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FABRICATION AND SURFACE MODIFICATION OF POLY L-LACTIC ACID NANOSTRUCTURES FOR GROWTH FACTOR IMMOBILIZATION AND NEURAL STEM CELL DELIVERY

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Ce mémoire intitulé:

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présenté par : HADDAD Tanit

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RÉSUMÉ

Les accidents vasculaires cérébraux, le cancer et la maladie d'alzheimer sont les principales causes de décès au Canada. Les lésions nerveuses ou les nerfs endommagés dans le système nerveux central peuvent détruire la qualité de vie des survivants, présentent de grands couts pour la société et peuvent entraîner la mort. Il n'existe actuellement aucun traitement efficace encore pour aider à la régénération des tissus nerveux.

Les stratégies de régénération des tissus nerveux ont été étudiées, cependant, de nombreuses limites ont été rencontrées. Par exemple, l'injection directe de cellules souches neuronales dans le système nerveux central a donné lieu à la formation de tumeurs. L'injection directe de facteurs de croissance n'a révélé aucun bénéfice avant sept jours de traitement continu. En outre, l'encapsulation des facteurs de croissance a été précédemment étudiée et possède des inconvénients majeurs. Des limites telles que, la difficulté à maintenir leur livraison à long terme sur une gamme de concentration définie, leur courte demi-vie ainsi qu'une courte distance de migration ont été observées. La production *in situ* et la distribution de facteur de croissance par l'intermédiaire de cellules telles que les fibroblastes modifiés génétiquement pour exprimer le BDNF ainsi que les cellules fabriquées en coculture ont aussi démontré des inconvénients majeurs. En effet l'immunosuppression était constamment requise et le taux de survie des cellules très faible.

Les biomatériaux tels que les hydrogels ont été largement étudiés et présentent des difficultés de manipulations, une faible adhérence, des couts élevés, des difficultés à incorporer des médicaments sur leur structure ainsi que de présenter des difficultés lors de leurs stérilisations.

Cette étude vise donc à optimiser les stratégies de régénération des tissus nerveux existants. Les biomatériaux électrofilés ont montré des résultats prometteurs dans la littérature en raison de leur porosité, haut rapport surface-volume, interconnexion des pores ainsi que de leur topographie imitant la matrice extracellulaire (ECM) du cerveau. Ces biomatériaux électrofilés

ont donc été utilisés dans ce projet. Le procédé d'électrofilage a été utilisé dans cette étude afin d'obtenir des fibres dans la gamme nanométrique présentant une topologie à haute porosité, le diamètre des fibres idéales ainsi qu'une résistance mécanique adéquate. L'acide poly lactique-L (PLLA) est étudié dans cette recherche; il s'agit d'un polymère couramment utilisé dans l'ingénierie tissulaire neurale étant donné qu'il est autorisé par la FDA et a été utilisé sous la forme de nanofibres électrofilées.

Des études antérieures ont utilisé d'autres protéines pour l'immobilisation tels que la laminine et le collagène. Ces résultats, cependant, n'étaient pas prometteurs et nécessitaient l'addition de facteurs de croissance dans les médias. Par conséquent, dans ce travail, les nanofibres de PLLA électrofilées ont été optimisées par greffage de manière covalente du facteur de croissance épidermique (EGF) puisque EGF a montré des résultats prometteurs dans des études précédentes.

Les nanofibres de PLLA électrofilées ont d'abord été fonctionnalisées avec polyallylamine pour introduire des groupes amine. Ensuite, le greffage de l'EGF par l'intermédiaire d'un ester de glycol de bis-N-succinimidyle pentaéthylène (PEG) espaceur est effectué. Le substrat est resté physiquement intact et le diamètre moyen de fibres ainsi que la porosité est restés inchangés après la fonctionnalisation de groupes d'amine. Ceci est en contraste frappant avec les protocoles d'amination se fondant sur un traitement au plasma qui a rapporté une dégradation du PLLA ou aminolyse au moyen de la petite molécule ethlynediamine (EtDA).

Des cellules souches d'ingénérie ressemblant à des cellules souches neuronales (NSLC) ont ensuite été ensemencées sur les substrats modifiés et se sont avérées viables jusqu'à 14 jours. Leur prolifération ainsi que leur propagation ont été observées. L'adhérence cellulaire et la prolifération sont supérieures lorsque les substrats ont été greffés avec de l'EGF en comparaison à des substrats qui ont été seulement aminés. Comme témoin positif, la prolifération des NSLC a été caractérisée sur des nanofibres de laminine dans du milieu dépourvu de EGF. Aucune différence significative dans la prolifération des cellules entre les substrats de type EGF greffé et le témoin positif n'a été observée. Par conséquent, ce nouveau biomatériau fonctionnalisé et

greffé du EGF démontre une adhésion cellulaire efficace, une prolifération ainsi que la viabilité des cellules jusqu'à 14 jours et présente une avenue prometteuse dans le traitement de la régénération de cellules souches.

ABSTRACT

Damaged nerves and nerve injuries in the Central Nervous System (CNS) diminish the quality of life of survivors, are costly to society, and can cause death. Strokes, cancer, and Alzheimer's Disease (AD) are the leading causes of death in Canada. There is currently still no efficient treatment to aid in the neural tissue regeneration of damaged nerve cells.

Previous nervous tissue regeneration strategies have been studied, however, many limitations were encountered. For example, direct injection of neural stem cells into the central nervous system (CNS) has resulted in tumor formation. Direct injection of growth factors showed no benefits until after seven days of continuous treatments. Furthermore, the encapsulation of growth factors was previously studied and demonstrated major drawbacks. Some limitations, such as the difficulty to maintain long-term delivery within a defined concentration range, as well as the short half-life of the growth factors and short migration distance were observed. Additionally, the *in situ* production and delivery of growth factors using cells, such as the transplantation of genetically modified fibroblasts to express BDNF, as well as the co-culturing of cells have also seen some drawbacks. Immune suppression was constantly needed and low cell survival rate was observed. Furthermore, biomaterials such as hydrogels have been widely studied and proved to be difficult to handle and sterilize, load drugs and nutrients, are non-adherent, and expensive.

This study thus aims at optimizing existing nervous tissue regeneration strategies. Electrospun biomaterials have shown promising results in literature due to its high porosity, high surface area-to-volume ratio, interconnected pores, and topography that mimic extra cellular matrix (ECM) in the brain. The electrospinning process was used in this study to obtain the desired qualities mentioned, as well as to obtain fibers in the nano-metric range. Poly L-lactic acid (PLLA) is a polymer commonly used in neural tissue engineering, since it is FDA-approved and was used as a form of electrospun nanofibers in this study.

Previous studies have used other proteins for immobilization such as laminin and collagen. These results, however, were not promising and most often required the addition of growth factors in the media as well. Therefore, in this work, the electrospun PLLA nanofibers were optimized by covalently grafting epidermal growth factor (EGF) since EGF has shown promising results in previous studies.

The electrospun PLLA nanofibers were first functionalized with polyallylamine to introduce amine groups, before EGF grafting via a bis-N-succinimidyl-pentaethylene glycol ester (PEG) linker. The substrate remained physically intact, the average fiber diameter (AFD) and porosity also remained unchanged following amine functionalization. This is in stark contrast with amination protocols relying on plasma treatment that has been reported to degrade PLLA or aminolysis using the small molecule ethylenediamine (EtDa).

Engineered neural stem-like cells (NSLC) were then seeded onto the modified substrates and were shown to be viable up to 14 days, while proliferating and spreading. Cell adhesion and proliferation was improved when substrates were grafted with EGF, when compared to substrates that were only aminated. As a positive control, NSLC proliferation was also characterized on laminin-coated mats in EGF-free medium where no significant differences in cell proliferation were observed between the EGF-grafted substrates and the positive control. Therefore, this new functionalized and EGF-grafted biomaterial has achieved efficient cell adhesion, proliferation as well as cell viability for up to 14 days and has promising use in stem cell regeneration therapy.

