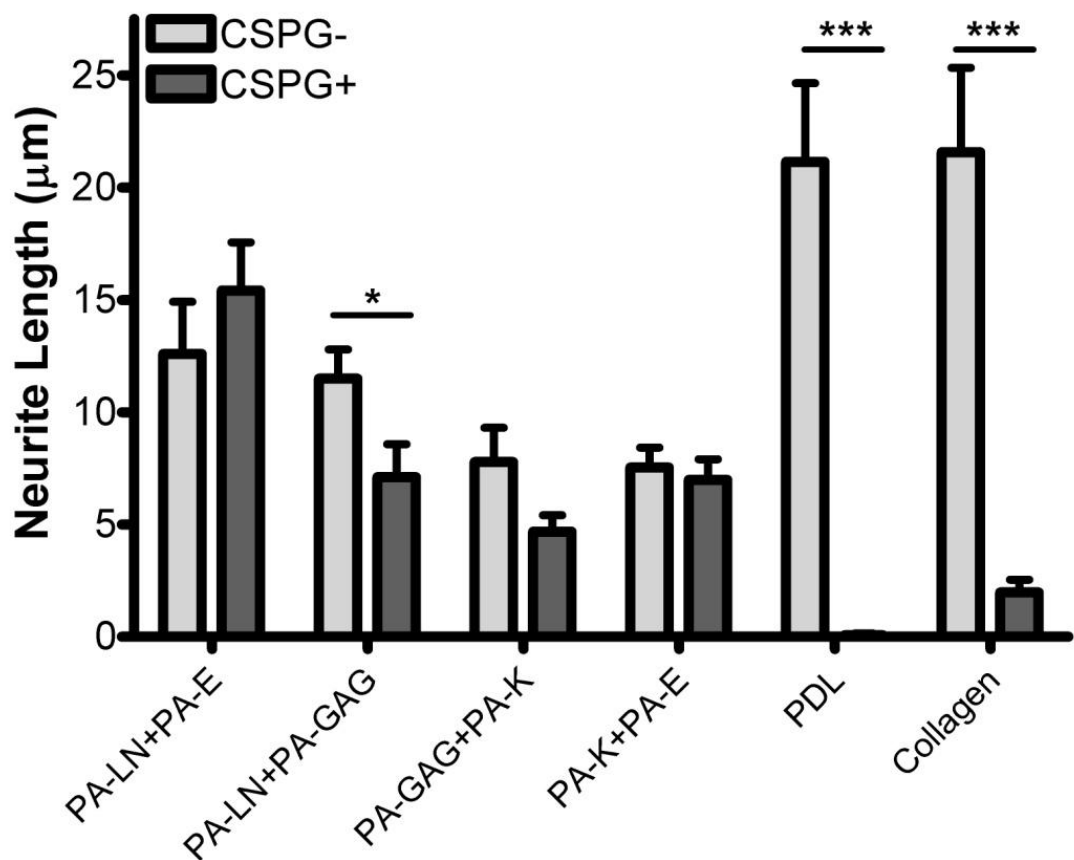


Figure 2. 27. Effect of CSPG inhibition on neurite length at day 2. PA scaffolds are beneficial in terms of overcoming inhibitory effect of CSPGs. Neurite length is not dramatically reduced on PA substrates in the presence of CSPGs in opposite to that on Collagen and PDL. Student's t-test is performed within each substrate group.

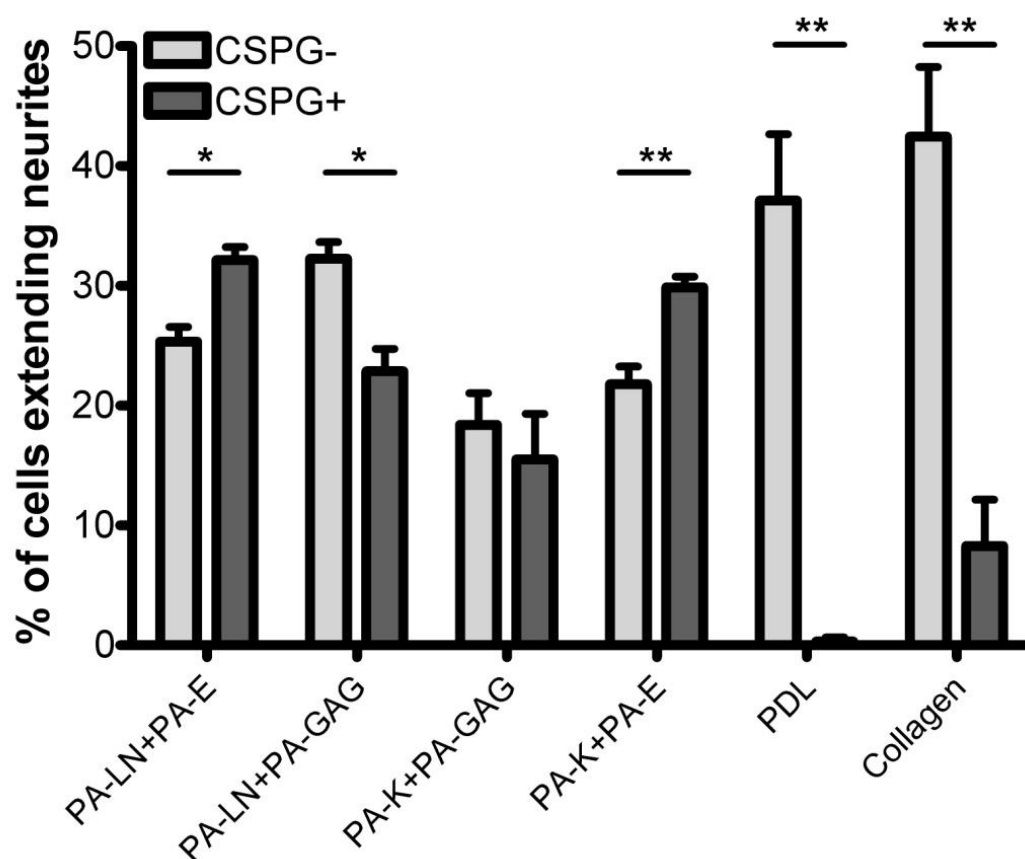


Figure 2. 28. Effect of CSPG inhibition on percentage of cells extending neurites at day 2. Percentage of cells extending neurites is not dramatically reduced on PA substrates in the presence of CSPGs in opposite to that on Collagen and PDL. Student's t-test is performed within each substrate group.

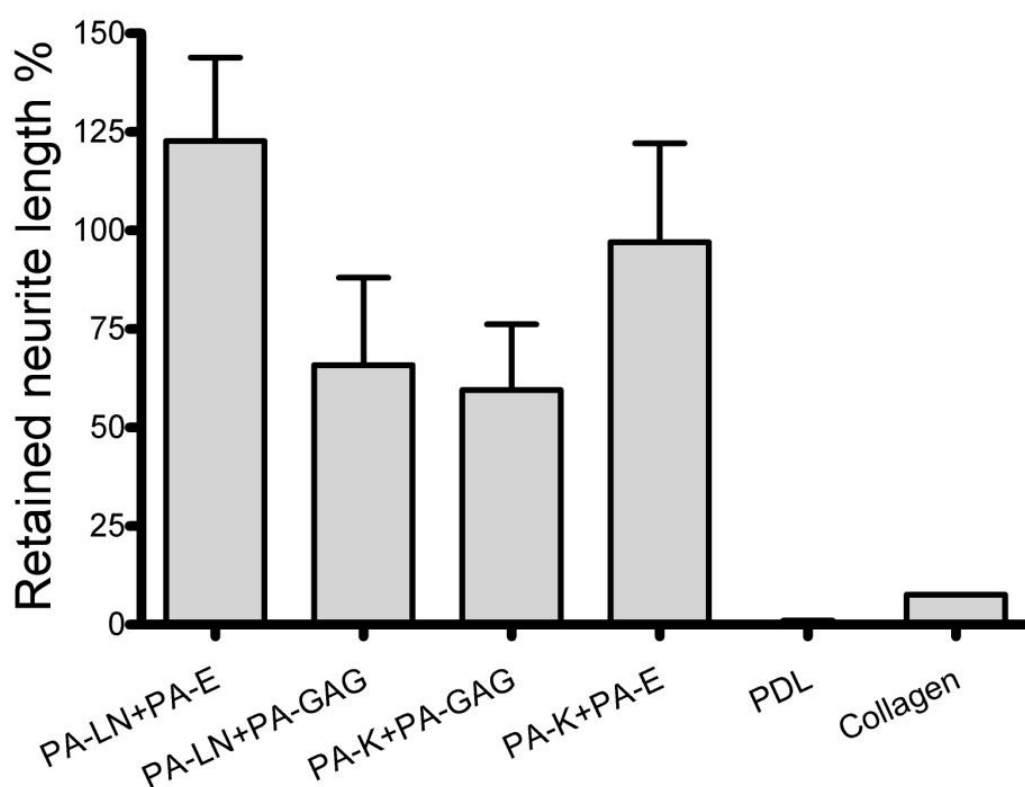


Figure 2. 29. Percentage of neurite length retained after CSPG treatment for 2 days. Much longer neurites are retained in the presence of CSPGs on PA substrates when compared to that on Collagen and PDL.

2.4. CONCLUSIONS

In this chapter, we studied a synthetic peptide nanofiber system mimicking neural ECM in terms of its neurite outgrowth promoting ability. These nanofibers contain laminin derived peptide signals along with heparan sulfate mimicking groups interacting with growth factors active in neuronal regeneration. These two bioactive components of this scaffold were found to collectively act and promote neurite

outgrowth of PC-12 cells effectively. In accordance with previous reports on full-length laminin-HSPG complexes, our completely synthetic composite scaffold induces much longer neurite formation when compared to the scaffold with laminin-derived signals alone. PA nanofiber scaffolds carrying laminin epitope alone were previously found to induce neural differentiation of stem cells as well as promoting axonal growth and serotonergic fiber plasticity besides inhibiting glial scar formation after spinal cord injury^{82, 88-89}. With the novel dual inducer peptide nanofiber scaffolds that are presented in this study, the effect of such nanofiber systems on neuroregeneration can be improved. Peptide scaffolds used in this study also aided cells to overcome the inhibitory effect of CSPGs on axonal growth. These results show that this newly developed peptide scaffold provides a promising novel therapeutic approach for neuroregeneration studies due to its neurite outgrowth promoting ability, even in the presence of inhibitory components that are abundant in injured nervous system.

CHAPTER 3

**BIOFUNCTIONALIZATION OF POLYMERIC
NERVE GUIDANCE CONDUITS WITH PEPTIDE
NANOFIBERS FOR SCIATIC NERVE
REGENERATION**

3.1. INTRODUCTION

Peripheral nerve injury (PNI) incidences are high in the society leading to a high number of patients suffering from neuropathic pain and bringing an economic burden resulting from health care costs. Peripheral nerve surgeries are carried out for approximately 10^5 patients per year in United States and Europe⁸. 20 million patients are known to suffer from peripheral neuropathy and approximately \$150 billion is spent annually for health care due to nerve injuries in United States⁷. Even worse is the low success of current therapies leading to less than half of the patients being able to fully recover and gain sufficient motor and sensory function⁷. If not treated appropriately, PNI result in painful peripheral neuropathies leading to lifelong disability. Depending on the length of the nerve gap formed by the injury, different therapeutic approaches are considered by clinicians. Coaptation, though being easier to carry out, can only be applied to very short lesions under 4 mm. If the gap distance is longer, such end-to-end suturing brings extra tension to the nerve leading to a secondary damage. Besides the gap distance the delay in the repair also affects the therapeutic success in coaptation due to inelastic recoil in nerve stumps and excess connective tissue formation due to embedding of nerve stumps in scar tissue¹²⁷. Other therapeutic options should be considered in long distance gaps and for delayed repair. Autografting is considered as the clinical gold standard for treatment of injuries with larger gap distances. A nerve segment harvested from another site of the body, preferably from a region this nerve loss can be tolerated, is sutured to the nerve ends formed by the injury. Peripheral nerves have endogenous regenerative potential in contrast to the central nervous system. Axonal growth rate in human peripheral

nerves is approximately 1 inch monthly¹²⁸. However, lack of guidance molecules can make the process chaotic. Unless therapeutically guided, randomly growing axons might have difficulty in path finding to reach the distal stump resulting in the formation of a painful neuroma and can even lead to amputation. Neuromas are the main cause of chronic neuropathic pain after trauma and the surgical removal of the benign tumor tissue is the most commonly used treatment. However, even after multiple surgeries, painful symptoms are not relieved in one in five patients¹²⁹. Hence, guidance cues are essential for pain free regeneration of the peripheral nerve through the end target organ. Transplanted nerve section in the autograft provides these guidance cues including endoneural tubes as well as Schwann cells. Schwann cells provide guidance both physically, by Büngner bands, and chemically through inductive soluble factors they secrete. Though reducing the neuroma formation risk and providing inductive cues for axonal regeneration make them to be considered as gold standards, autografts also possess several drawbacks. Donor site morbidity is inevitable due to denervation induced by harvesting the nerve segment. Application of the technique is also limited by the length of the nerve section required. Harvesting long nerve segments to treat very long gap distances or multiple injury sites is impossible. Besides, mismatch between the anatomy of sensory and motor nerve fibers between the host and donor nerves is known to lead to insufficient regeneration in some cases¹³⁰. An alternative to autograft is nerve guidance conduits (NGC) which can offer even a better regeneration when designed smartly. Nerve guidance conduits are hollow tubes used to bridge transected nerve stumps for enhancing the axonal regeneration. Considering that materials used for production, fabrication methods and functionalization options of the interior surface are limitless,

NGCs are promising candidates for perfect regeneration of peripheral nerve injuries offering gain of function. They can also be synthesized in any length and size, relieving the gap distance limit in regeneration after PNI. NGCs provide a barrier against scar tissue infiltration and allow axonal elongation through the distal stump. Besides providing an isolation chamber to axonal stumps for proper path finding, they can also present neuroinductive signals to regenerating axons resembling that in the peripheral nervous system (PNS). These signals may include but not limited to physical guidance cues, extracellular matrix elements of basal lamina and neuroinductive soluble factors or small molecules tethered to the internal surface of the conduit. Functionalization with multiple cues can better mimic the permissive environment of the PNS. NGCs can also be produced from biodegradable materials which undergo complete degradation some time after regeneration without any adverse effects.