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antiNgR	Nogo-66 receptor- antibody
AD	Alzheimer's disease
AFD	Average fiber diameter
APP	Amyloid precursor protein
BDNF	Brain derived neurotrophic factor
Bis(NHS)PEG_5	Bis-*N*-succinimidyl-(pentaethylene glycol) ester
BSA	Bovine serum albumin
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
DCM	Dichloromethane
DRG	Dorsal root ganglion neurons
ECM	Extra cellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EtDA	Ethylenediamine
EtOH	Ethanol
FGF	Fibroblast growth factor
GDNF	Glial cell-line derived neurotrophic factor
HA	Hyalorunic acid
HCl	Hydrochloric acid
hESCs	Human embryonic stem cells

LC-SPDP	Succinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexanoate
MAG	Myelin-associated glycoprotein
MeOH	Methanol
MSC	Mesenchymal stem cell
NDC	Normal donkey serum
NGF	Nerve growth factor
NPMM	Neural progenitor maintenance medium
NSC	Neural Stem Cell
NSLC	Engineered Stem Like Cell
NSPC	Neural stem/progenitor cells
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin – 4/5
OGmp	Oligodendrocyte myelin glycoprotei
OPCs	Oligodendrocyte precursor cells
PAAm	Polyallylamine
PBS	Phosphate buffered saline
PC-12	Pheochromocytoma cells
PCL	Poly (e-caprolactone)
PD	Parkinson’s disease
PDGF	Platelet-derived growth factors
PET	Polyethylene terephthalate
PHEMA	Poly(2-hydroxyethylmethacrylate)
PLA	Poly lactic acid
PLGA	Poly lactic co-glycolic acid

PLL	Poly-L-lysine
PLLA	Poly L-lactic acid
PMMA	Poly(methyl methacrylate)
PNS	Peripheral Nervous System
PVam	Polyvinylamine
SCI	Spinal cord injury
SEM	Scanning electron microscopy
STAT-3	Signal transducer and activator of transcription
TUJ-1	β-III-tubulin staining
TFA	Trifluoroacetic acid
TH	Tyrosine hydroxylase
VEGF	Vascular endothelial growth factor

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INTRODUCTION

Strokes, cancer, trauma, degenerative diseases in the brain (ex. Parkinson's (PD) and Alzheimer's diseases (AD)), and nerve injuries are some of the many sources of damage or diseases inflicting change in the nervous system that cause cell death. They can destroy the quality of life of survivors, are costly to society and are the leading causes of death and injuries in North America. [1] For example, 16 million first-ever strokes occur annually worldwide causing 5.7 million deaths with an estimated cost of $65.5 billion in 2008 in the United States only. [2] 1.4 million Americans endure brain injuries annually of which 50,000 patients die. [1]

It is widely known that when damaged, there is almost no regeneration or repair of cells that occur in the central nervous system (CNS) since the neuron's cell body has been affected (unlike in the peripheral nervous system (PNS) where injured axons are able to self-repair and restore function). Damaged axons in the PNS regenerate and recover by proliferating Schwann cells and phagocytosing myelin by macrophages or monocytes. [1] Damaged neurons in the CNS (brain and spinal chord), however, have difficulty in restoring function due to the lack of Schwann cells. Schwann cells are glial cells present in the PNS; they contain neurotrophic and growth factors that form myelin sheaths on axons. They range in length from 220-400 micrometers with a thickness of 2-5 micrometers. [3] In a cross-section (Figure 1-1), several unmyelinated axons are embraced by Schwann cells, while surrounded by basal lamina. This complex structure is the result of overlap and regrouping of adjoining Schwann cells. [3]

Furthermore, the CNS lacks macrophages and monocytes, therefore are unable to clear the myelin debris when an axon injury occurs. Additionally, after CNS injury, glial scar tissue form due to the presence of astrocytes and act as a barrier to regenerating neurons. Inhibitory molecules such as myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OGmp) are also present in the CNS that inhibits axon regeneration. [4-8] A schematic diagram of CNS and PNS injury is shown in Figure 1-2. [9]

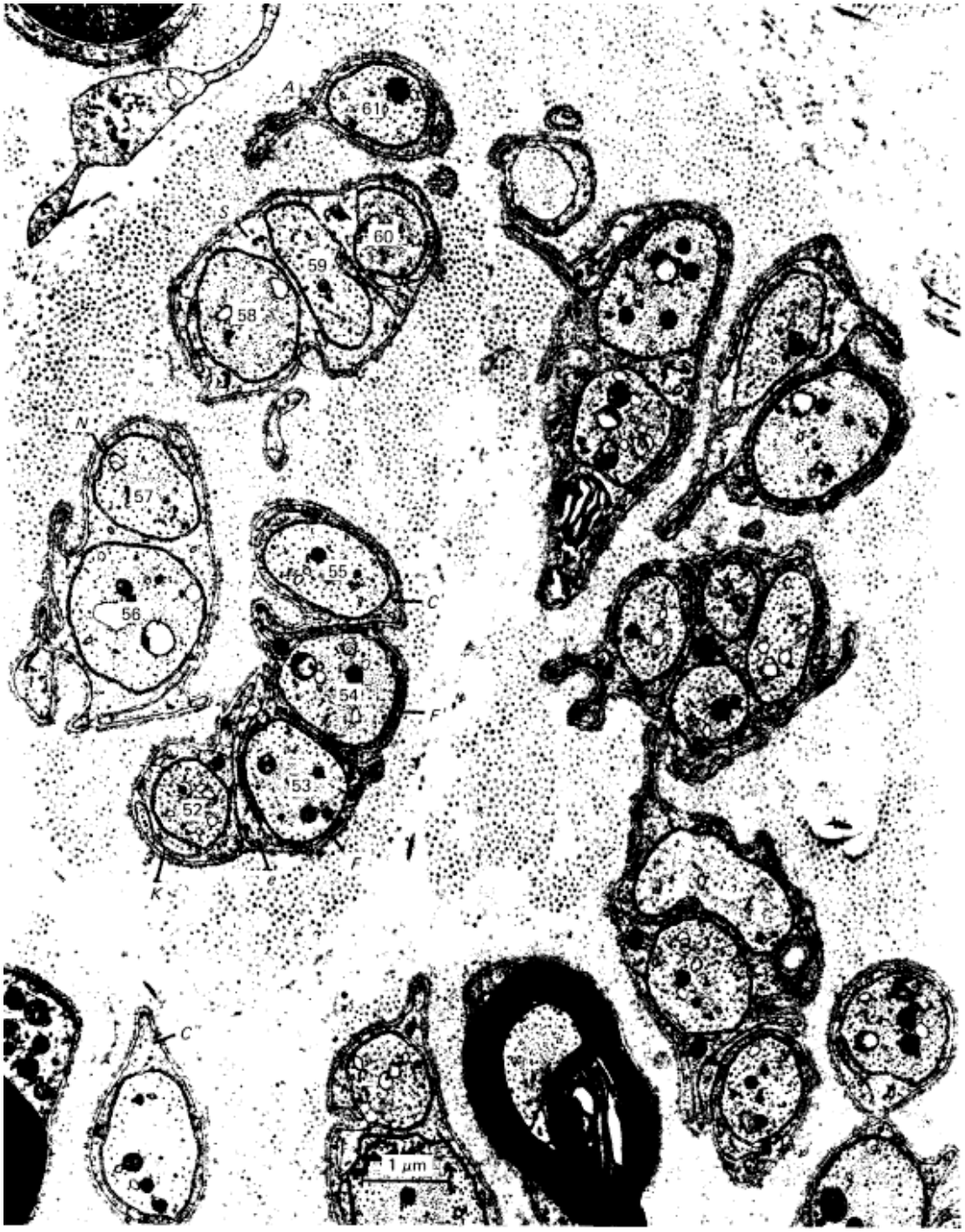


Figure 1-1: Cross-section of unmyelinated axons being embraced by Schwann cells. Axons are marked by numbers and Schwann cells by letters. A capital letter denotes a Schwann cell in contact and a lower case letter a Schwann cell out of contact with an axon. [3]

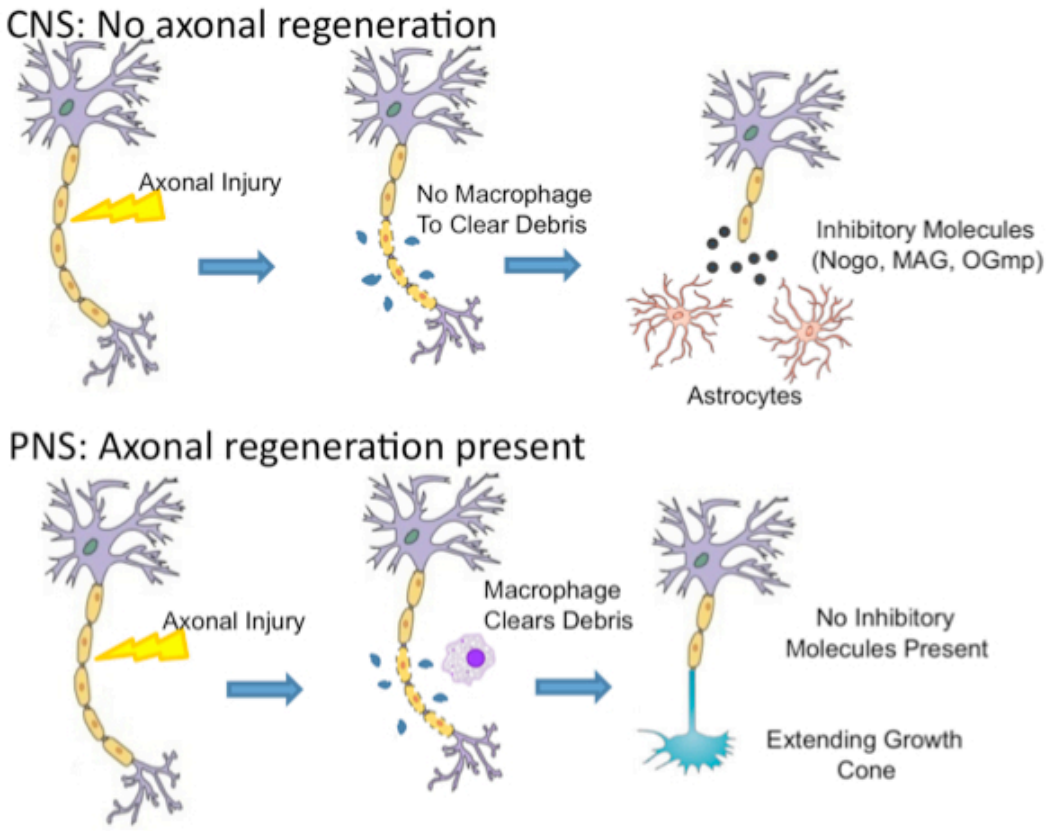


Figure 1-2: Schematic representation comparing injury at the CNS vs. PNS. [9]

The possibility of using biomaterials as a cell-scaffold construct in neural tissue engineering has been explored as an alternative to current therapeutic strategies. The ideal material should have excellent cytocompatible properties, otherwise they may induce severe inflammation or infection and may fail to improve neural cell growth. [1] Electrospun nanofibers have shown to be cytocompatible while stimulating cell proliferation. [10, 11] Haibin et al [11] have seeded MC3T3-E1 cells (cells commonly used to assess cytotoxicity of potential substrates for cell growth) on poly lactic acid (PLA) fibrous scaffolds, and after a 48 hour incubation, have seen that the cells were able to spread and extend on the scaffolds as well as seep through the meshes on the scaffolds.

The use of polymer fibers, such as polyesters, as scaffolds has been tested and has given promising results for tissue regeneration. [12-14] Li et al [14] demonstrated that BALB/c C7 mouse fibroblasts adhered and spread on the surface of the PLGA fiber network while cells grew

in the direction of fiber orientation. This favorable cell–scaffold interaction supports the biocompatibility of the structure. Ishuag et al [13] have investigated the use of 3-D poly (DL-lactic-co-glycolic acid) foam scaffolds for transplanting autogenous osteoblasts to regenerate bone tissue. The authors reported that the scaffolds supported the proliferation and differentiation of cells.

Among polyesters, poly L-lactic acid (PLLA) seems to be an ideal FDA-approved biomaterial due to its non-toxicity and resemblance to the extra-cellular matrix in the brain, thus its potential in neural cell regeneration. [15, 16] An image of glial cells and extra-cellular matrix is shown in Appendix C (Figure C5). PLLA is biodegradable; PLLA is naturally degraded to L-lactic acid via hydrolysis of the ester linkages (Figure 1-3). L-lactic acid is eventually metabolized in the body to carbon dioxide and water. [17]

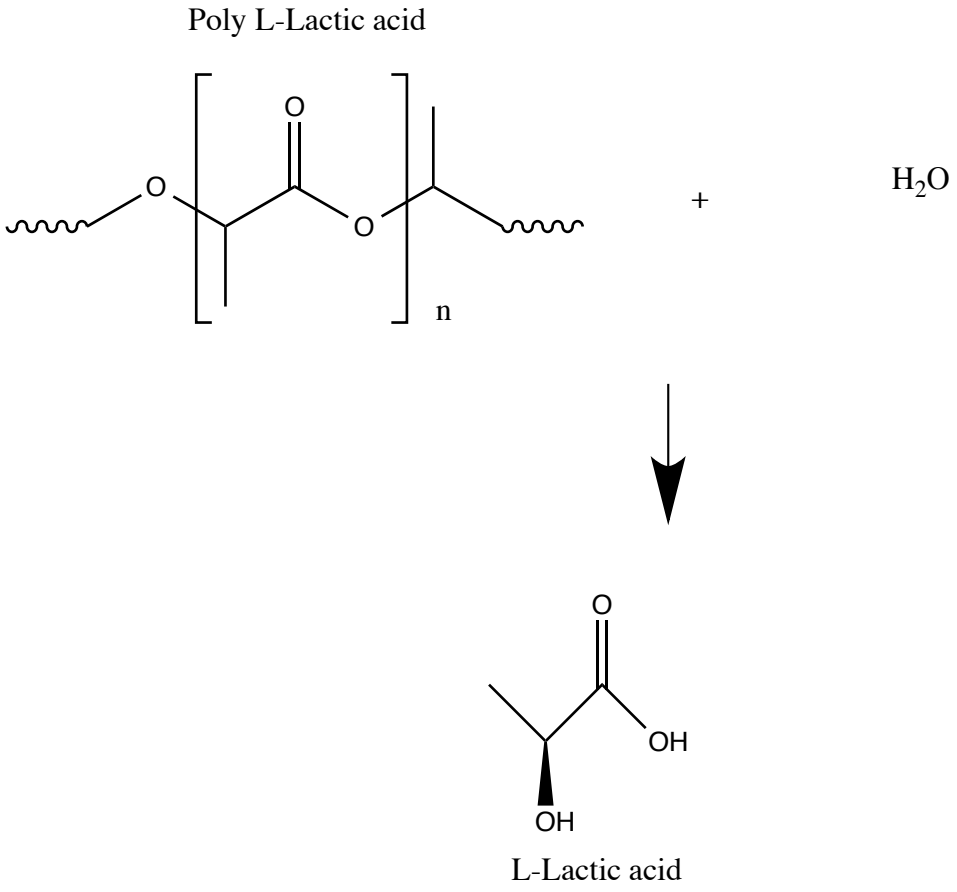
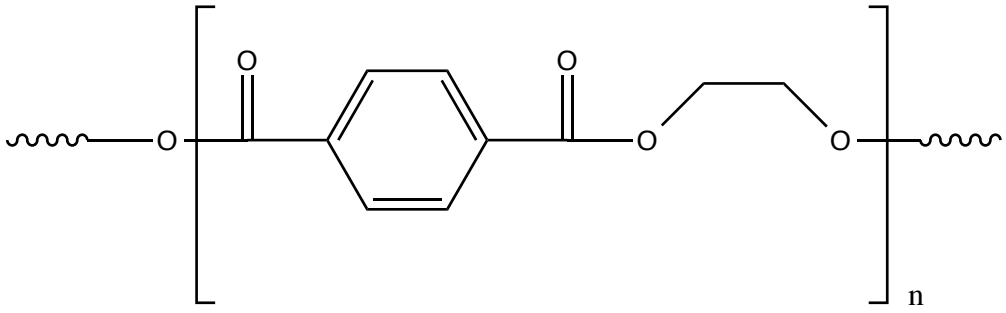
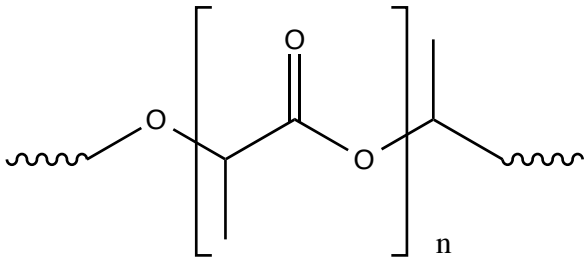


Figure 1-3: Hydrolysis of Poly L-Lactic acid to L-Lactic acid

Furthermore, growth factor signaling has been shown to have a significant role in tissue repair. In the last few decades, our understanding on the role of growth factors on the CNS has also greatly increased. [18-20] Furthermore, the inclusion of growth factors within scaffolds made of biodegradable and biocompatible polymers is desirable in order to retain them at the site of transplantation and control their spatiotemporal delivery to the damaged region of the CNS. [21-23] Epidermal growth factor (EGF), a soluble 6kDa polypeptide that is naturally present in the CNS, is known to promote the oligomerization and phosphorylation of cell surface EGF receptors (EGFRs) leading to cell proliferation and was used in this study. [24-28] Of interest, when tethered onto a scaffold, EGF has been shown to promote rapid expansion of neural stem cells (NSCs). [24, 29, 30] Amine functionalization on polyethylene terephthalate (PET) with ethylenediamine (EtDA) and polyvinylamine (PVam) has been previously reported [31] PLLA and PET are two different polymers with different chemical structures as shown in Figure 1-4. In this sudy, PLLA nanofibers were found to be very fragile and the necessary adjustments were performed and adapted from the above-mentioned protocol.