Providing inductive cues in the NGC is crucial for elongation of the axon from the proximal stump beyond distal stump to reach the end target organ. Extracellular matrix (ECM) of PNS is quite informative for NGC functionalization and mimicking signals produced in peri-axonal space by Schwann cells is helpful for enhancing regeneration. In this manner, we used self assembling peptide nanofiber gels containing neuroinductive signals for biofunctionalization of clinically used polymeric NGCs. These gels are made of nanofibers consisting of laminin derived and heparan sulfate mimicking peptide sequences and were used to fill the interior lumen of the NGC. Laminin is an ECM protein abundantly present in basal lamina of peripheral nerves which is secreted by Schwann cells¹³¹. Basal lamina production is

upregulated upon PNI and it has a significant role in the regenerative potential of the PNS⁴⁸. Besides its role as a positive regulator of neurite outgrowth, laminin is also important in Schwann cell differentiation after injury and its ablation impairs axon regeneration severely⁵⁷. Heparan sulfate proteoglycans (HSPG) are also PNS basal lamina components⁵⁶. Heparan sulfate (HS) is known to bind to growth factors as well as cell surface receptors. Binding of HS increase the local concentration of growth factors, improve their stability as well as enhancing cell signaling by those growth factors¹³²⁻¹³³. Schwann cells secrete HS binding FGF-2, expression of which is also upregulated upon injury and has an inductive effect on axon growth¹³⁴. Depletion of the basal lamina in large gap injuries leads to loss of guidance molecules including laminin and FGF-2 and it is a limiting factor in axonal regeneration. Overcoming this limitation can be possible by mimicking basal lamina through use of synthetic nanofibers. Short peptide sequences presented on these nanofibers can bind to cell surface receptors and growth factors to induce axonal regeneration in the lack of basal lamina. Such scaffolds presenting laminin derived signal along with HS mimicking moieties were previously found to improve neurite outgrowth *in vitro*⁸¹. In this study, peptide nanofiber biofunctionalization of a polymeric hollow nerve guidance conduit was carried out. Neurolac[®] TW (Polyganics, Netherlands) is a biodegradable synthetic NGC made of poly-DL-lactide- ϵ -caprolactone. It is resorbed within 24 months after insertion¹³⁵. Guidance molecules provided by peptide nanofibers were used within the lumen of Neurolac[®] TW to achieve an enhanced neural regeneration after complete transection of sciatic nerve. Regeneration was proved to be quite better according to functional outcome

analysis as well as histological analysis when peptide nanofiber biofunctionalized NGCs were used.

3.2. MATERIALS AND METHODS

3.2.1. Materials

9-fluorenylmethoxycarbonyl (Fmoc) and tert-butoxycarbonyl (Boc) protected amino acids, [4- α -(2',4'-dimethoxyphenyl)Fmoc-aminomethyl]enoxy]acetamidonorleucyl-MBHA resin (Rink amide MBHA resin), Fmoc-Asp(OtBu)-Wang resin and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from NovaBiochem and ABCR. The other chemicals were purchased from Fisher, Merck, Alfa Aesar or Aldrich. Neurolac TW nerve guidance conduits (NG02-015/03) were obtained from Polyganics and used as directed. Antibodies against S-100 (04-1054) and β -III-tubulin (04-1049) and HRP conjugated secondary antibody (12-349) were purchased from Millipore.

3.2.2. Synthesis and purification of peptide amphiphiles

Synthesis and purification of PA molecules was carried out as described in the section 2.2.2.

3.2.3. Scanning electron microscope and transmission electron imaging of peptide nanofibers

SEM imaging was carried out for observation of PA nanofiber networks. Oppositely charged PA solutions of 1 wt% were mixed in appropriate volume ratio (final volume

being 50 μ L) to produce neutral charge gels. Gels were then dehydrated by transferring to 20%, 40%, 60%, 80% and 100% v/v ethanol sequentially. Dehydrated PA gels were critical-point-dried afterwards by using Autosamdri®-815B equipment from Tousimis. Dried PA networks were coated with 6 nm Au/Pd and SEM (FEI Quanta 200 FEG) images were taken by using an Everhart–Thornley Detector (ETD) at high vacuum mode with 15 keV beam energy.

3.2.4. Surgical procedure

Ten weeks old male Sprague-Dawley rats were used under the approval of Animal Ethics Committee of Gulhane Military School. Twenty eight rats were divided into four experimental groups with seven rats each. Rats were anesthetized before surgery with xylazine and ketamine (80 mg/kg). Sciatic nerves were exposed by skin and muscle incision and full transection injury was performed by excision of 10 mm nerve tissue. 15 mm nerve conduit was sutured to transected nerve ends by inserting 2.5 mm nerve segments from both proximal and distal end into the tube and leaving a 10 mm gap (Figure 3.1, a-c). After suturing the conduit, PA gels with neutral charge were formed *in situ* by injecting 100 μ L/PA solution with a total volume of 200 μ L. Two oppositely charged PA solutions were injected into the tube with two different injectors at the same time (Figure 3.1d). PA-LN+PA-GAG gel was used for biofunctionalization of biologically inert conduits while PA-K+PA-E gel was used as an epitope-free control. To obtain a neutral charged PA gel, 4 mM PA-LN was mixed with 4 mM PA-GAG, and 4 mM PA-K was mixed with 2 mM PA-E at equal volumes. PAs were dissolved in isotonic sucrose (0.25 M) solution. Nerve conduits filled with sucrose was used as non-treated conduit control (S). For autograft treated

animals (A), excised nerve tissues were reversed and sutured back to nerve stumps. Surgical operations were performed on the sciatic nerve of right hind limbs and left limb sciatic nerves were used as healthy control.

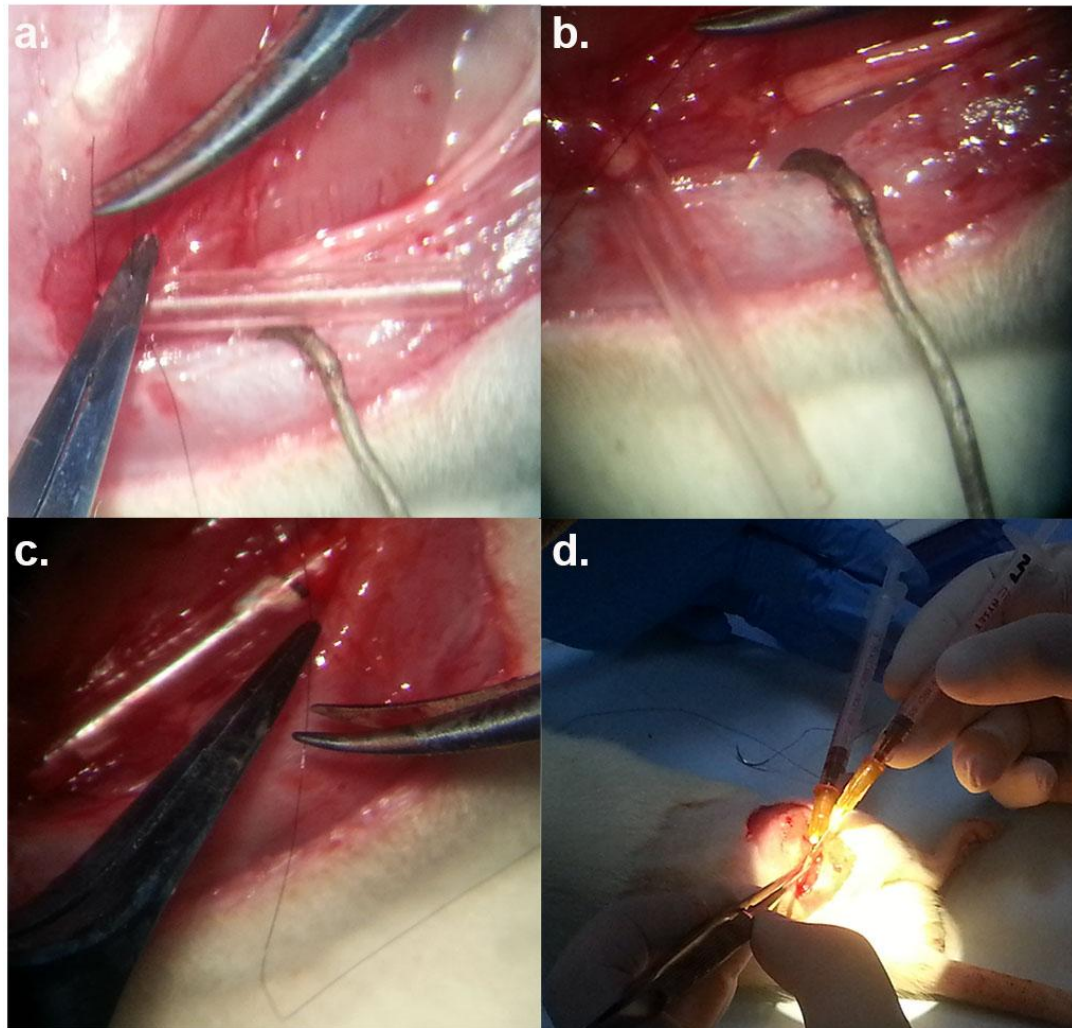


Figure 3. 1. Images of surgical operation shows conduit sutured to the excised sciatic nerve (a-c) and application of the PA gel in the conduit (d).

3.2.5. Walking track analysis

Twelve weeks after the surgery, walking track analysis was performed as described previously¹³⁶. Briefly, hind paws were ink-stained and rats were allowed to walk in an 8 cm x 43 cm wood walking path with a dark box at the end of the path. Footprints were analyzed to calculate sciatic functional index from the following parameters; PL (paw length), the distance between the top of the third toe and the bottom of the heel, TS (toe spread), the distance between the first and the fifth toe and IT (internal toe spread), the distance between the second and fourth toe. All measurements were carried out from both experimental (right) and normal (left) limbs. Parameters obtained from experimental limb were denoted as EPL, ETS, and EIT while those from the normal limb were called as NPL, NTS, NIT, and sciatic functional index (SFI) was calculated for each animal by using the following formula:

$$SFI = 109.5(ETS-NTS)/NTS-38.3(EPL-NPL)/NPL+13.3(EIT-NIT)/NIT-8.8.$$

3.2.6. Electrophysiological measurements

12 weeks after the surgery, rats were anesthetized after which sciatic nerves were exposed. A bipolar stimulating electrode was placed to proximal to the injury site under sciatic nerve. Recording electrode was placed in the gastrocnemius muscle. Compound action potentials and latency of signals were recorded and recovery in amplitude along with the increase in latency time was calculated by comparing the operated nerve with the healthy nerve of the same rat.

3.2.7. Histological analysis of sciatic nerves

Sciatic nerves were harvested after EMG measurement at 12 weeks and each harvested nerve was dissected into three parts named as proximal, mid-tissue and distal (Figure 3.8). Mid-tissue was a 2 mm segment taken from the middle part of the graft and it was fixed in 2.5% gluteraldehyde. After post-fixation with 2% OsO₄ for 4 h, mid-tissues were embedded into Araldite resin. Semi-thin (1 µm) transverse sections were used for toluidine blue staining. Proximal and distal segments were fixed with 10% formalin and embedded into paraffin after dehydration of tissues in increasing concentrations of ethanol and xylene. 4 µm longitudinal sections were used for hematoxyline-eosin staining and immunohistochemistry (IHC).

Hematoxyline eosin staining was performed on sections incubated overnight at 58 °C for deparafinization. Rehydration was performed by serial incubations of tissues in decreasing concentrations of ethanol after which nuclei are stained with Mayer's hematoxyline solution for 45 sec. After immersing tissues in 0.1% (v/v) HCl, washing under running water, rinsing in dH₂O and 95% ethanol were carried out. Cells were counterstained with eosin Y solution for 50 sec. After dehydration steps in 95%, absolute ethanol and xylene, slides were mounted on coverslips with xylene based mounting medium.

Immunohistochemistry was performed on deparafinized and rehydrated tissues. After rehydration, tissues were incubated with pepsin for antigen retrieval at 37 °C for 10 min after which they were washed under running tap water for 3 min. After rinsing

with TBST (tris buffered saline with 0.025% Triton X-100) 2 times, 5 min blocking was carried out. 10% goat serum and 1% BSA dissolved in TBS were used as blocking buffer in which tissues were incubated for 2 h at RT. Primary antibodies were diluted in TBS with 1% BSA. 1:100 dilution of β -III-tubulin and 1:250 dilution of S-100 were used and slides were incubated overnight at 4 °C in humidified chamber. For negative control of staining tissues were incubated in antibody dilution solution. After two rounds of 5 min wash with TBST, tissues were incubated in 1:500 diluted goat-anti-mouse HRP conjugated secondary antibody for 1 h at RT. DAB staining was carried out after 3 washes with TBST. DAB substrate was used warm (RT) and after incubation with substrate slides were rinsed in dH₂O. Counterstaining with hematoxyline was carried out after which slides were mounted for imaging.

3.3. RESULTS

3.3.1. Characterization of peptide nanofiber gels

Synthetic nerve guidance conduits are hollow tubes that provide isolation of injured nerve ends from the surrounding connective tissue, and prevent fibroblast infiltration and scar tissue formation. Besides, they function as a guidance channel for the newly elongating axons from the proximal stump to find the distal stump which is crucial for prevention of painful neuromas that are common in untreated peripheral nerve injuries¹³⁷. Although NGCs are considered as an alternative therapy for long gap nerve transections without harvesting a nerve from a donor site, therapeutic success is not as high as desired yet. Incorporation of biological functional groups can

provide additional benefit for axonal recovery considering that the axonal development occur not in a hollow tube but in an environment full of biological signals that work in a collective manner. These signals are normally provided by ECM molecules, growth factors and cytokines and nearby cells secreting these factors in the peripheral nerves. To achieve an enhanced axonal recovery, Neurolac[®] TW conduits' lumens were filled with peptide nanofiber gels providing native tissue like biological signals. Two different peptide amphiphiles (PA) with collaboratively working bioactive epitopes were used for biofunctionalization of NGCs. PA-LN contains a laminin derived short peptide "IKVAV", while PA-GAG mimics growth factor binding ability of heparan sulfate glycosaminoglycans through hydroxyl, carboxylic acid and sulfonate groups presented on side chains of the amino acids in the sequence. These two PA molecules are oppositely charged and form a self supporting PA-LN+PA-GAG gel upon mixing by charge neutralization and hydrophobic collapse. As physical guidance is also important in axonal elongation and path finding, a control peptide nanofiber gel, named as PA-K+PA-E, was also used. PA-K+PA-E consists of two peptide amphiphile molecules without bioactive epitopes. PA-K contains only a lysine and PA-E contains a glutamic acid for charge incorporation, instead of bioactive epitopes present in PA-LN and PA-GAG. Both PA-LN+PA-GAG and PA-K+PA-E gels consist of peptide nanofibers with a 8-10 nm diameter as imaged by TEM (Figure 3.2, c-d) and nanofiber networks within these PA gels have a physical structure that resembles that of natural ECM as revealed by SEM imaging (Figure 3.2, a-b)¹³⁸.

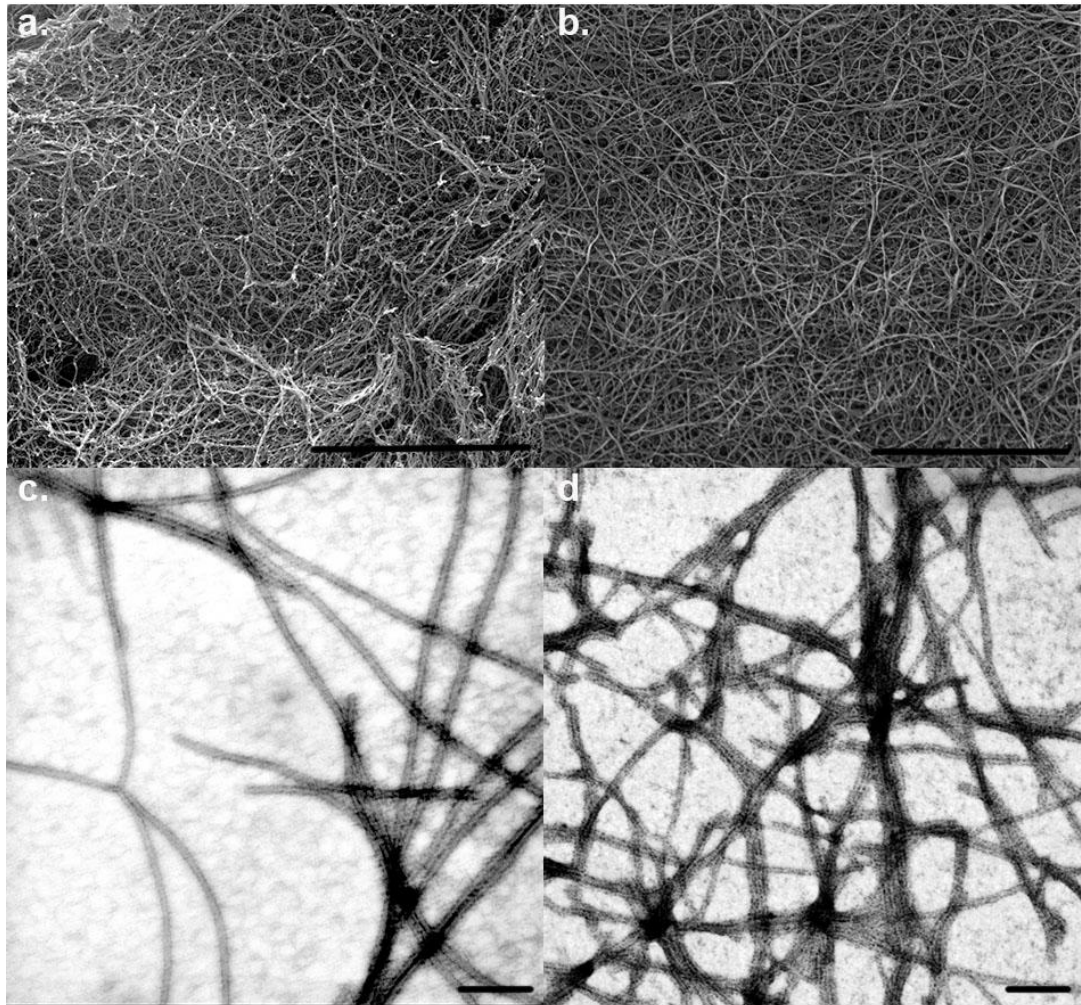


Figure 3. 2. SEM (a, b) and TEM (c, d) images of PA-LN+PA-GAG (a, c) and PA-K+PA-E (b, d).

3.3.2. Functional evaluation of transected sciatic nerves after repair

Functional outcome of the repair after full transection of the sciatic nerve was assessed 12 weeks after operations. Motor behavior recovery was determined by sciatic functional index (SFI) measurements deduced from walking track analysis. Fully recovered sciatic nerve transmits motor signals to the paws allowing toe

opening and full pressure applied by the paw leading to a normal walking pattern of footprints. The better the opening of toes, the higher is the distance between toes; and the higher the pressure applied by paw to the surface, the longer is the paw length. Less negative SFI values are obtained for rats whose distance between toes and paw length are longer. SFI values closer to zero indicates better functional regeneration while those close to -100 indicate worse regeneration if any at all. Biofunctionalized nerve conduit treatment resulted in best functional outcome (Figure 3.3), although the results were not significant due to high variation within the groups (Figure 3.4). When nerve conduits were filled with epitope free peptide nanofiber gel, mean results were similar to those from the bioactive gel filled conduits, both being better than empty conduit and autograft. However, analyzing SFI values from each rat indicates quite well recovered individual rats within PA-LN+PA-GAG filled conduit treated group (Figure 3.5). SFI values of these individuals are much closer to zero and quite better than those within other groups. Number of rats eliminated from walking track analysis due to self-chewing leading to digit loss is an indirect measurement of lack of sensory recovery. Digit loss was observed in a high number of animals treated with empty conduits and autograft while almost none was observed in animals treated with peptide nanofiber filled conduits. These results indicate that filling the conduit with peptide nanofibers leads to better sensory and motor recovery when compared to the use of empty conduit and even to the autograft treatment.

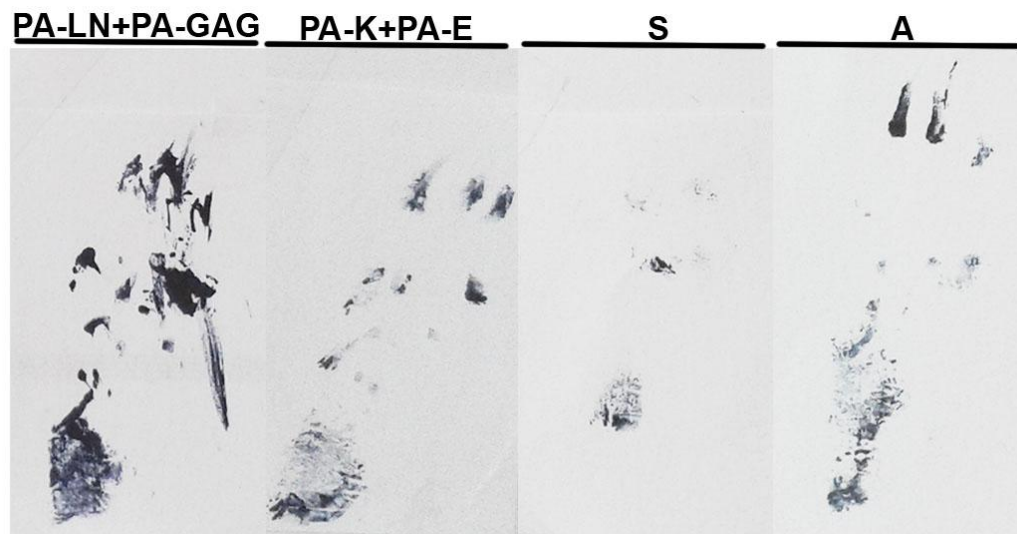


Figure 3. 3. Footprints of rats obtained during walking track analysis at week 12 after operation.

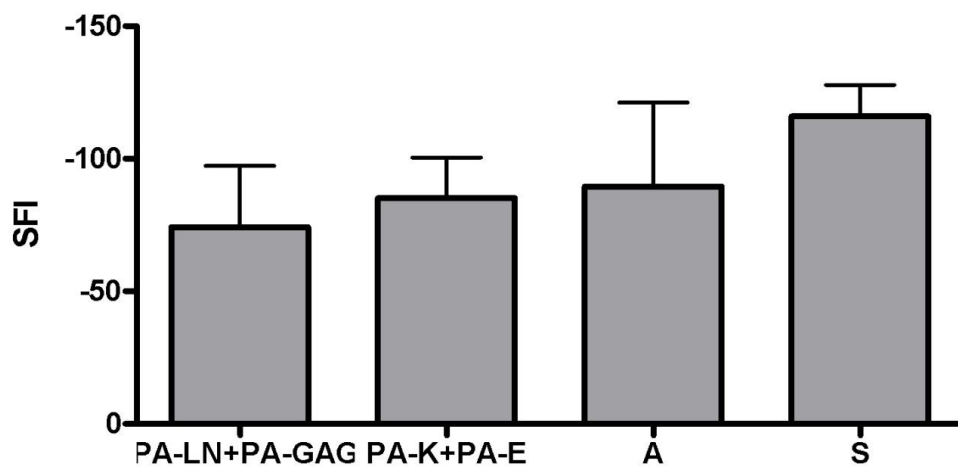


Figure 3. 4. Sciatic functional index measurement results showing better, yet nonsignificant scores in rats treated with PA-LN+PA-GAG biofunctionalized nerve guidance conduits.

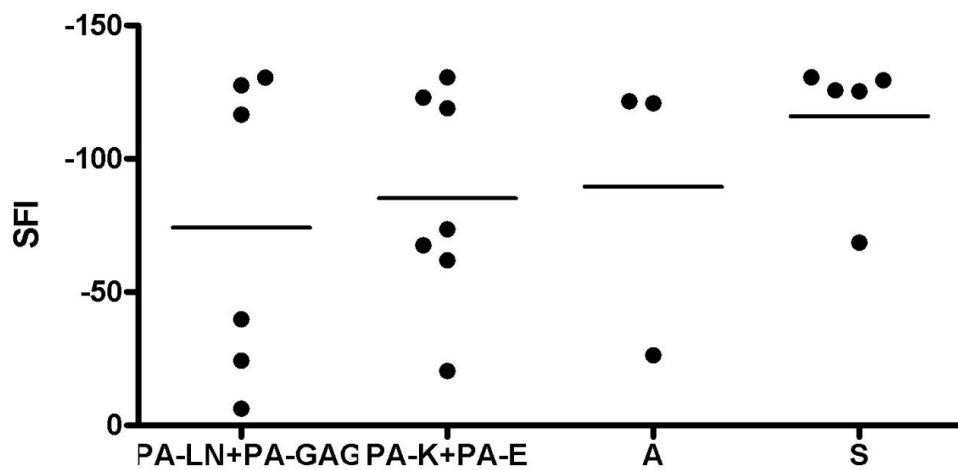


Figure 3. 5. Sciatic functional index measurement results showing performance of individual rats within groups.

Electromyography was also performed as a quantitative measure of nerve signals transmitted from regenerated axons. Stimulating muscles proximal to the transected nerve section results in receiving signals from end target muscles if the regenerating axons reach to the target organ and properly myelinated. Two measures are used to detect functional recovery by EMG. Amplitude shows the size of the action potentials transmitted through the regenerated axon fibers while latency shows the time it takes for signals to reach the end target. The higher the amplitude and the shorter the latency, the better is the electrical signal transmission through the nerve. EMG results are in good coherence with SFI measurements. Use of conduits biofunctionalized with PA-LN+PA-GAG gels show significant improvement in amplitude when compared to use of empty conduits and autograft treatment. Epitope free control PA-K+PA-E gel, when used in conduits, also lead to higher amplitudes

than empty conduits and autograft but the differences were not significant. Latency measurements indicated a delay in signal transmission in nerves repaired with empty conduits and autografts, and a faster transmission when conduits were filled with peptide nanofibers.

Considering the results of EMG and SFI measurements together, we can conclude that a better functional outcome was obtained when transected sciatic nerves were treated with peptide nanofiber filled conduits. Use of bioactive PA gels results in better SFI values and slightly higher amplitudes, when compared to the use of control PA gels. This indicates that signal transmission is better in nerves treated with PA-LN+PA-GAG biofunctionalized conduits. Axonal recovery is suggested to be further improved with the help of biological signals provided by PA-LN+PA-GAG nanofibers in the conduit lumen according to the functional outcome measurements. Laminin derived IKVAV peptide sequences, along with the growth factor binding epitopes from GAG mimetic sequences, might lead to a faster rate of axonal elongation and induce recovery in a higher number of axons. Laminin is not only important in neurite outgrowth but also in Schwann cell differentiation and myelination of newly elongating axons¹³⁹⁻¹⁴⁰. It is also known that ablation of laminin impairs Schwann cell proliferation¹⁴¹. Higher number of Schwann cells can dedifferentiate and turn into proliferating state in the presence of laminin derived IKVAV sequence resulting in higher number of Schwann cells for initiation of Wallerian degeneration. This in turn might recruit macrophages in higher numbers and leads to faster and better clearance of axonal debris. Myelin products from axonal debris are known to be inhibitory for axonal elongation and their clearance by

Wallerian degeneration paves the way for restoration of the nerve conduction pathway. Besides, Büngner bands formed by proliferating Schwann cells is an important path finding element for growth cones of elongating axons¹⁴². Dedifferentiation of Schwann cells into myelinating state is also important. Myelination of higher number of axons and/or thicker myelin deposition around axons leads to a better insulation of elongated axons. As myelin insulation will keep the signal being transmitted without loss, amplitude of the signal will be higher in myelinated axons. Besides, insulation also increases the rate of transmission resulting in shorter latency as desired with the therapy. Besides the potential role of IKVAV peptide sequence, mimicking sulfated GAG-growth factor interaction is also expected to contribute to the peripheral nerve regeneration. FGF-2 secretion is known to be upregulated after PNI. FGF-2 is a heparin binding growth factor with a neurite inducing capability besides its role on Schwann cell proliferation¹³⁴. It was previously proved that FGF-2 also binds to PA-GAG with high affinity¹⁴³. Better preservation of FGF-2 along with the increased local concentration on peptide nanofibers and better presentation to cell surface receptors could also contribute to the better functional outcome in PA-LN+PA-GAG functionalized conduit treated rats.