PET



PLLA

Figure 1-4: Chemical structures of Polyethylene terephthalate (PET) and Poly L-Lactic acid (PLLA)

The main objective of this work is to fabricate a PLLA scaffold by electrospinning, to establish the optimum topological features of nanofibrous mats for neural stem cells and to subsequently functionalize it for improved neural stem cell transplantation. More specifically, we will investigate EGF grafting onto the PLLA scaffold after surface modification via aminolysis. This strategy will combine the advantages of both the PLLA polymer as a cell-scaffold (mechanical strength, high porosity, biodegradability and biocompatibility, etc) as well as the benefits of tethered EGF. The impact of EGF-grafted PLLA nanofiber scaffolds upon neural stem-like cells (NSLC) adhesion, viability and proliferation will be tested. Neural stem-like cells (NSLC) were provided by NewWorld Laboratories Inc. They were created from somatic cells through reprogramming by NWL. Neural Stem-Like Cells (NSLCs) were engineered from human fibroblast cells by transient reprogramming using defined factors. These reprogrammed cells are epigenetically stable. They have global gene- and protein-expression profiles that are similar to Neural Stem Cells.

A literature review is presented in the first part of this thesis showing the current strategies documented for neural stem cell regeneration.

Based on the literature review, the second part of the thesis presents, in our opinion, the most promising strategy for fabricating and modifying a substrate to promote cell proliferation for neural tissue regeneration. An extensive cell culture study was performed on fibers grafted with EGF under different media conditions.

CHAPITRE 1 GENERAL PROCEDURE

A literature review summarizing all pertinent work performed in neuronal tissue engineering is presented. The literature review consists of the different methods currently reported for tissue regeneration. Such strategies include direct injection of stem cells and/or growth factors, encapsulated growth factors, hydrogels as scaffolds, etc as well as the surface modification of polymeric scaffolds using different bio-macromolecules. The advantages and disadvantages of the strategies presented in the literature review are analyzed in an attempt to develop an optimized strategy.

In a second section, an article entitled 'Fabrication and Surface modification of poly L-lactic acid (PLLA) scaffolds for growth factor immobilization and neuronal stem cell delivery' is presented. This article was submitted to the Journal of Biomedical Materials Research - Part B, a highly interdisciplinary peer-reviewed journal whose common focus is on biomaterials applied to the human body and covers all disciplines where medical devices are used.

The second part of this thesis presents the step- by- step process of the fabrication of PLLA electrospun scaffolds, followed with an optimized amine functionalization, and EGF grafting. An extensive cell culture study was performed with EGF-grafted fibers under different media conditions. Negative controls involving aminated nanofibers and pristine nanofibers were performed for comparison, as well as positive control (nanofibers that were only coated with laminin). These cell culture assays were used to test for cell adhesion, viability and proliferation using our new strategy.

Finally, future work directions and improvements are proposed such as a grafted-EGF density-dependent study, EGF tethering to the PLLA scaffold in an oriented fashion for improved bioactivity, and the study of progenitor cell differentiation to specific and appropriate lineages.

CHAPITRE 2 LITERATURE REVIEW

2.1 Direct injection of stem cells for *in situ* tissue regeneration

Contrary to what was once believed, studies in the last two decades have shown that the CNS does indeed contain neural stem cells and has potential for extensive cellular replacement. The discovery of neural stem cells has therefore opened up the development of therapeutic strategies for CNS using stem cells. [32] Even though neural stem cells are present in the CNS, the quantity of active neural stem cells seems to be very low. Neural stem cells range in size from 4-50 µm. [33-38] Schematic drawing [39]of NSC and its environment is shown below.

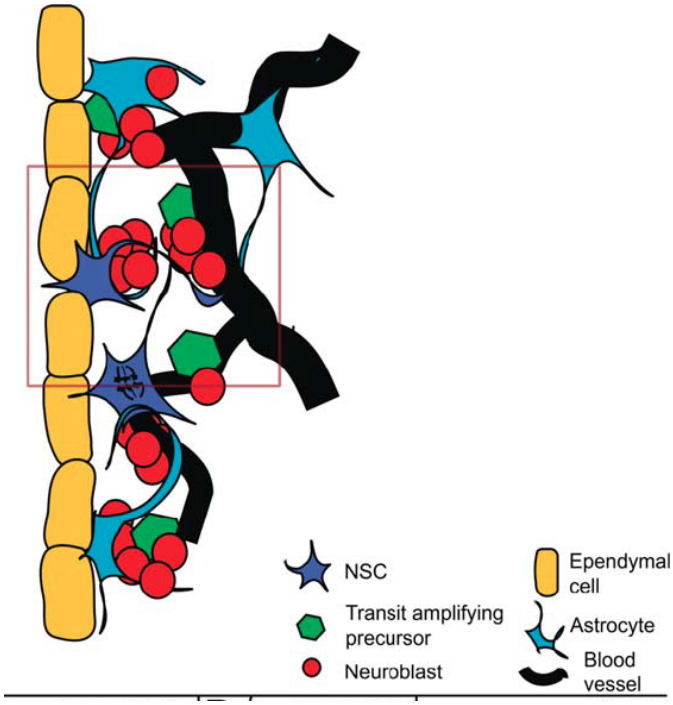


Figure 2-1: Neural stem cell niche with NSC (in blue) [39]

The injection of additional stem cells at the site of injury for cell regeneration has thus been studied. In an animal model where aspects of multiple sclerosis were reflected, adult neural stem cell cultured neurospheres were injected in the CNS. The injected cells migrated to the damaged areas of the CNS and the animals displayed significant functional recovery. [32, 40]

However, the animal experiment may not be translated to humans since multiple sclerosis in the human CNS is a chronic and inflammatory disorder that involves both glial and axonal changes and its treatment is different from the animal experiment. [32] Another obstacle for the implementation of treatments based on direct cell injection is the lack of appropriate factors in the host tissue to accommodate the injected stem cells. Altered pathological tissue due to Alzheimer's disease is no longer able to present factors that promote proper differentiation hence making it difficult for the injected stem cells to treat the disease. [41, 42] Furthermore, Hansmann et al [43] have implanted immortalized oligodendrocyte precursor cells (OPCs) where it has been reported that cell injection may result in tumor formation. That is, it has been suggested that a failure of molecular control mechanisms in murine glial precursor cell line BO-1 cells, responsible for a shift from differentiation to proliferation, led to tumor formation. [43] The signal transducer and activator of transcription (STAT)-3 (member of the Janus Kinase (Jak)/STAT-pathway downstream of the epidermal growth factor receptor (EGFR)) had been used as a tumor suppressor in the PNS but its function in glial tumors is still unknown. [43, 44] The presence of CNS stem cells was first discovered when growth factor responsive cells from both embryonic and adult CNS stem cells were isolated. [19, 20] Furthermore, studies on stem cells have shown that growth factors play a significant role in controlling the fundamental process of development. [32] It has been previously reported that direct implantation of human mesenchymal stem cells (MSCs) stimulated the synthesis of neuronal survival factors; increased endogenous expression of nerve growth factor (NGF), vascular endothelial growth factor (VEGF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor-2 (FGF-2) as well as cell proliferation. [45] These studies showed that the benefits coming from direct stem cell injections may be due to their ability to directly produce growth factors and thus provide the appropriate cues for proper development/repair. This suggests that the direct injection of growth factors may be sufficient instead of stem cell injection. [46] This could explain why, in contrast to the CNS that lack Schwann cells, injured peripheral neurons are able to regenerate their axons because of their Schwann cells that provide neurotrophic factors. [46]

2.2 Direct injection of growth factors for *in situ* tissue regeneration

Growth factors are able to maximize the intrinsic regenerative potency of endogenous progenitor cells as well as aid exogenous stem cell proliferation and differentiation. [21, 47] It is now known that peripheral nerve bridges contain large quantities of NGF, BDNF, neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF) and glial cell-line derived neurotrophic factor (GDNF) secreted by Schwann cells, which is the cause of the stimulation of axon growth regeneration. [48] Tuszynski et al [49, 50] have genetically modified suspensions of autologous fibroblasts to secrete NGF and these modified cells were injected into the central gray matter of the non-lesioned thoracic spinal cord. The results demonstrated that axons grew in significantly larger numbers when presented with an environment containing growth factors. [49, 50] Kobayashi et al [51] have shown that brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) prevented atrophy of rat rubrospinal neurons and promoted axonal regeneration. Furthermore, Boyd and Gordon [52] have shown that long-term continuous treatment with exogenous GDNF significantly increased the number of motoneurons that regenerate their axons. However, no significant benefits were observed from the combined GDNF and BDNF treatment on the axonal regeneration of motoneurons until after seven days of continuous treatment. Vejsada et al [53] have also shown that continuous neurotrophic factor delivery was essential in order to promote the long-term survival of axotomized neonatal motoneurons. Furthermore, the short half-lives of growth factors, their relatively large size, slow tissue penetration and potential toxicity at systematic level exposures limits their use by direct injections. [54] A summary of the various growth factors mentioned is presented in Appendix B.