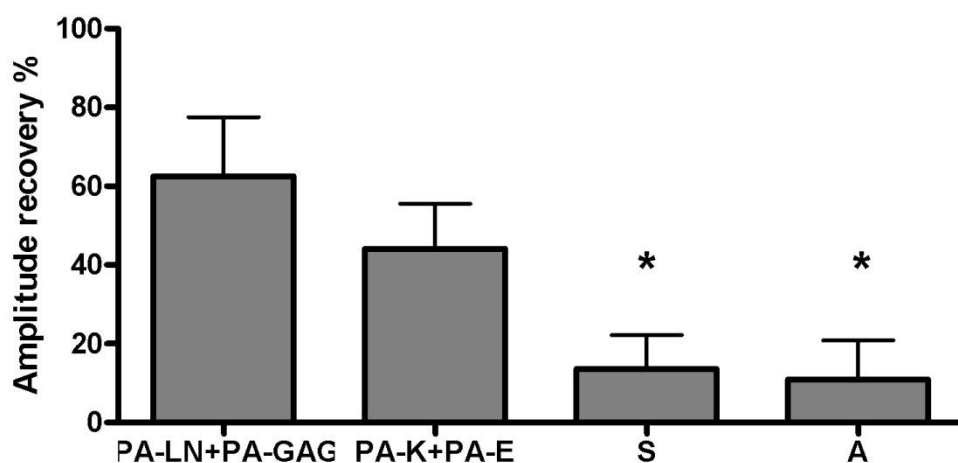


Figure 3. 6. Recovery of lost amplitude at week 12 as measured by EMG.

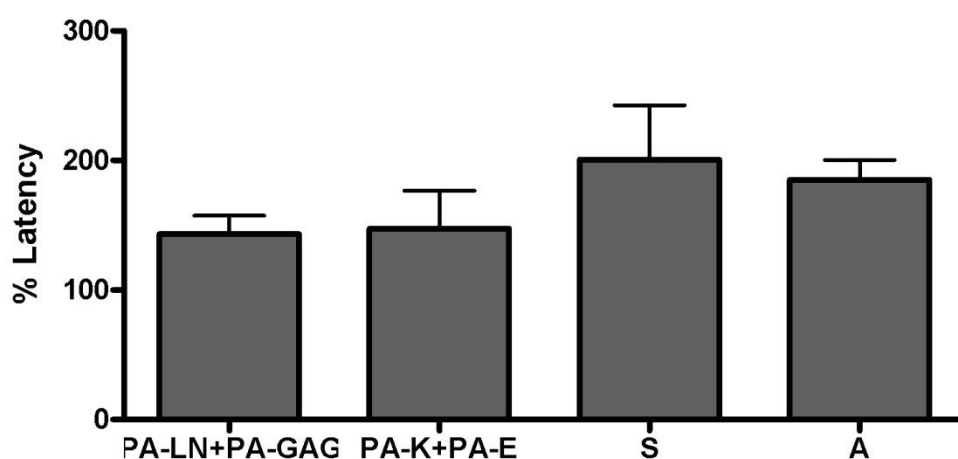


Figure 3. 7. Percentage increase in latency in operated nerves when compared to the healthy unoperated nerves at week 12, as measured by EMG.

3.3.3. Histological analysis of regenerating nerve morphology

In order to understand the effect of PA-LN+PA-GAG functionalization of the conduit on regenerating tissue, a detailed histological analysis was carried out.

Excised tissues were dissected into three parts (Figure 3.8). 3 mm tissue segment from the center of conduit and autograft was processed for ultra structure imaging by TEM and toluidine blue staining for detailed analysis of myelination. Proximal and distal tissues were used for analyzing the extent of axonal elongation and density of the elongated axons. Besides, Schwann cell population of elongated axons was also investigated due to its importance in myelination of newborn nerves.

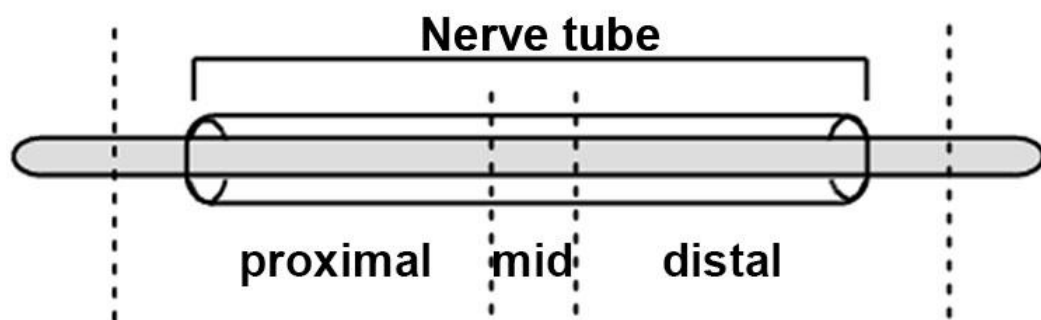


Figure 3. 8. Excised nerve tissue parts used for different histological analysis. Proximal and distal tissues are embedded in paraffin while mid tissue is embedded in resin for ultra structure analysis.

Overall morphology of the repaired nerves was deduced from observation of hematoxyline-eosin (HE) stained tissues (Figure 3.9). Good linear ordered structure was observed in all treatment groups in the proximal segment. This indicates a healthy axonal elongation from the proximal stump of the transected nerve to the center of the 10 mm nerve gap produced by transection. However when the nerve segments closer to the distal stump were observed, it was clear that nerves elongated

further and reached to the distal stump when conduit lumen was filled with peptide nanofibers. This is suggested to occur with the help of physical guidance provided by nanofiber network that fills the way through the conduit starting from the proximal nerve stump and reaching the distal stump. This is evidenced by the lack of reintegration at the distal stump when empty conduits were used. Besides the physical guidance provided by the continuous nanofiber network, bioactive signals provided in PA-LN+PA-GAG filled conduits proved to enhance axonal elongation. This became more obvious in closer view of the nerve beyond distal stump (Figure 3.10). Axon density and integrity was better in PA-LN+PA-GAG filled conduits in these regions when compared to that in epitope free control PA-K+PA-E filled conduits.

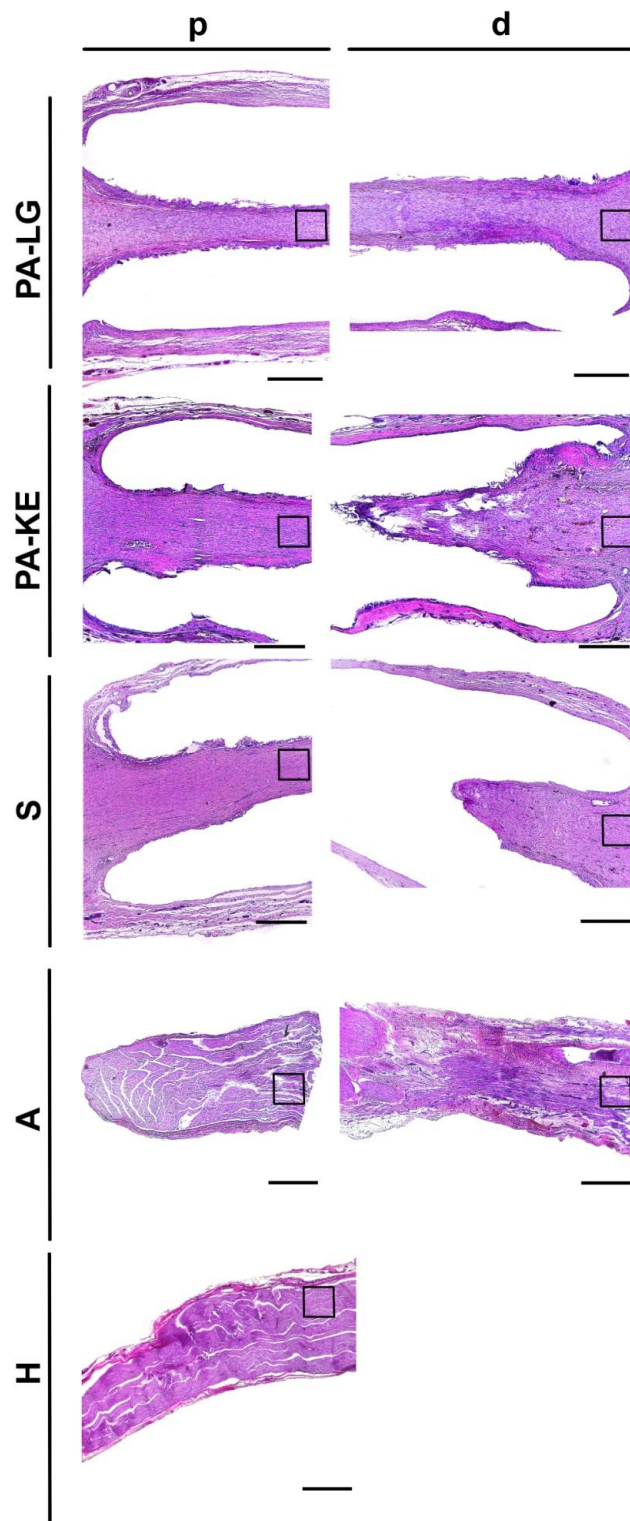


Figure 3. 9. H&E stained nerve tissues harvested at 12 weeks after operation. Scale bars are 200 μm .

Images obtained at higher magnification (Figure 3.10) also provide information about the integration of the newborn nerves at the far distal region. Signs of Wallerian degeneration are clear in nerves at far distal region for all nerves except those treated with PA-LN+PA-GAG filled conduits. Though not as perfect as those in the proximal segment, nerve fascicles show linear orientation at distal segment, too, when PA-LN+PA-GAG biofunctionalized conduits were used. This linear orientation seemed to be hampered in all other treatments applied. Besides, there was no sign of gaps caused by denervation in PA-LN+PA-GAG filled conduit treated nerves which was clearly present in those treated with all other treatments including autografts. This indicates that after the initial clearance of nerve fragments in the distal part by Wallerian degeneration, elongated nerves could reach the distal stump at sufficient numbers and form a healthy continuous path to the end target organ with the help of laminin and GAG mimetic groups presented on PA-LN+PA-GAG nanofibers. In the lack of these groups, although there were some axons that reached their destination, it was not enough to completely reinnervate the denervated tissue.

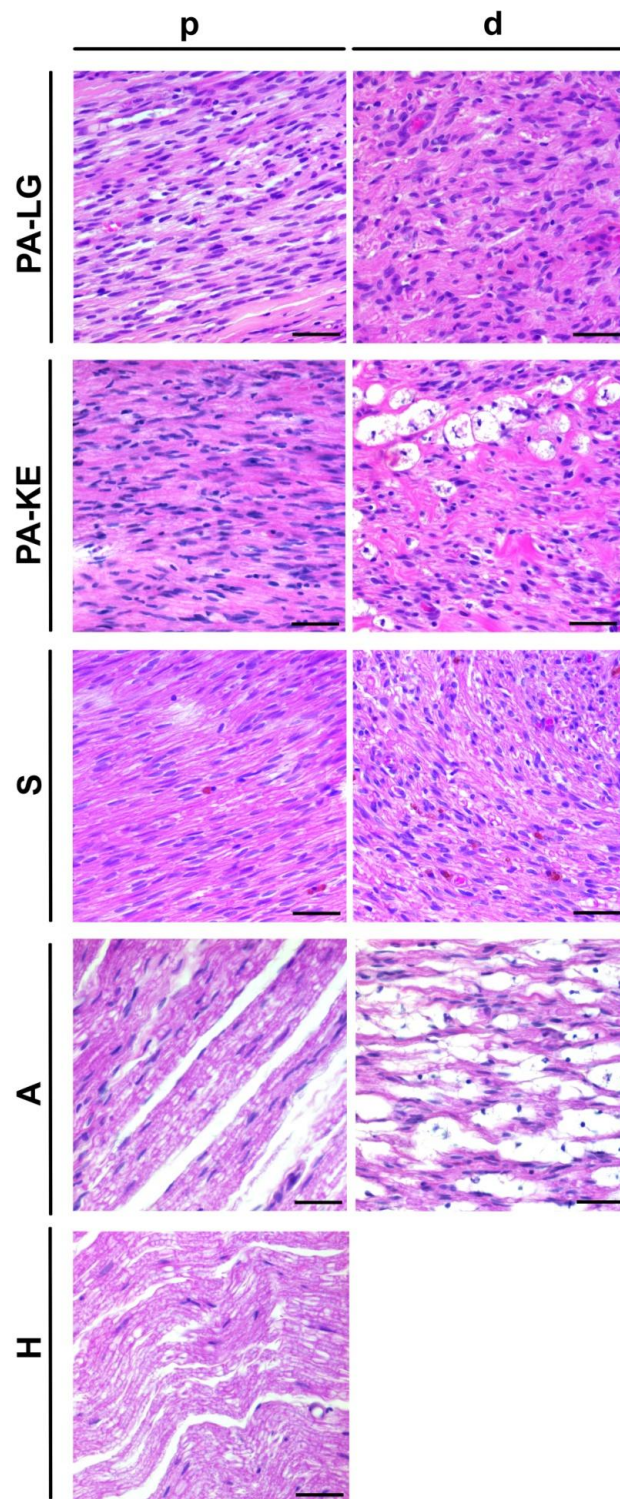


Figure 3. 10. Magnified images of H&E stained nerve tissues harvested at 12 weeks after operation. Scale bars are 40 μm .

Staining nerves against β -III-tubulin and S100 further proved the better regeneration in nerves treated with PA-LN+PA-GAG biofunctionalized conduits. A more condensed β -III-tubulin staining pattern in these nerves indicates that a higher number of axons recovered and elongated in the presence of laminin and GAG mimetic cues (Figure 3.11). This is observed not only in the proximal segment but also in the far distal segment showing that elongating axons could achieve to reach far beyond the distal injury site. Insufficient reinnervation and discontinuity in the nerves treated with autografts, empty or control PA-K+PA-E filled conduits observed are in good correlation with HE staining results but more apparent in β -III-tubulin stained tissues. Reinnervation at distal region is crucial for the end target organ functionality. Histology results are in good coherence with the EMG and SFI measurements in this manner as both pointed out improved functionality upon treatment with PA-LN+PA-GAG biofunctionalized conduits. Inability to achieve reinnervation at distal region leads to a muscle atrophy which eventually leads to improper, even if any, gain of function in long term¹⁴⁴.

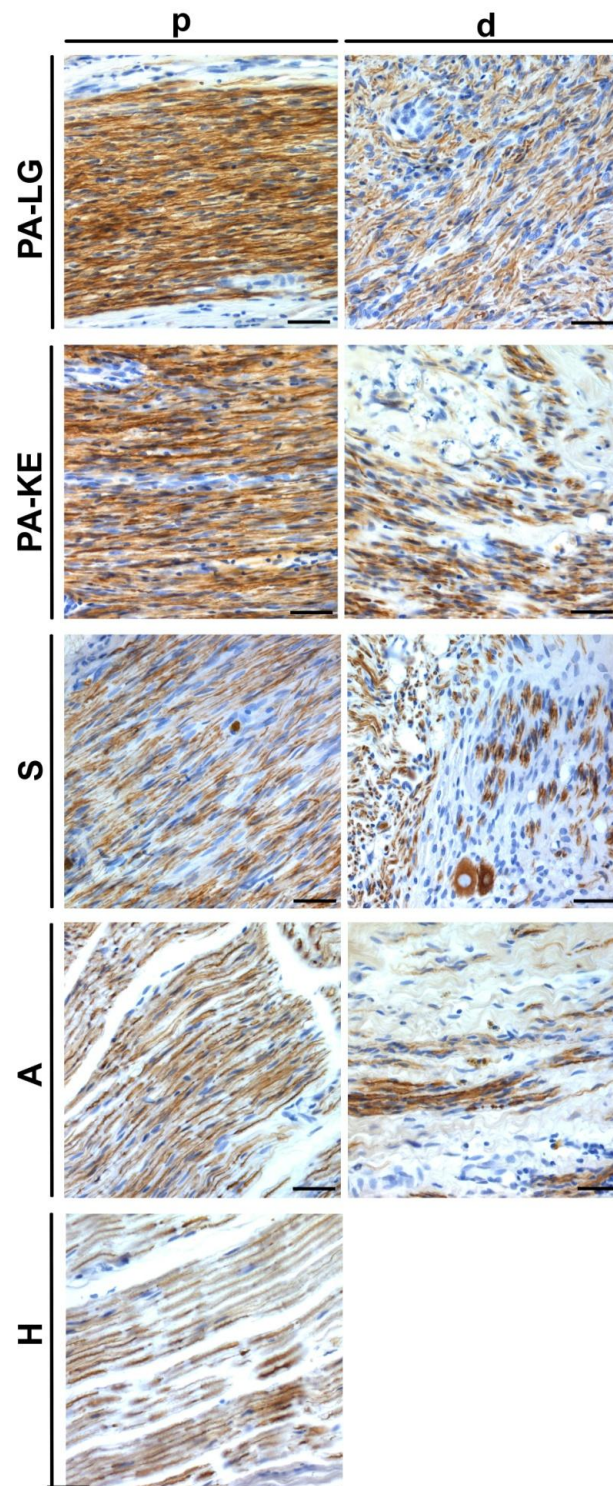


Figure 3. 11. Nerve tissues harvested at 12 weeks after operation are immunostained against β -III-tubulin. Scale bars are 40 μ m.

Presence of Schwann cells on axons is an important characteristic structure of healthy peripheral nerves for proper myelination. Any disruption in the myelination hampers the motor and sensory functions of the nerve leading to a loss of function in the target organ. Schwann cells in regenerating nerves were visualized by staining against S100 (Figure 3.12). Newborn nerves at proximal segment for all groups were found to be populated by Schwann cells showing an aligned pattern, a characteristic of peripheral nerves. This indicates that elongated axons can potentially be myelinated providing a healthy signal transmission. However, the continuity in Schwann cell alignment on nerve fibers is crucial for the restoration of the nerve conduction pathway. The distal segment of the nerve should also hold properly positioned Schwann cells for this to happen. When S100 stained tissues are analyzed it can be clearly seen that even if the Schwann cells were placed on axons at proximal regions, this could not be achieved at distal regions except PA-LN+PA-GAG treated nerves. Schwann cells could not populate the distal regions where elongating nerves also could not integrate successfully in other treatment groups. This is most clear in autograft treatment for which the proximal segment is almost similar to the healthy tissue both in β -III-tubulin stained nerve fascicles and S100 stained Schwann cells on the nerves, but the distal segment is quite far from the healthy tissue. Overall histology analysis clearly pointed out a better improvement in sciatic nerve repair when treated with PA-LN+PA-GAG biofunctionalized conduits. Axons elongated from the proximal end of injury reached beyond the distal stump; axonal density was higher with no sign of denervation at far distal region; good linear ordered tissue structure was constituted; and Schwann cells populated newborn nerves efficiently. These histological findings explain the better functional outcome

in rats treated with PA-LN+PA-GAG nanofiber functionalized conduits containing laminin derived and GAG mimetic epitopes. Physical guidance of elongating axons for path finding towards the distal stump also proved to be important as can be seen from the better regeneration in PA-K+PA-E filled conduit repaired nerves when compared to the nerves repaired with empty conduit. However, physical guidance in the conduit lumen alone was apparently not sufficient. Though providing a better regeneration when compared to the empty conduit, lack of biological signals in PA-K+PA-E obviously lead to an incomplete reinnervation of the tissue beyond the distal injury site.

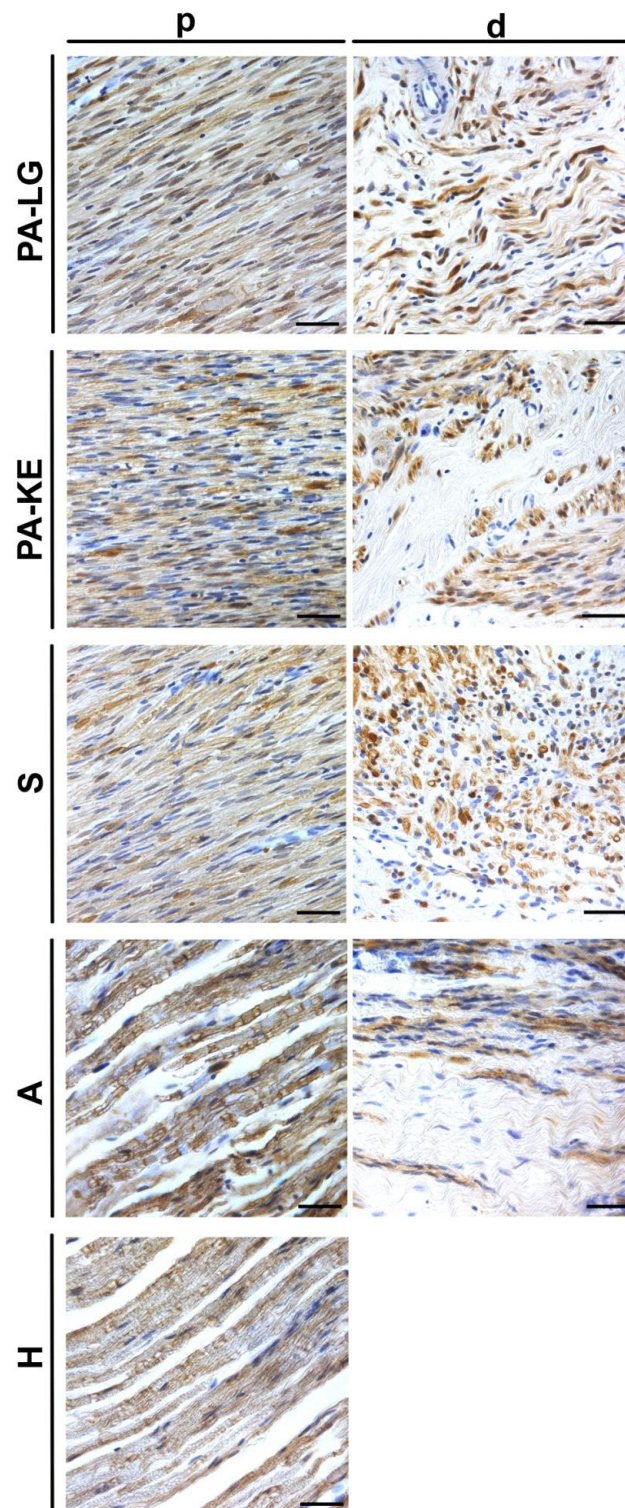


Figure 3.12. Nerve tissues harvested at 12 weeks after operation were immunostained against S100. Scale bars are 40 μm .

3.4. CONCLUSION

Guidance of regenerating axons for healthy path finding towards distal stump is essential in peripheral nerve injury, especially those with full transection. Transected nerves with long gaps between the proximal and distal ends have poor regenerating ability due to the lack of guidance that results in very little number of axons reaching to the distal end target organ. This results in lack of gain of function after injury and there occurs a possibility of neuroma formation by unguided growth of axons from the proximal stump. Polymeric nerve guidance conduits are used clinically to provide an isolation to regenerating axons making it easier for them to extend towards their target. However lack of bioactive signals limit the success of these guidance tubes resulting in worse regeneration than expected. Peptide nanofibers mimicking the healthy ECM around neural cells are used to introduce bioactivity into the lumen of a clinically used nerve guidance conduit (Neurolac TW[®]) made of poly(DL-lactide- ϵ -caprolactone). When injected into the Neurolac TW[®], PA-LN+PA-GAG nanofibers resulted in very good regeneration in the transected sciatic nerve with 10 mm gap distance. Besides having very good linearly ordered axonal bundles that is stained intensely against β -III-tubulin and S100 at proximal region, nerves treated with bioactivity introduced nerve tubes were the only group showing regeneration at the distal end. Denervated areas that both lack axons and Schwann cells were not observed for this group. These histological findings were supported by functional output at 12 weeks after injury, as measured by walking pattern analysis and electromyography. Signal transmission was much better in nerves treated with PA-LN+PA-GAG filled conduits. Laminin has an essential role in peripheral nerve

regeneration with its effect on both the axon growth cones and Schwann cells. Inducing laminin-integrin signaling by IKVAV sequence presented on peptide nanofibers along with improved growth factor signaling by binding of endogenous growth factors on peptide nanofibers through GAG mimetic groups is suggested to boost regeneration in this study.

CHAPTER 4

**ENCAPSULATION OF OLIGOTHIOPHENE
DERIVATIVE IN PEPTIDE NANOFIBER
SCAFFOLDS FOR ELECTRICAL STIMULATION
OF NEURAL CELLS**

4.1. INTRODUCTION

Neural cells are electrically active cells and they communicate with each other through electrical impulses. They not only convey an incoming signal to the nearby cell in the circuit but also respond to it through intracellular molecular mechanisms. Electrically triggered neural cell responses include but not limited to a change in survival, secretion profile, growth cone activity leading to neurite outgrowth and synaptic activity¹⁴⁵⁻¹⁵³. Besides, nervous system communicates with internal organs through this language of electrical impulses, named as action potentials. Different patterns of electrical impulses with differences in frequency, duration and waveform of the stimulus convey a different signal to the end target organ¹⁵⁴. Neural control over internal organs includes a wide variety of functions. Insulin secretion from pancreas, urinary control over bladder through sacral nerve, appetite control through feeling full with the help of vagus nerve and even the secretion of inflammatory molecules by immune cells are some examples¹⁵⁵⁻¹⁵⁶. Given the wide variety of electrically controlled functions over the body including the internal organs through peripheral nervous system and the central nervous system, scientific interest in the area is high with a desire to use electrical signals to treat a variety of disorders. Mapping the electrical signal language of neural cells and using this decoded information to provide the mimicking impulses is the current goal to develop effective electroceuticals. NIH has opened up an initiative entitled as ‘Stimulating Peripheral Activity to Relieve Conditions (SPARC)’ starting from 2015 and GlaxoSmithKline(GSK) is currently funding the research in bioelectronics showing the high interest in the area¹⁵⁶.

Treating diseases by use of electrical impulses is actually being used for several decades with success. The pacemakers and cochlear implants are currently saving millions of people's life and making life of many others by allowing them to hear. Besides, deep brain stimulation is also being used clinically with a high success in symptomatic relief for patients suffering from Parkinson's Disease¹⁵⁵.

An incoming electrical signal leads to a change in the membrane potential by opening of voltage gated Na^+ channels. If the signal is sufficient to pass the action threshold, internalized Na^+ ions derive opening up more channels and it derives more Na^+ influx leading to a sharp increase in the membrane potential from -70 mV to +30 mV. This depolarization in turn derives the opening of voltage gated K^+ channels which reverse the full process. K^+ efflux leads to repolarization of the membrane into negative values making the cell available to any following signal¹⁵⁷. Such changes in membrane polarizations are transferred through the membrane towards the presynaptic nerve terminals where electrical signal is converted to the chemical signal and conveyed to the nearby cell in the circuit. This is how neural cells communicate with each other and transmit signals to the end target organ through electrical and chemical signals.

Besides being the code to be transmitted, electrical signals also have some direct effect on cells leading to a variety of cellular responses depending on the pattern of the stimulation and the type of the cell to be stimulated. This occurs through a number of suggested molecular mechanisms. Upon electrical stimulation, opening of voltage gated calcium channels results in an elevation in the intracellular Ca^{2+} levels (Figure 4.2)¹⁵⁸. Elevated levels of Ca^{2+} in turn induce activation of calcium/calmodulin dependent protein kinases (CaMKs) by releasing the inhibitory domains and allowing the catalytic domains for autophosphorylation. CaMKs in turn modulate gene expression perhaps by NF κ B activation. MEK/ERK signaling pathway is also found to be activated upon membrane depolarization and electrical stimulation. Besides these two pathways, yet another one is known to come into effect upon electrical stimulation. cAMP levels are increased after electrical stimulation which is proposed to be linked with calcium sensitive adenylyl cyclases. cAMP in turn activates protein kinase A and higher levels are linked with regenerative potential of neurons through higher survival rates and better axonal growth¹⁵⁴.