2.3 Encapsulated Growth factors

One of the most common approaches used to get controlled drug release is to embed the drug into a hydrophilic or hydrophobic matrix; a matrix defined as a three-dimensional network containing the drug, polymer, solvents, etc. [55] The drug is then released and the chemical kinetics are affected by many factors such as polymer swelling, polymer erosion, drug dissolution (re-crystallization), drug diffusion characteristics, particle size distribution, drug-polymer interaction, drug distribution inside the matrix, drug/polymer ratio and geometry. [55, 56] Biocompatible and biodegradable biomaterials are excellent for the gradual release of a drug or

for tissue scaffolds. When designing the biomaterials to be used, many factors are taken into consideration such as the degradation rate and the appropriate materials, conditions, dimensions, and geometries are chosen for specific applications. [57] Wang et al reported the encapsulation of vascular endothelial growth factor (VEGF) and BDNF in PLGA microspheres using the *water-in-oil-in-water* emulsion technique. The PLGA microspheres were then embedded within a cross-linked hyaluronic acid (HA) hydrogel as a delivery system. [58] Although hydrogels are widely used for encapsulating growth factors, they release their growth factors in an initial outburst that is higher than physiological levels, therefore affecting the duration of the delivery. [46, 59] Lam et al [60] have encapsulated and stabilized NGF into Poly (lactic-co-glycolic) (PLGA) acid microspheres by spray-freeze-drying method. First, the human NGF formulations were prepared in two different buffer solutions and lyophilized: one buffer system consisting of histidine and the other consisting of sodium bicarbonate and zinc acetate. For the spray-freeze drying technique, the human NGF formulations were pumped into an ultrasonic spray nozzle with a peristaltic pump. The protein was then sprayed into a flask containing liquid nitrogen and merged into a liquid nitrogen bath. The frozen protein droplets were poured into a stainless steel tray and dried by lyophilization (primary temperature at -25°C for 30 h, followed with secondary drying at 20°C for 10 h). The NGF released from the PLGA microspheres degraded at physiological temperature. In order to prevent NGF degradation and aggregation during microencapsulation and release, PEG or surfactant (pluronic F68) was added to the formulation, however, the integrity of the NGF released was not improved.

2.4 *In situ* production and delivery of growth factors using cells

The grafting of primary fibroblasts that are genetically modified *ex vivo* to produce BDNF or NT-3 has been previously reported.[61, 62] However, immune suppression was needed in order to prevent the rejection of the grafts. In order to overcome this need for immune suppression, previous reports have developed a method based on alginate encapsulation for grafting non-autologous BDNF producing fibroblasts into the injured spinal chord.[63] Tobias et al [64] have also shown that alginate encapsulation can protect non-autologous BDNF producing fibroblasts (Fb/BDNF) from rejection after transplantation into a site of injury and promoted behavioral recovery. They have further shown that the grafting of encapsulated Fb/BDNF containing cells into a subtotal cervical hemi-section resulted in partial recovery of forelimb

usage as well as axonal growth.[64] These cell-containing scaffolds have shown their capability to survive transplantation into the injured spinal cord for at least one month in the absence of immune suppression.[63] Furthermore, alginate encapsulation was able to protect the Fb/BDNF for two months after grafting.[64, 65]

Layers of cells may be used where the bottom layers are first cultured and can provide the requirements needed to the cells of interest (cultured on top) resulting in the regeneration of neuronal structures ex vivo. [46] Zeng et al [66] have cocultured cells from the bone-marrow derived stromal cell line PA6 to induce differentiation of human embryonic stem cells (hESCs). The production of dopaminergic neurons at a high frequency from hESCs was observed as well as the survival of transplanted, tyrosine hydroxylase (TH)-positive cells after 3 weeks of differentiation. A bottom layer of PA6 cells was also used as a feeder layer to initiate the neural differentiation of BG01 cells. An outgrowth of elongated cells was observed after 6 days in culture. However, the survival of dopaminergic neurons after transplantation was limited suggesting this method is not ideal. It has also been shown that the co-culturing with PA6 cells is simple and fast and can be used with cells from subhuman primates.[67] Other studies reported the administration of NGF to the brain using *ex vivo* gene delivery. [68-72] Genetically modified autologous fibroblasts were used to produce and secrete NGF. This method of delivery sustained NGF production for at least 18 months, prevented cholinergic degeneration, stimulated cholinergic function, and improved memory.[68-72] Tuszynski et al have also applied this procedure to humans where genetically modified NGF-producing fibroblasts were injected into the brains of 8 subjects suffering from early-stage probable Alzheimer disease.[73] Evidence showed that growth factor delivery in this approach has the potential to modify neurological disease progression.

2.5 Scaffolds for stem cell therapy

In the case of neurodegenerative diseases, the injected stem cells cannot properly differentiate because the pathological tissue does not contain the necessary factors.[46] NSC transplantation may not be effective because the amyloid precursor protein (APP) metabolism is altered and might lead to excessive gliogenesis. Stem cell therapy is challenging due to the blood-brain barrier that makes it difficult for the neurotrophic factors to diffuse. Also, other causes that may explain why the NSCs are not able to regenerate properly in the damaged brain are hostility

of the environment; inflammation, glial scar formation, release of inhibitory molecules, and the absence of growth-guiding astrocytes. [74] As a result, and because of the fragility of the cells, a biomaterial to be used as a scaffold to transplant the stem cells into the host target area appears to be highly desirable. Figure 2-2 shows a schematic of a classic tissue engineering process in which cells can be isolated from the patient (from a biopsy) and expanded.[75] Two distinct strategies are described where one corresponds to the direct injection of cells whereas the other is the use of a polymer scaffold. Hydrogels can also be directly injected, in combination with cells, into the host environment.[75]

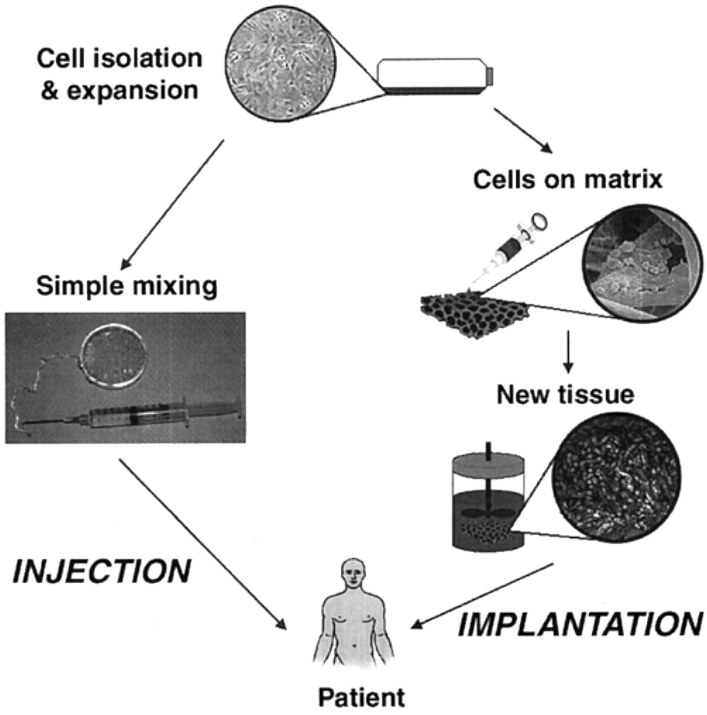


Figure 2-2: Schematic illustration of typical tissue engineering approaches. Cells are obtained from a small biopsy from a patient, expanded in vitro, and transplanted into the patient either by injection using a needle or other minimally invasive delivery approach, or by implantation at the site following an incision (cut) by the surgeon to allow placememnt. [75]

2.5.1 Hydrogel as a scaffold

Hydrogels have shown promising characteristics as they are relatively easy to create, have a 3D structure, and the best material can be chosen based on the desired mechanical structures as shown in Figure 2-3. [76]