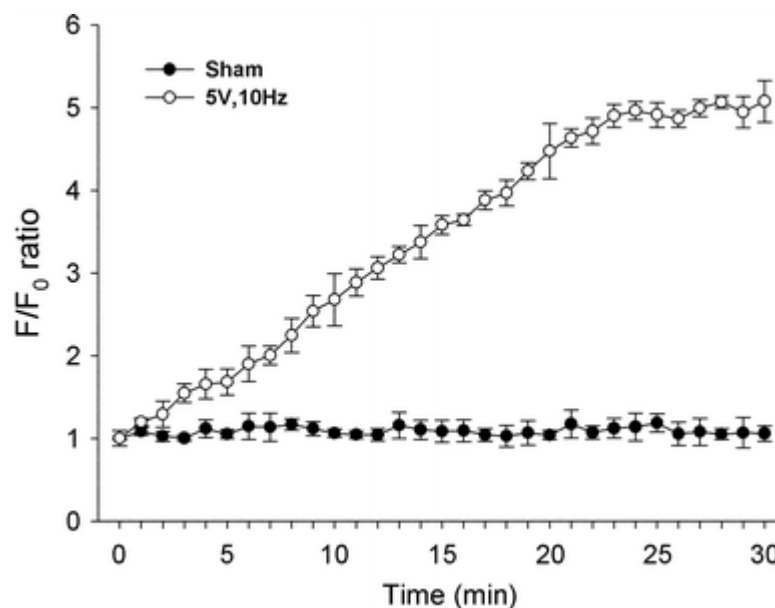


Figure 4. 1. Increase in intracellular Ca^{2+} concentration in DRG neurons. Electrical stimulation of 5 V, 10 Hz for 30 min lead to a 5 fold increase in intracellular Ca^{2+} level. F/F_0 ratio shows fold increase in concentration, F_0 being the initial concentration just before stimulation. Reproduced from Yan X.D. et al, Electrical Stimulation Induces Calcium-Dependent Neurite Outgrowth and Immediate Early Genes Expressions of Dorsal Root Ganglion Neurons. *Neurochemical Research* 2014, 39(1): 129-141 with kind permission from Springer Science and Business Media¹⁵³.

External application of electrical signals in proper range is thought to kick of an action potential over cells to which electrical stimulation is carried out. To enable an artificially elicited action potential, conductive materials suitable for cell culture are required. Conductive polymers of different types are commonly used for this purpose (Figure 4.3). These polymers are biocompatible and they are useful for transmission of the electrical signals to the cells cultured on.

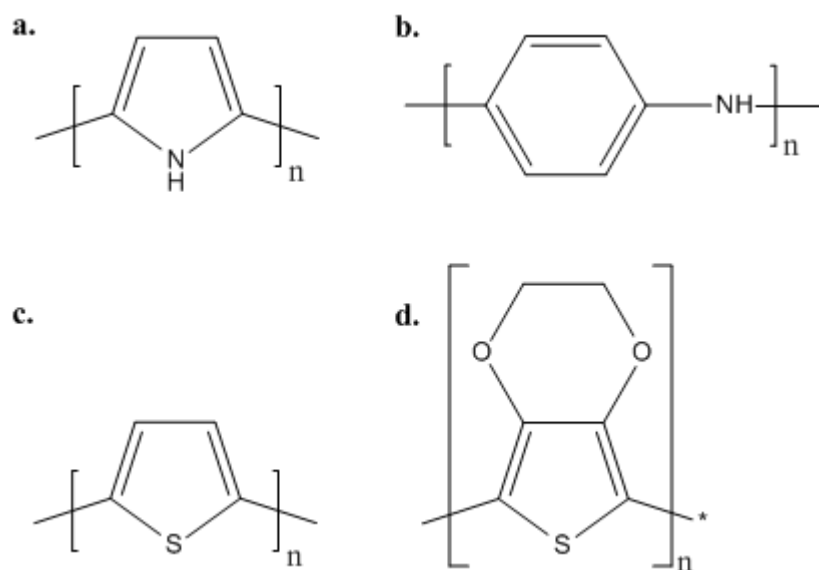


Figure 4.2. Chemical structure of commonly used conducting polymers. (a) polypyrrole, (b) polyaniline, (c) polythiophene, (d) poly(3,4-ethylenedioxythiophene).

PC12 cells cultured on polypyrrole substrates were found to extend much longer neurites upon electrical stimulation. Neurite length was doubled in cells electrically stimulated for 2 h when compared to those of non-stimulated cells¹⁵⁰. Electrical stimulation of PC-12 cells on gold surfaces also lead to an improvement of neurite outgrowth similar to that happens on conductive surfaces¹⁴⁹. Besides the improved neurite outgrowth, increased level of neurotrophin expression was found to take place upon stimulation by electrical impulses. BDNF expression was found to be upregulated in spinal neurons in a Ca^{2+} and Erk dependent manner. Cytosolic Ca^{2+} levels were also found to increase upon electrical stimulation and the presence of a voltage gated calcium channel inhibitor (nifedipine) or Erk inhibitor completely blocked upregulation in BDNF¹⁵². Increased level of intracellular Ca^{2+} in spinal neurons is correlating well with previous findings where growth cones were shown

to have an increased level of Ca^{2+} ¹⁴⁷. Upregulation of BDNF secretion was also observed when transected sciatic nerves were treated with electrical stimulation. Besides the increased level of neurotrophins, sensitivity to neurotrophins was also improved. BDNF receptor trkB was found to be expressed more in nerves that are electrically stimulated. Sciatic nerve regeneration after a delay in treatment was found to be better when treated with an external electrical current applied through copper electrodes inserted. Not only the number of regenerated nerve fibers were higher, but the EMG results showed higher amplitudes and shorter latencies in stimulated nerves¹⁵⁹. Nerve fiber density was again found to be better in another study when transected sciatic nerves were stimulated with an electrode type conduit¹⁶⁰.

However, these conductive polymers lack some desired properties to mimic the natural tissue close enough. Their mechanical properties are quite far away from the very soft nature (around 1 kPa) of the nervous system tissues. Mechanical signals are another driving force in regeneration. Adjusting the stiffness of the culture surface to nearby values of the natural tissue allows a greater success over neural differentiation. Soft hydrogels with stiffness near to that of brain was found to induce highest level of neural marker β -III-tubulin in NSCs when compared to the stiffer hydrogels^{68, 70-71}. This indicates that differentiation into neurons is favored on soft surfaces. Mesenchymal stem cells cultured on soft matrices with optimum level of elasticity, were found to undergo neural differentiation, while those on stiffer matrices preferred to differentiate into muscle or bone cells depending on the level of stiffness⁶⁹. In another study, it was found that laminin coated soft gels favors growth

of neurons and suppress astrocyte growth while astrocytes overgrow neurons in mixed cortical cultures¹¹⁶. Hence, mechanical property of the substrate should essentially match with that of the CNS tissues for efficient culture and differentiation of neural cells.

Besides their unsuitable mechanical properties, conducting polymers are not biodegradable and they are not resorbed after the desired regeneration takes place, therefore not allowing the complete formation of a new natural tissue at the site regeneration. Hence, novel scaffold materials that are conductive, biodegradable and hold mechanical properties similar to soft neural system tissues are welcomed for effective electrical stimulation of cells either in culture or in the body.

In this study, we introduced electrical conductivity to peptide nanofiber scaffolds which we previously found to promote neural regeneration. DH4T (3,3'''-Dihexyl-2,2':5',2'':5'',2'''-quaterthiophene) is a semiconductive oligomer of thiophene with alkyl tails at both ends (Figure 4.4) and by encapsulating DH4T in peptide nanofibers through hydrophobic interactions, we obtained a hybrid scaffold suitable for electrical stimulation of cells.

4.2. MATERIALS AND METHODS

4.2.1. Optimization of DH4T amount for encapsulation into PA molecules

1.5 mg PA-GAG was dissolved in 100 μ L of HFIP for each vial of different DH4T concentration. 0.5 mg /0.1 mg /0.05 mg /0.025 mg DH4T in 1 mL of THF were

prepared by dilution from 1 mg/mL DH4T stock in THF. To avoid effect of solvent evaporation during sample preparation all of the DH4T in specified concentration was mixed with one vial of 1.5 mg PA in HFIP. Mole ratio of PA:DH4T was 1.2:1 when 0.5 mg DH4T was used and 1.2:0.5/0.25/0.125 in lower amounts of DH4T. Volume ratio between solvents was kept fixed as 1:10 (HFIP:THF). After mixing PA and DH4T by vortexing for 1 min, vials were sonicated at 40 °C for 30 min. Solvent evaporation was carried out by rotary evaporation and overnight drying under vacuum. Dried material was scratched from the walls of vials with a metal spatula and 1 mL ddH₂O/vial was added. After 1 min vortex, vials were incubated at 40 °C water bath for 30 min and another 1 min vortex was carried out. Contents of glass vials were transferred into separate 1.5 mL eppendorf tubes and they were centrifuged at 3000 g for 30 min. 800 µL of supernatant (80% of initial volume) was transferred to fresh tubes avoiding the DH4T precipitate. Supernatants were centrifuged again at 3000 g for 30 min and 650 µL of supernatant (80% of the volume before second centrifuge) was transferred to a fresh tube.

4.2.2. Calculation of encapsulated DH4T amounts from UV measurements

In order to determine the DH4T amount in samples, samples were diluted 15 times with DMF making DMF: ddH₂O mixture (14:1 volumetric ratio) as the final solvent of measurement. DMF was used to release encapsulated DH4T and make it available for measurement. Standard samples of DH4T with known concentration were prepared by 5 fold dilutions from 25 mM DH4T stock dissolved in THF. Dilutions were made by using DMF: ddH₂O mixture at 14:1 ratio. The same mixture was used

as blank and absorbances were measured between 200-800 nm. A standard curve plotted from absorbance of standard DH4T samples at 410 nm was used to derive DH4T concentration in samples.

To check the encapsulation by shift in absorbance spectrum, samples diluted in water (to keep DH4T in encapsulated state) 15 folds were also measured in the same range.

4.2.3. Fluorescence measurement of DH4T encapsulated PAs

DH4T encapsulated PA samples prepared by UV measurements were excited at 400 nm and the emission spectrum between 400-600 nm was obtained.

4.2.4. Viability assay of PC-12 cells on PA substrates

PA gels were prepared in 96 well plates by using the protocol described in section 2.2. 2 mM PA solutions were used at equal volume for preparation of scaffolds with no net charge. DH4T concentration between different scaffolds was adjusted to be equal to 0.069 mM after mixing two components with different DH4T amounts (Table 4.1).

Table 4. 1. Properties of scaffolds used for viability assay

Scaffold	Components	PA charge	PA conc.	DH4T conc.
PA-LN+PA-GAG-DH4T	PA-LN-DH4T	+3	2 mM	0.003 mM
	PA-GAG-DH4T	-3	2 mM	0.135 mM
PA-LN+PA-GAG	PA-LN	+3	2 mM	-
	PA-GAG	-3	2 mM	-
PA-KK+PA-EE-DH4T	PA-KK-DH4T	+2	2 mM	0.092
	PA-EE-DH4T	-2	2 mM	0.046
PA-KK+PA-EE	PA-KK	+2	2 mM	-
	PA-EE	-2	2 mM	-

10^4 cells/well were seeded on UV sterilized dried substrates (n = 3/group) and Live/Dead assay was performed 24 h later as described in section 2.2.14.

4.3. RESULTS

A method for encapsulation of semiconductive DH4T molecule into PA molecules is developed in this study. Chemical structures of DH4T and PA molecules used are given in Figure 4.3-4.4. This method allowed us to remove any unencapsulated DH4T and quantify the amount of DH4T molecules encapsulated. Such quantification is quite important as it allows control over the amount of semiconductive material in the hybrid matrix. Such a control in turn provides experimental repeatability for *in vitro* studies in terms of scaffold preparation. By having information on the amount of the DH4T in the PAs, it is possible to adjust the same amount of DH4T in all experimental groups and in each different experiment.

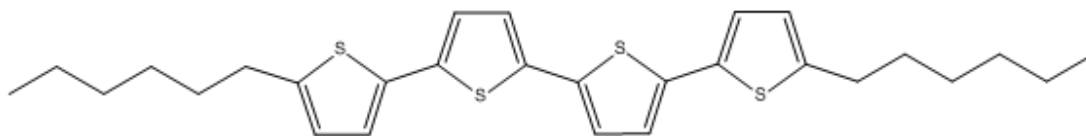


Figure 4. 3. Chemical structure of DH4T molecule.

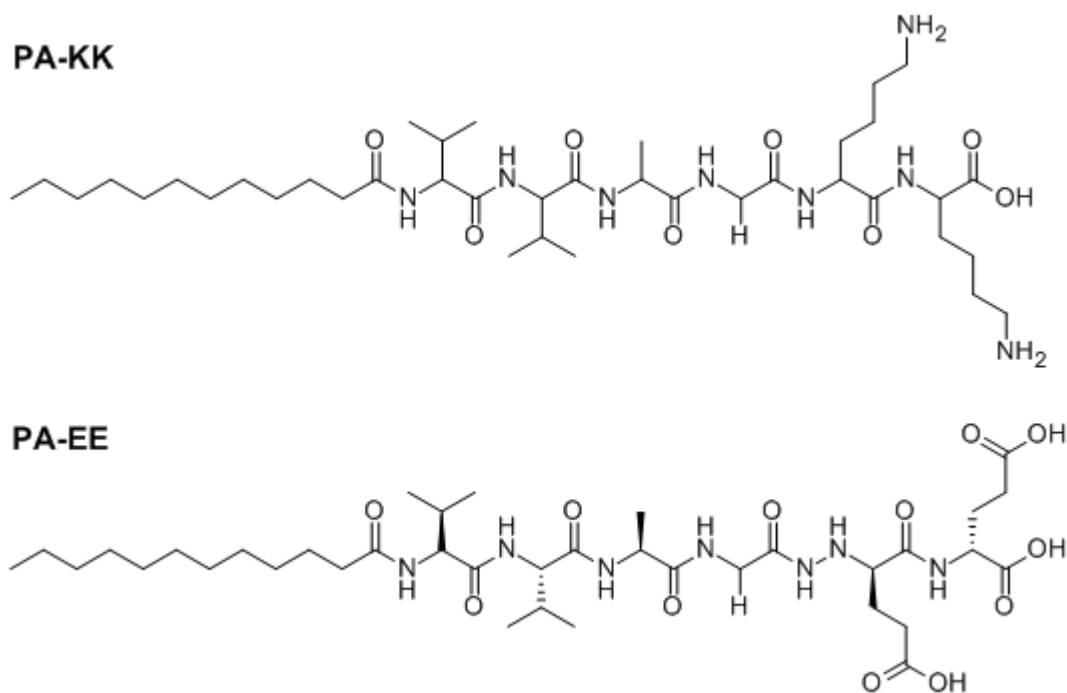


Figure 4. 4. Chemical structure of PA-KK and PA-EE molecules. Structures of PA-LN and PA-GAG are given in Figure 2.1, page 41.

4.3.1. Optimization of DH4T amount for encapsulation

Quantification of encapsulated amount is based on the absorbance spectrum of DH4T, which gives a characteristic peak positioned at 410 nm. Serial dilutions of DH4T were used to obtain a standard curve. The absorbance at 410 nm linearly increases with increasing DH4T concentration (Figure 4.5) which allowed us to

prepare a standard curve (Figure 4.6). Formula driven from the slope of the standard curve is used for DH4T quantification of encapsulated PA samples.

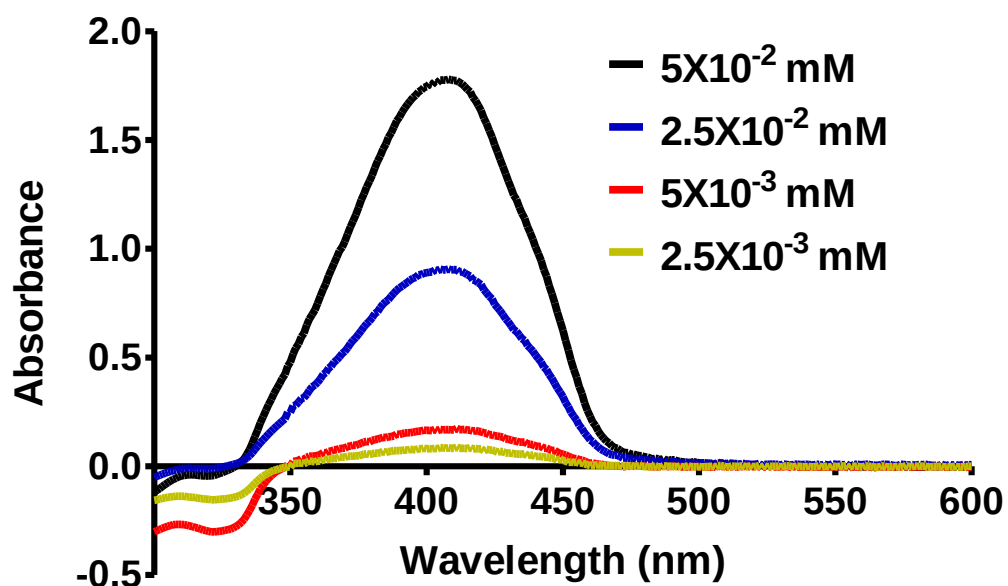


Figure 4. 5. Absorbance spectrum of standard DH4T samples of known concentrations.

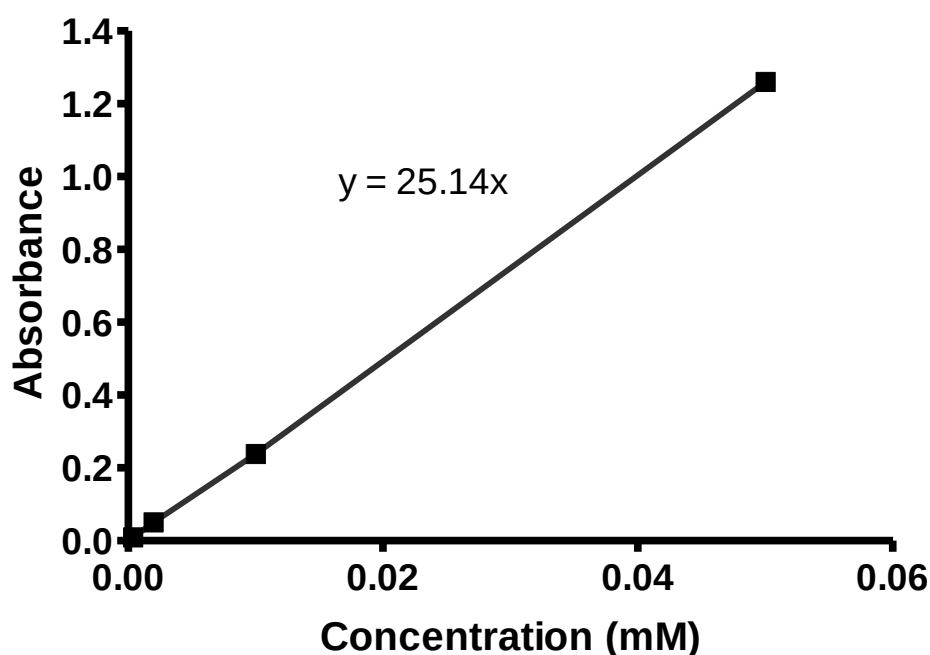


Figure 4. 6. DH4T standard curve plotted by using absorbance values at 410 nm.

Initial DH4T amount was optimized by using increasing amounts of DH4T amounts to obtain an optimal amount of DH4T in PA nanofibers after encapsulation. PA amount was fixed at 1.5 mg for all conditions and by reducing the amount of DH4T different mole ratios between PA:DH4T were used for encapsulation. As can be seen in the Figure 4.7, absorbance at 410 nm increased with increasing concentration of DH4T. Concentration of the encapsulated DH4T and encapsulation ratio between PA:DH4T are given in Table 4.2. 1.2:1 mole ratio between PA:DH4T was selected for future encapsulations according to the results of this experiment.

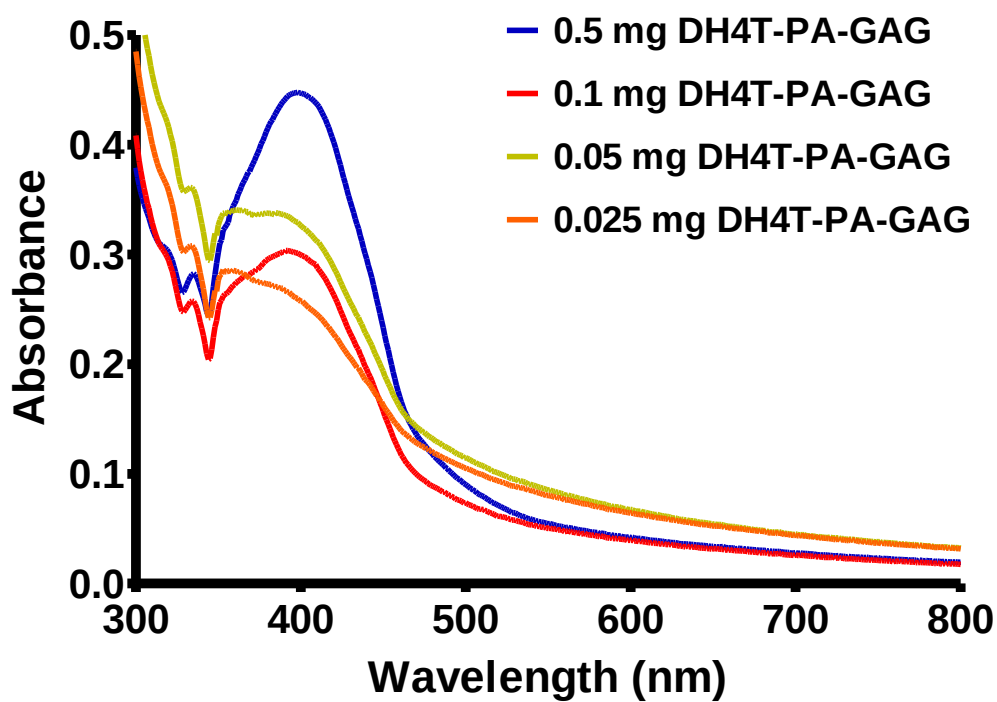


Figure 4. 7. Absorbance spectrum of samples with different DH4T amounts used for encapsulation.

Table 4. 2. Optimization of DH4T amount for encapsulation.

Initial DH4T amount (mg)	Initial PA:DH4T mole ratio	Encapsulated DH4T amount (μ moles)	Final PA:DH4T mole ratio
0.5	1.2	0.180	6.1 : 1
0.1	1.2:0.2	0.119	9.3 : 1
0.05	1.2:0.1	0.129	8.6 : 1
0.025	1.2:0.05	0.101	10.9 : 1

Encapsulation was verified by the shift of absorption spectra. When samples were diluted in DMF, disorganization of secondary structures leads to release of encapsulated DH4T. Released DH4T showed the same characteristic peak at 410 nm that was observed with DH4T standard solutions. However, when samples were diluted with water meaning that encapsulation state is maintained, the spectrum shifted toward lower wavelengths (Figure 4.8). In addition to UV spectrum results, fluorescence measurements also verified the encapsulation. Samples where encapsulated DH4T molecules were released by DMF disorganization of PA secondary structures showed higher emission when compared to the samples in water (Figure 4.9). Such a reduction in emission upon encapsulation is thought to occur by quenching of DH4T molecules by each other due to the close packing in encapsulated state. Emission spectra also showed the same type of shift as it was observed in absorption spectra.

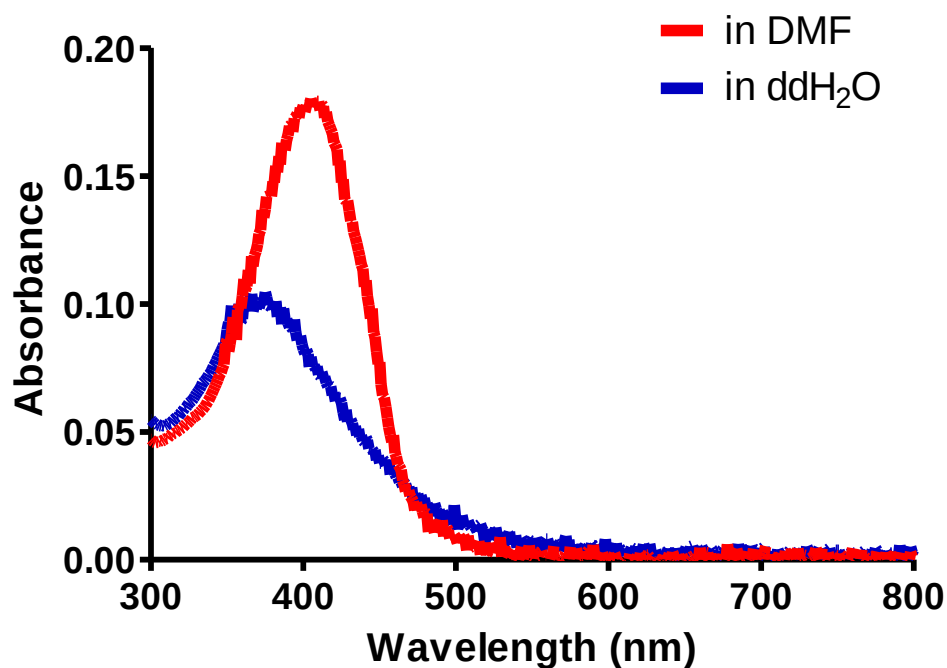


Figure 4. 8. Absorbance spectrum of 0.5 mg DH4T encapsulated PA-GAG diluted in DMF or ddH₂O showing the shift absorption spectra upon encapsulation.

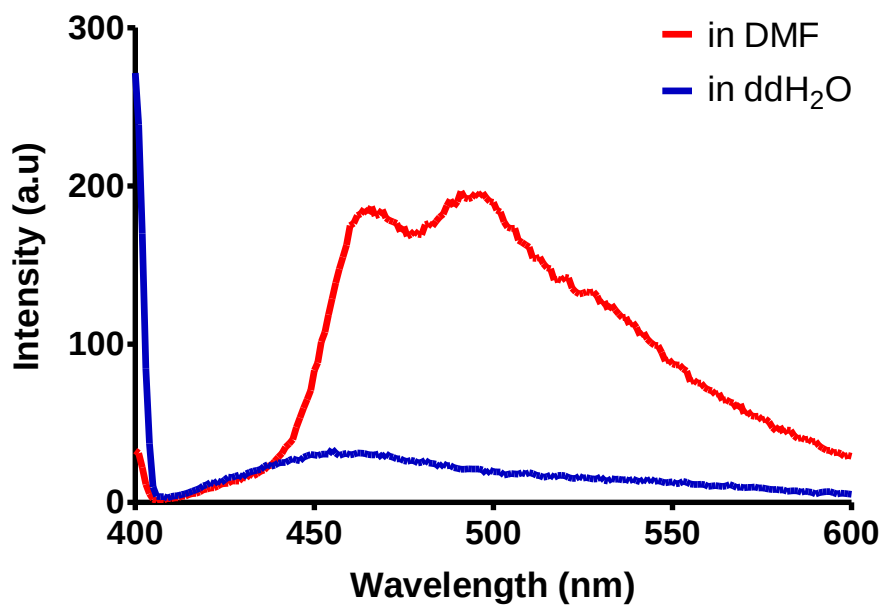


Figure 4. 9. Fluorescence measurement of 0.5 mg DH4T encapsulated PA-GAG samples excited at 400 nm.

4.3.2. Encapsulation of DH4T in different PA molecules

After optimization studies, DH4T encapsulated PAs for cell culture studies were prepared for four different PA molecules; PA-LN, PA-GAG, PA-KK and PA-EE. PA-LN+PA-GAG nanofibers were already shown to have effective contribution to the neurite outgrowth by PC-12 cells and by introduction of electrical conductivity, it was aimed to be further improved. PA-KK+PA-EE nanofibers lack any epitope sequence and instead they contain either two lysines or two glutamic acids as charged residues required for nanofiber formation. These epitope-free nanofibers were used as control scaffolds having the same physical structure with PA-LN+PA-GAG nanofibers without having the biological signals. Encapsulation of DH4T could be successfully achieved for all PAs verified by the shift in the spectra towards lower wavelengths upon encapsulation (Figure 4.10-4.11). Encapsulation efficiencies of different PAs were varied though the most efficient encapsulation being achieved for PA-GAG and the lowest efficiency occurred for PA-LN. Encapsulated DH4T concentrations are given in Table 4.3 for each PA molecule.

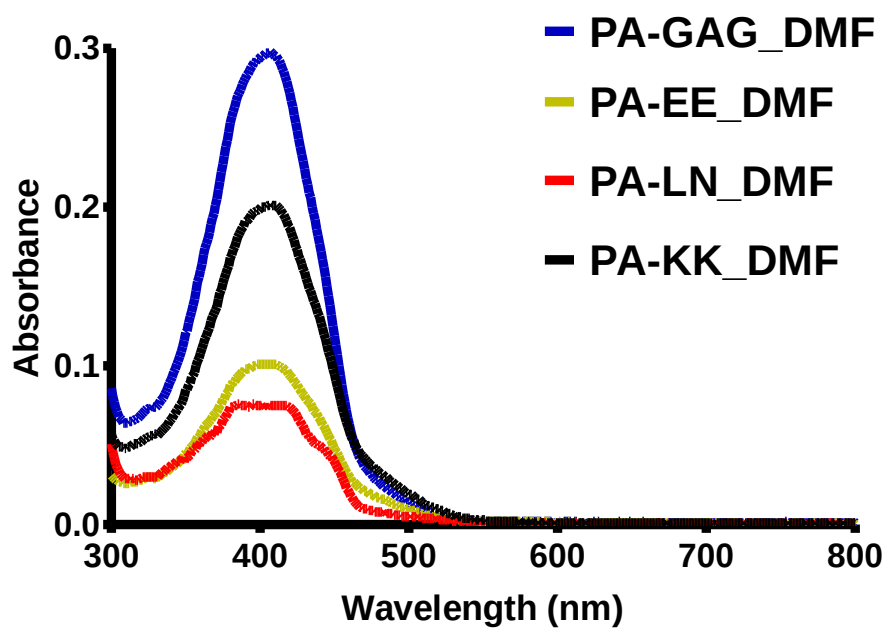


Figure 4. 10. Absorbance spectrum of DH4T encapsulated PA samples diluted with DMF.