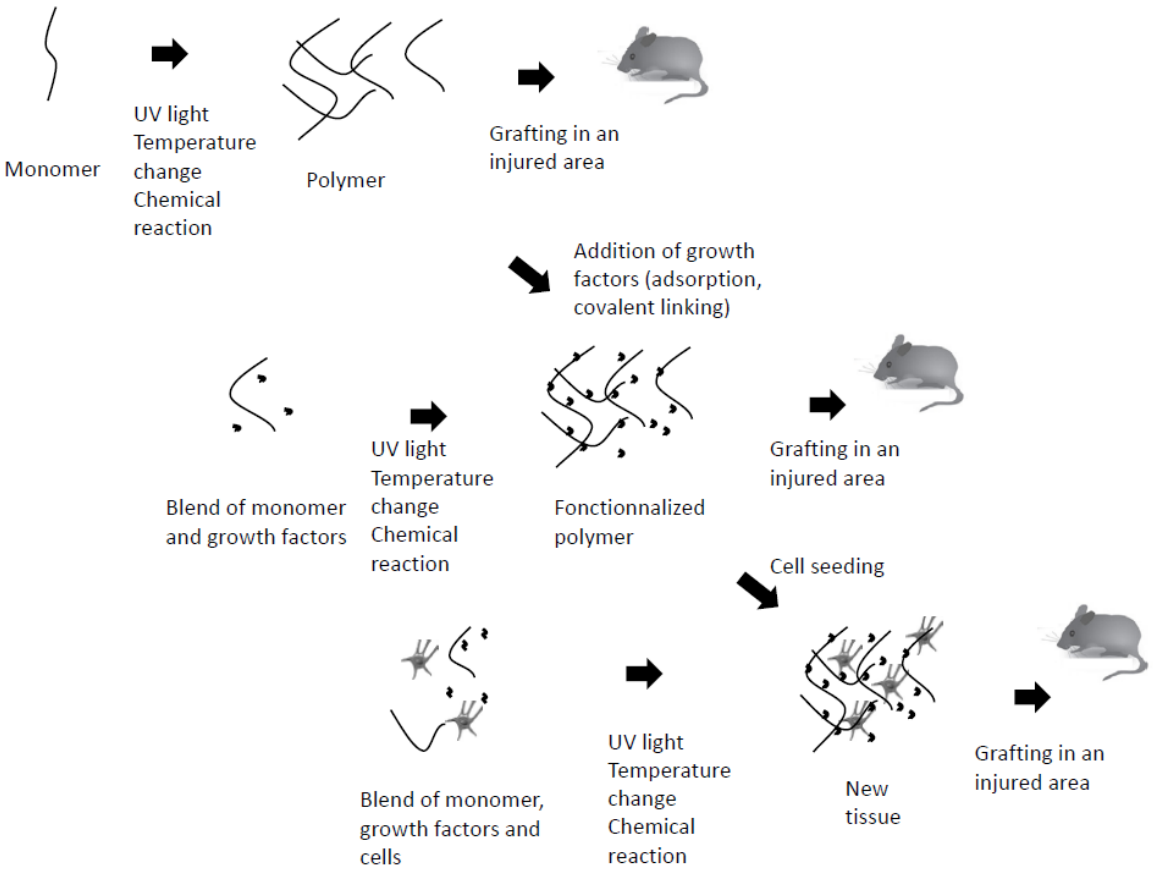


Figure 2-3: Hydrogel fabrication and application. [76]

Numerous studies have been performed on the use of hydrogels as a cell scaffold. Hyaluronic acid (HA) hydrogels modified with laminin, harboring similar mechanical and rheological properties as brain tissue were shown to inhibit glial formation and support angiogenesis. [77, 78] Wei et al have [79] demonstrated that when hyaluronic acid (HA)-based hydrogels modified with poly-L-lysine (PLL) and a nogo-66 receptor- antibody (antiNgR) fusion protein (the whole structure being referred to HA-PLL/antiNgR) was administered to rats,

inhibition of glial scar formation was observed. HA, however, is highly hydrophilic; in order to undergo cell proliferation, cells need a substrate that is neither too hydrophilic nor too hydrophobic. [80] Woerly et al [81] have evaluated the application of Poly[N-(2-hydroxypropyl) methacrylamide] (PHPMA) hydrogels for the promotion of axonal regeneration in the transected rat spinal cord. Following implantation, the hydrogel was able to bridge tissue defects, favored cell ingrowth, angiogenesis and promoted axonal growth. Their similarity in viscoelastic properties to neural tissue (as well as large surface area) contributes to their success as a strategy for neural tissue regeneration. Type I collagen has been used as an injectable hydrogel while inhibiting glial scar formation after spinal cord injury. [82] However, telopeptide regions are present during gel formation for crosslinks between molecules. These telopeptide regions are implicated in antigenicity and render the gels weaker when removed. [83]

Furthermore, some limitations of hydrogels as potential scaffold for neural tissue engineering include their high cost and low mechanical strength. [75, 84]

2.5.2 Electrospun Fibers

A variety of methods exist for the fabrication of PLLA nanofibers; they can be fabricated by liquid-liquid phase separation, [85] or thermally induced phase separation method [86], as well as melt spinning method (melt extrusion and hot draw). [87] Recently, electrospun scaffolds have been assayed as a substrate to support cell regeneration. [10, 16, 88, 89] Electrospun fibers have been fabricated for the generation of scaffolds destined to neural tissue engineering because of their interconnected pores, high porosity, high surface area-to-volume ratio, and topography that mimic the natural extracellular matrix (ECM). Electrospun fibers have been demonstrated to support the attachment and differentiation of various neuronal cells, such as dorsal root ganglion neurons (DRG), Schwann cells, hippocampal neurons, as well as PC12 cells. [90]

2.6 Electrospinning

Electrospinning is a fabrication technique that produces polymeric fibers using electrostatic force. It is possible to obtain fibers from a wide variety of materials such as poly (e-caprolactone) (PCL), poly lactic-co-glycolic acid (PLGA), poly lactic acid (PLA), etc. Fiber properties such as diameter, porosity, and alignment can also be tuned during the electrospinning process. This technique consists of placing a polymer solution in a syringe where a strong

difference of potential is applied between a metallic collector and the metallic needle. A droplet charged with static electricity in the shape of a Taylor cone is then created. This droplet is attracted by the electric field in the direction of the collector where charge repulsion exceeds the surface tension, [91] and evaporates during its flight, creating fibers with a diameter varying from tens of nanometers to a few micrometers. [92] Electrospun fibers are attractive for scaffold production due to the relative simplicity of its fabrication, (summarized in Figure 2-4), its tunability and its cost-effectiveness.

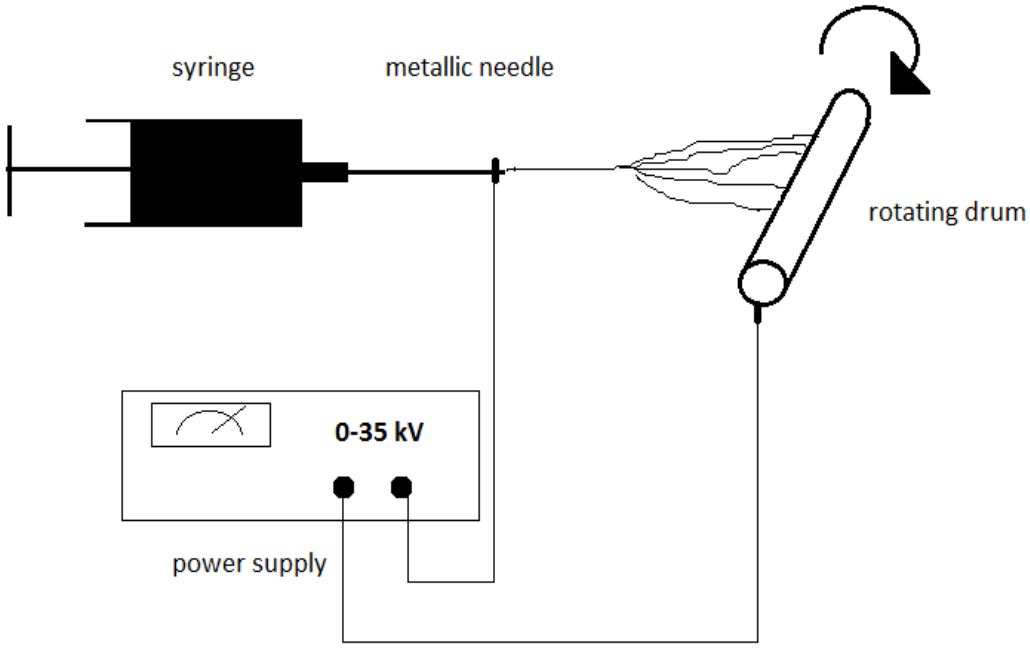


Figure 2-4: Electrospinning set-up. [93]

2.6.1 Polymer solution conditions

A suitable solvent that is able to dissolve the polymer while possessing suitable vapor pressure is usually chosen. A good solvent is one that evaporates quickly enough for the fiber to maintain its integrity when it reaches its target but not too quickly in order to allow the fiber to harden before reaching the collector. Furthermore, the quality of the solvent [94], as well as the temperature of the solution has an effect on fiber diameter. [95] There are also many other conditions that can influence the nanofiber topography and morphology (e.g., beaded, random, or aligned, etc) such as polymer concentration, molecular weight, solution viscosity, surface tension,

solution conductivity, dielectric effect of solvent, voltage, feed rate, collector speed, distance between tip and collector, ambient temperature and humidity.

2.6.1.1 Polymer concentration, molecular weight, and solution viscosity

Polymer concentration has an effect on fiber diameter as well as fiber morphology. Generally, higher polymer concentrations yield nanofibers of larger average diameters. [96] Another factor affecting the viscosity of the solution is the molecular weight (Mw) of the polymer. The molecular weight represents the length of the polymer chain and determines the amount of polymer entanglements in a solution. Low Mw polymer solutions are not able to form fibers when electrospun. [93] Furthermore, Mw directly affects the viscosity, which is understandable since the amount of polymer chain entanglements in the solvent are determined by polymer length. Usually, a low viscosity solution tends to result in bead formation in the fiber structure. [93, 97] Also, when the viscosity of the solution is low, there may be a secondary jet that erupts from the main jet and cause a variety of different fiber diameters. [93, 98] When too viscous, solutions can also dry at the tip of the needle, preventing electrospinning. [10, 99] Additionally, at very low humidity, the solvent evaporation rate is higher causing clogging to occur at the needle tip. [93]

2.6.1.2 Surface tension

The surface tension is defined by the tractional force, γ, acting across any unit length of line on the interfacial membrane (i.e. liquid and vapour). [100] To begin the electrospinning process, the charged solution must first overcome its surface tension, allowing the stretching of the polymer solution to reach the collector without the formation of beads. [93] The Coulomb repulsion between the charged ions in a polymer solution favors the creation of a jet, whereas the surface tension of the solution favours a sphere-like shape. When the electrical potential of the surface is increased, the electrical forces dominate the surface tension of the solution and a charged jet of fluid (referred to as the Taylor cone) is released as shown in Figure 2-5 where the Taylor cone is shown at the top of the illustrations. [101]

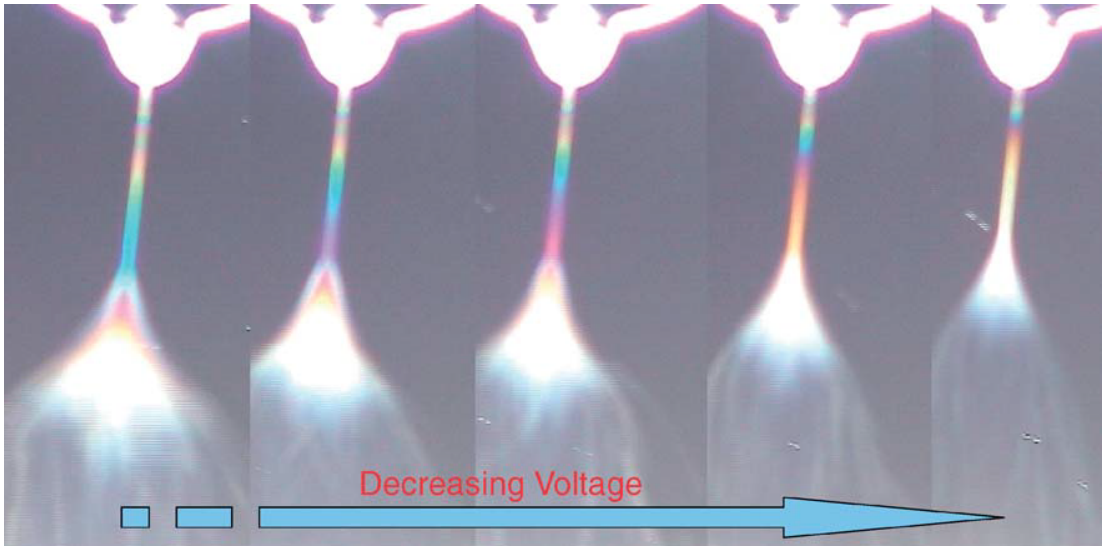


Figure 2-5: Taylor cone shown at top of images. Lower voltages result in thinner fluid jets as well as less fluid being drawn from syringe where the interference colors provide information on jet diameter. [102]

2.6.1.3 Solution conductivity

The conductivity of a solution is a measure of its ability to conduct electricity. The presence of ions has an effect on the conductivity; many of the solvents used in electrospinning possess a certain level of conductivity. The stretching of the polymer solution from the syringe is caused by the repulsion of the charges at the polymer surface. An increase in the stretching of a solution results from the increase of charges, leading to bead-less fibers. An increase in polymer solution stretching also results in fibers with smaller diameters. [93, 99]

2.6.1.4 Dielectric effect of solvent

The dielectric constant (a quantity measuring the ability of a substance to store electrical energy in an electric field) of a solution plays a major role in electrospinning. A solvent with a high dielectric constant will result in a fiber with smaller diameters and less beads. [93]

2.6.2 Electrospinning process conditions

External factors such as voltage, feed rate, temperature of solution, ambient temperature, humidity, type of collector, diameter of needle, and distance between tip and collector also play a role in the outcome of fiber morphology.

2.6.2.1 Voltage

Taylor cones define the onset of extensional velocity gradients in the process of forming fibers. [103] The polymer jet exits the syringe needle and forms a Taylor cone when a high voltage is applied and the electrostatic force overcomes the surface tension of the solution; the coulombic repulsive force in the jet then stretches the solution. Generally, a high voltage leads to smaller fiber diameters; this is due to the fact that a higher voltage will lead to an increase in stretching of the solution. Lower voltages result in thinner fluid jets and less fluid is drawn from the syringe as shown in Figure 2-5 where the interference colors provide information on jet diameter. [101]

A higher voltage can cause instability of the jet and Taylor cone, causing them to recede into the syringe needle, resulting in beaded fibers. [93]

2.6.2.2 Feed rate

The feed rate cannot be too high in order to give enough time for the solvent to evaporate during flight. When the feed rate is increased, the fiber diameter or bead size is also increased. [93]

2.6.2.3 Effect of collector

The collector plate is usually made out of a conductive material such as aluminum foil and/or a metal plate, which is electrically grounded. Once the voltage is turned on, there is an electric field between the source and the collector, hence attracting the polymer solution towards the collector. [93]

2.6.2.4 Distance between tip and collector

The distance between tip and collector has an effect on resulting fibers. For example, beads can be observed when the distance is too low. [104] This is due to the fact that decreased

distance causes an increase in field strength. A distance that is too high may either decrease or increase the fiber diameter [93] therefore an optimal distance is necessary in order to achieve the desired fiber morphology.

2.6.2.5 Ambient humidity and temperature

Water condensation can form on fibers when the humidity is too high and this can affect the fiber morphology. [93, 104] The humidity can affect the diameter and porosity of the fibers. [105, 106] The humidity and temperature have a significant effect on average fiber diameter. At high ambient humidity (higher than the optimal range of 20-40 % relative humidity), the fiber diameter of a polymer can decrease and form beads as represented in Appendix C (Figure C3), which can pose a potential challenge. [107] Furthermore, the solution absorbs ambient water at high humidity leading to the fusion of fibers at the collector [107] as demonstrated in Appendix C (Figure C4).

2.7 Tissue engineering

Tissue engineering is the application of the principles of engineering as well as life sciences to make a biological substitute, which often contains cells and biodegradable scaffolds co-cultured *in vitro,* that can restore, maintain or improve tissue function. In tissue engineering procedures, artificial extracellular matrices (ECM), i.e. biomaterials, are often used as scaffolds for cells to grow in. Cells interact with the biomaterials where their initial interaction is determined by the chemical composition, surface energy, roughness, and surface topography that are in direct contact with cells.[108] Cell adhesion, migration, differentiation and proliferation depend on biological cues such as growth factors and cytokines; they also depend on chemical and physical characteristics of the biomaterial surface.[108] Polyesters such as poly lactic acid (PLA) are FDA-approved; they are biodegradable, biocompatible, and non-toxic. Polyester surfaces have poor hydrophilicity, however, and lack functional groups that may ease chemical tailoring of their surface. Hence, the introduction of such functional groups is usually needed. Many different surface modification methods have been reported such as plasma treatment[109, 110], surface hydrolysis[111], chemical grafting[112], physical adsorption[113] and self-assembly technology.[114]

2.7.1 Biological response to biomaterials

In order to develop an efficient biomaterial, the understanding of cell interactions with the extracellular matrix (ECM) is important. In a normal biological situation, cell-cell interactions are mediated and modulated by signaling complexes that occur through adhesion receptors on cell surfaces when cells interact with the ECM. Cells adhere to their ECM through their cytoskeleton actin filaments. Cell membrane receptors, called integrins, are what mediate this adhesion. [108] The ECM also acts as a reservoir for growth factors whose diffusion (and thus availability) is controlled by the ECM physical properties. The ECM protects the growth factors from degradation and regulates their synthesis. [115]