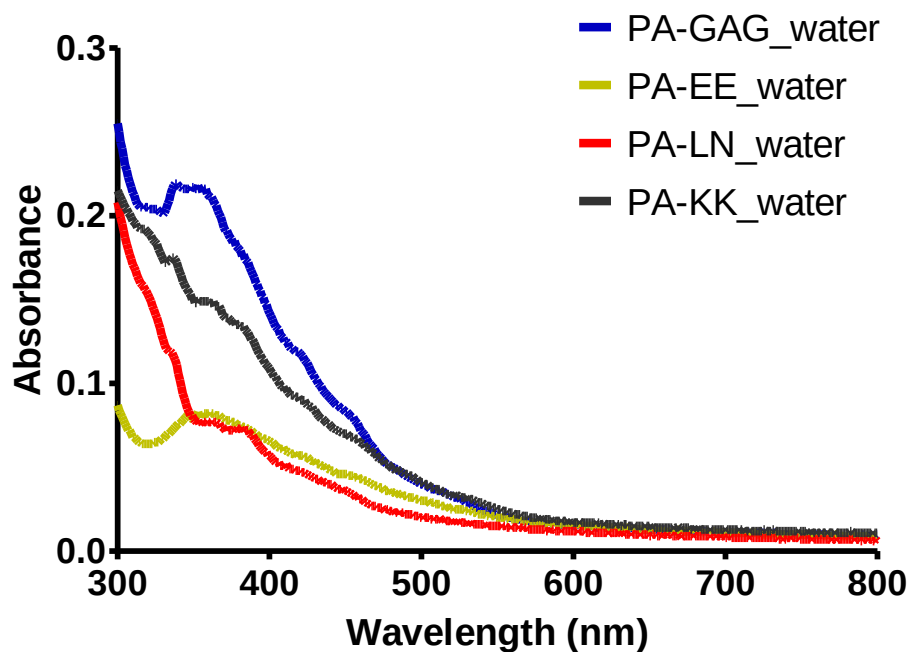


Figure 4. 11. Absorbance spectrum of DH4T encapsulated PA samples diluted with ddH₂O.

Table 4. 3. Results of DH4T encapsulation in different PA molecules.

PA molecule	Encapsulated DH4T amount (μmoles)	Final PA:DH4T mole ratio
PA-LN	0.403	58.2 : 1
PA-GAG	2.169	14.8 : 1
PA-KK	1.316	21.7 : 1
PA-EE	0.663	43.2 : 1

Although encapsulated amount of DH4T was found to be different for different PA molecules, DH4T amount in the nanofiber scaffolds could be fixed between different scaffolds and different rounds of experiments. To achieve this, PA-LN-DH4T was used by diluting it with PA-LN (without DH4T) 11.3 folds so that final DH4T concentration would be 0.003 mM. By mixing this diluted PA-LN-DH4T with PA-GAG-DH4T at equal volumes for nanofiber formation, nanofibers with DH4T concentration of 0.069 mM were obtained. Mixing PA-KK-DH4T with PA-EE-DH4T at equal volumes also resulted in the same final concentration of DH4T.

4.3.3. Viability assay for PC-12 cells

In order to investigate if encapsulated DH4T exerts any toxic effect on cells, PC-12 cells cultured on PA substrates for 24 h were analyzed for cell viability. Calcein AM-Ethidium homodimer 1 based live-dead assay was used for viability analysis. Nonfluorescent calcein AM incubated with cells is converted to a fluorescent dye by

cellular esterase activity present in cells while ethidium homodimer 1 internalized by dead cells enter cell nuclei and give red fluorescence. Results given in Figure 4.12 indicate that there is no toxic effect introduced by DH4T encapsulation and DH4T encapsulated PAs are still biocompatible without any interference to cell viability.

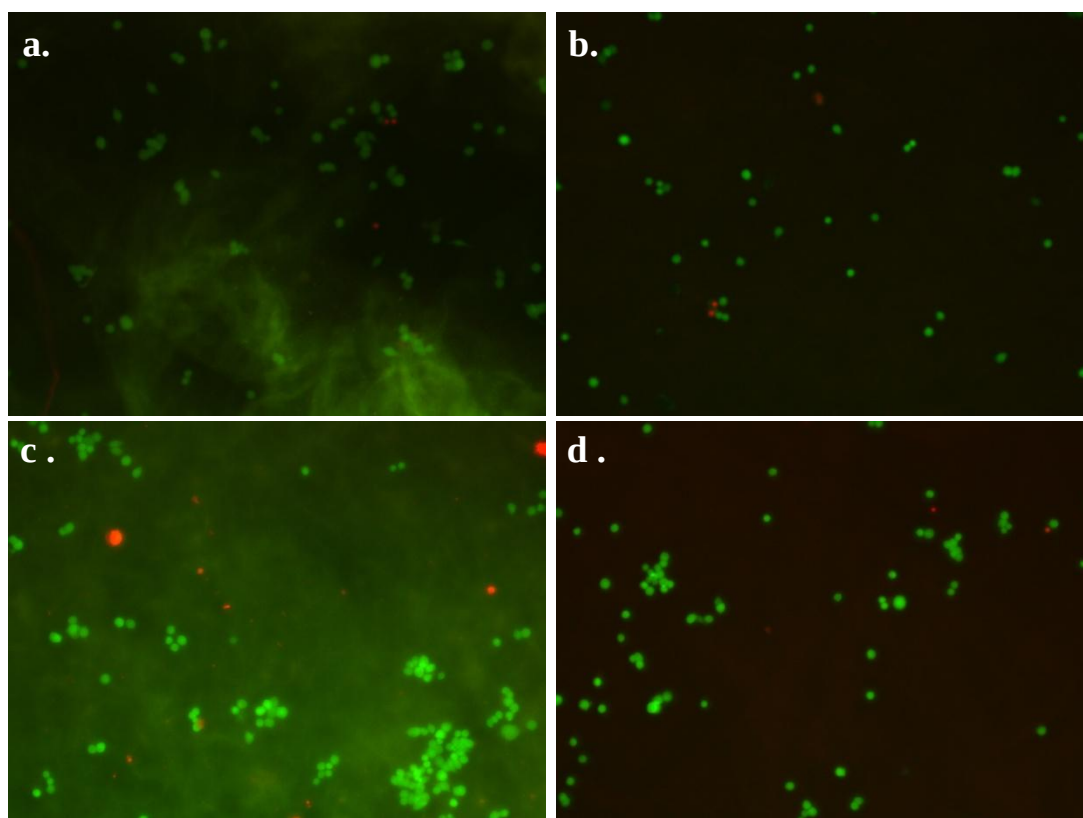


Figure 4. 12. Viability assay results of PC-12 cells cultured on PA substrates for 24 h. a. PA-LN+PA-GAG-DH4T(+), b. PA-LN+PA-GAG-DH4T(-), c. PA-KK+PA-EE-DH4T(+), d. PA-KK+PA-EE-DH4T(-). Live cells are stained with Calcein (green) and dead cells are stained with ethidium (red). Images are obtained at 200x magnification.

4.4. CONCLUSION

Neural cells benefit from external electrical stimulation as evidenced by an increase in cell survival, improved neurite outgrowth and neurotrophin secretion as well as increased responsiveness to neurotrophins. Commonly used substrates for application of electrical current over cells are conductive polymers. Though having advantage of bearing conductivity these polymers are far from elasticity of natural environment of neural cells which is around 1 kPa in brain. Besides, they are not biodegradable which also limits their use in clinics. Hence new materials suitable for electrical stimulation of neural cells and designed considering such issues are required to be developed. Bioactive peptide nanofiber scaffolds, which already proved to improve neural differentiation and regeneration, were modified in this study to make them a suitable platform for electrical stimulation of cells. A semiconductive oligothiophene molecule, DH4T, is encapsulated into peptide nanofibers to introduce conductivity. It was possible to remove any nonencapsulated material by centrifugation and quantify the amount of DH4T encapsulated. Though with differing efficiency, this method allows a quantifiable amount of DH4T encapsulation in different peptide nanofibers. Scaffolds formed by using these nanofibers have the potential use for electrical stimulation of neural cells.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

Nervous system disorders affect an increasing number of people disabling them, leaving them in need for life-long health-care, and bringing an economical burden along with suffering from pain and loss of motor, sensory and cognitive functions. Increase in life expectancy leads to an increase in aged population which in turn leads to an increase in the number of people suffering from neurodegenerative disorders such as Alzheimer`s disease and Parkinson`s disease. Besides neurodegenerative disorders, traumatic injuries of nervous system also lead to lifelong disabilities. Brain trauma, spinal cord injuries and peripheral nerve injuries are commonly observed in younger population in opposite to most neurodegenerative disorders. Loss of function in working age population introduces even a higher burden in society considering not only the loss in income of these people due to their disabilities but also the longer need of health-care.

Though initiated by different mechanisms, neurodegenerative disorders and nervous system traumatic injuries all lead to an eventual neural cell loss that is hardly tolerated. Complexity of nervous system arising from high numbers of specialized cell types and high numbers of cells along with the high numbers of interactions between them makes nervous system quite vulnerable to any damage that leads to cell loss. Mature neurons are unable to proliferate which makes regeneration even more impossible in the nervous system. Besides, in opposite to many other tissues, brain has a very limited number of resident stem cells confined to very specific locations. Hence, regeneration through endogeneous stem cell proliferation and differentiation is almost out of question. Caused by such problems and the reactive

inhibitory response of central nervous system to injury, effective therapies for nervous system disorders are not in clinics yet.

In order to induce effective neural regeneration, we exploited peptide nanofiber systems designed to mimic neural extracellular matrix. Peptide nanofibers exhibit high aspect ratio property which enables to introduce high number of biologically active epitopes in smaller areas. These epitopes can be derived from any functional domain of proteins from tissue of interest to induce a biological signal in cells. Cell surface receptor interacting short peptide sequences derived from extracellular matrix proteins, growth factors or any other inductive protein can be incorporated into peptide nanofibers for desired biological activity. Herein, we used a laminin derived five amino acid sequence “IKVAV” and a peptide sequence mimicking sulfated glycosaminoglycans through chemical functional groups in amino acid side chains (PA-LN and PA-GAG respectively). Laminin is present in basement membranes of both central and peripheral nervous systems and it is a positive regulator of neurite outgrowth. Besides, it is also required for proper Schwann cell differentiation and myelination of axons. Use of laminin derived IKVAV sequence is reported to exert a neuroinductive effect as well¹⁶¹. Sulfated glycosaminoglycans such as heparan sulfate have affinity for a variety of growth factors which makes them valuable for tissue engineering applications. Heparan sulfate-growth factor affinity enables localizing high concentrations of growth factors in close vicinity to cells, and protecting growth factors from degradation in addition to leading to improved presentation of growth factors to cell surface receptors. Heparan sulfate mimicking

PA-GAG also proved to have affinity to NGF. By combining two biologically active epitopes from ECM, we aimed to improve neural regeneration.

Scaffolds prepared with these dual active peptide nanofibers containing laminin and GAG derived epitopes lead to improved neurite outgrowth by PC-12 cells when compared to epitope free control peptide nanofibers. Both the neurite length and percentage of cells extending neurites were increased on these scaffolds. Besides, cells were found to express synaptophysin 1, an indicator of synaptic maturation. Neurite outgrowth on peptide nanofibers could even take place in the presence of inhibitory chondroitin sulfate proteoglycans with only up to 40% reduction, while neurite length was reduced 90% on collagen.

Neuroregenerative ability of PA-LN+PA-GAG nanofibers was also tested in a peripheral nerve injury model. Sciatic nerve transection with 10 mm gap distance was carried out in male Sprague-Dawley rats. Rats were treated with either autografts or a clinically used polymeric nerve tube, Neurolac[®]TW. PA-LN+PA-GAG gels were used for lumen biofunctionalization in nerve tubes. Epitope free peptide gels and isotonic sucrose solution filled nerve tubes were used as control. Functional recovery analysis at 12 weeks after surgery by EMG measurements indicated a better signal transmission from nerves treated with PA-LN+PA-GAG biofunctionalized nerve tubes. Besides, histological analysis revealed a better axonal regeneration in these nerves. Regenerated axons elongating from the proximal stump could reach to the distal stump in PA-LN+PA-GAG filled nerve tubes in opposite to the control PA or sucrose filled tubes. Denervation was clearly observed in far distal tissue in all groups except biofunctionalized nerve tube treated nerves. Nerve alignment was also

better in distal stump for this group. A denser staining of β -III-tubulin and S100 in PA-LN+PA-GAG filled nerve tube treated nerves also indicated an improved regeneration compared to other treatments.

Improved neurite length results obtained at *in vitro* culture, and increased functional outcome and axonal regeneration after PNS injury *in vivo* indicated PA-LN+PA-GAG nanofibers as neuroinductive materials. We further modified these nanofibers by encapsulation of a semiconductive oligothiophene derivative to allow electrical stimulation of neural cells on these scaffolds. Being electrically active cells, neurons can be excited by external electrical signals that can lead to functional improvement. Having both bioactive signals and physically appropriate properties, peptide nanofibers developed in this study are promising materials for neural regeneration applications.

Dual active scaffold system developed in this study can potentially be applied for treatment of central nervous system traumatic injuries. Being a permissive environment for neurite outgrowth even in the presence of inhibitory proteoglycans, these PA gels can improve regeneration after spinal cord injury. Axons damaged upon injury can elongate through the gel and it can lead to functional improvement in motor and sensory functions. Laminin and glycosaminoglycan double mimetic PA gels can also be applied for treating traumatic brain injuries. PA gels within the scar tissue can provide an environment which induces migration of nearby surviving cells that can establish new synaptic connections in turn to tolerate the trauma induced loss of neural networks.

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