Once the biomaterials are implanted in the body, the first step that occurs is water adsorption, followed by protein adsorption prior to cell attachment. The surface properties of biomaterials are important in that they determine the binding strength and structural arrangement of water molecules, which may affect later protein-surface, and thus cell-surface interactions. The porosity of a biomaterial also has an effect on cell behavior. Gugala et al reported the superiority of porous membranes to non-porous ones from poly (L/D-lactide) 80/20% for the growth and osteoblastic differentiation of rat bone marrow stromal cells *in vitro*. [108]

2.8 Poly lactic acid (PLA) and poly L-lactic acid (PLLA)

2.8.1 General

Poly(α-hydroxy acids) such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymers have been widely used as biomaterials. [116, 117] PLA is a thermoplastic, high strength, high-modulus aliphatic polyester made from renewable resources, more specifically α-hydroxy acids, which include polyglycolic acid or polymandelic acid. PLA is formed by direct condensation polymerization of lactic acid. Similarly, poly L-lactic acid is formed by polycondensation of the L enantiomer. The stereochemical structure of poly lactic acid can be modified by polymerizing a controlled mixture of the L- or D-isomers in order to yield either amorphous or crystalline, high molecular weight polymers. [118] Lactic acid has been first isolated in 1780 from sour milk and has many food-related applications. It is used as a buffering agent, acidic flavoring agent, acidulant and bacterial inhibitor in many processed foods and can

be manufactured by carbohydrate fermentation or chemical synthesis. [118] As mentioned earlier, lactic acid (2-hydroxy propionic acid) exists in two optically active configurations; the L(+) isomer is produced in humans and other mammals, whereas the D(-)- and L(+)-enantiomers are produced in bacterial systems. There are two different routes for PLA polymerization. The first route is through polycondensation from lactic acid to yield low molecular weight PLA. The second route is from lactide through ring-opening polymerization process to yield poly lactic acid as shown in Figure 2-6.

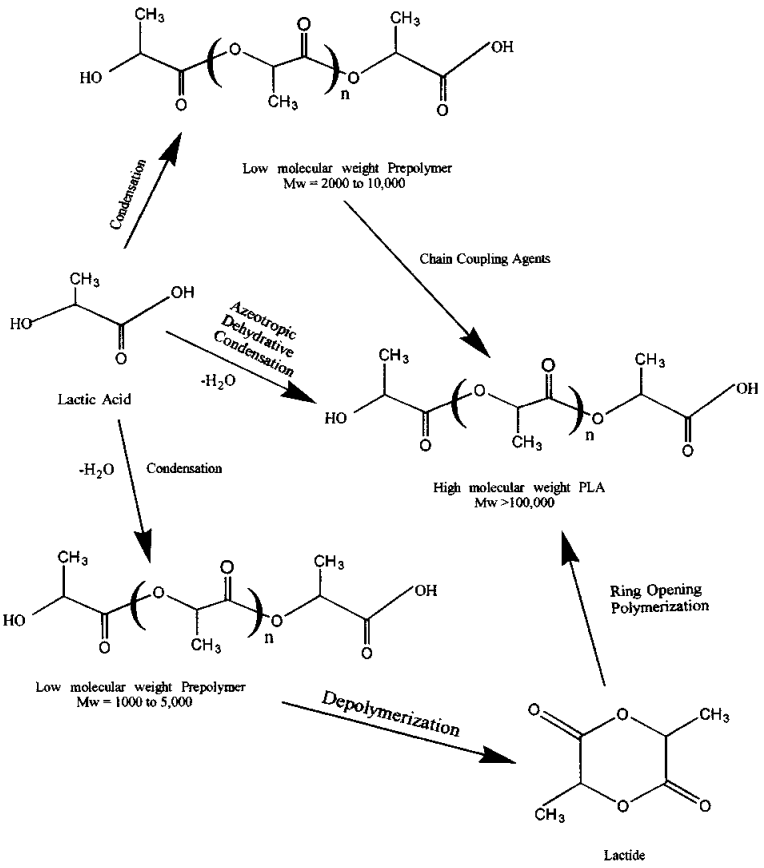


Figure 2-6: Synthesis of PLA [118]

2.9 Surface modification

The chemical structure, hydrophobicity, ionic groups, morphology, and topography of the biomaterial have an effect on the interaction with surrounding biological environment. Furthermore, the surface properties such as wettability, surface charge and topography affect cell adhesion. [108] Additional factors that influence the cell interactions with the scaffold include the structure of the biomaterial, porosity, pore size, geometry, distribution, surface texture such as roughness, pattern, orientation, and surface chemical properties such as free energy and surface micro-morphology. The porosity of a scaffold plays an important role in directing tissue formation and function. [119, 120] A high porosity (higher than 80%) [85] is necessary in a scaffold for homogeneous cell distribution and interconnection as well as diffusion of nutrients and oxygen. [121] The stiffness of a material also decreases as the porosity is increased. [121]

Pore size also has an impact on the amount of contraction a scaffold will undergo after implantation. [121] For example, a pore diameter ranging from 20–125 μm was needed for contraction-inhibiting activity to be observed in collagen–glycosaminoglycan graft copolymers used for dermal repair. [122] Choice of pore size is also related to the cell type to be cultured. [121] Furthermore, the maximum fiber thickness recommended for tissue engineering scaffolds is 150-200 μm due to the inability of and insufficient oxygen and nutrient transport within the deeper compartments of the biomaterial. [121, 123]

Surface modifications of biomaterials in an alkaline solution could be used for the production of hydrophilic and rough surfaces. [108] Cell adhesion to ECM proteins is mediated by integrins where they organize into complexes that contain structural and signaling proteins. There has been a large development to the modification of polyester surfaces. Several methods of surface modification are possible such as chemical grafting, surface coating, entrapment, and electrostatic self-assembly.

2.9.1 Aminolysis

Aminolysis can be used prior to the grafting of bioactive molecules where primary amines are introduced by thermally induced aminolysis. The aminolysis reaction is based on the nucleophilic attack of an amine onto the ester bonds of a polymer chain via polymer-chain scission and has been widely used in literature for the addition of amine groups. Fadeev et al have added amine groups on PET surfaces using polyamines. [124] Furthermore, Yang et al [125] reported UV-light-induced surface aminolysis reactions. The tertiary amines are functionalized onto the PET surface, are easily protonated and used for electrostatic immobilization of proteins. Studies have also reported the addition of amine groups using a small diamine, ethylenediamine (EtDA)[31, 126] as well as an aminated polymer, polyvinylamine (PVam). [31, 127] The aminated PET films were followed with the functionalization of Succinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexanoate, LC-SPDP linker, an amino-reactive molecule. The LC-SPDP functionalization of substrates could be followed with the oriented immobilization of cysteine-tagged proteins. [128, 129]

2.9.2 Surface coating/physical adsorption of proteins

Most materials need to be coated in order to improve cell adhesion, even electrospun fibers. [37, 130] This is due to the poor hydrophilicity of polyester surfaces. [108] Biomaterials as scaffolds are limited by the ability of attached cells to dynamically repopulate. [131, 132] Surface coating with, for example, collagen and laminin provide an adhesive interface between biomaterial surfaces and cells. Garic et al modified PLA surfaces by creating tiny cavities by sodium hydroxide, as well as coating with type-I collagen for comparison. [133] Results showed that human keratinocytes preferred growing on partially hydrolyzed surfaces. This study has suggested that other surface modifications can further improve keratinocyte culture on PLA. However, it has been reported that surface coating of proteins via adsorption may affect the topography of the scaffold and thus cell fate. [16] For this reason, other methods of incorporating proteins and growth factors have been explored.

2.9.3 Chemical grafting of proteins

Soluble growth factors have been used to help regeneration after spinal chord injury using mini pump technology. [134] This method has shown the importance of a stable gradient for guidance; it has also shown a limitation where the soluble growth factors need to be constantly replenished. Protein grafting was therefore attempted *in vivo*. Since surface immobilized growth factors are biomimetic [135-139], it has been hypothesized that they can guide neurites similarly to soluble growth factors. [134] Kapur et al [134] have photochemically bound nerve growth factors (NGF) via photoreactive PAAm onto microporous poly(2-hydroxyethylmethacrylate) (PHEMA) gels. Results showed that 30 ± 7% of the pheochromocytoma (PC-12) cell population responded to bound NGF, similarly in comparison to cells cultured on collagen in the presence of 40 ng/ml soluble NGF (39 ± 12%). Zhu et al showed a method for the photochemical immobilization of gelatin and chitosan onto polyesters. [140] Results showed that fibroblast cells did not spread on the chitosan-PLGA or pure PLGA surface after 2h but spread on the gelatin-chitosan-PLGA surface.

2.9.4 Proteins for surface modification

2.9.4.1 Collagen

Collagen adsorption

Some natural materials such as collagen, fibronectin, and some peptides have been reported as scaffold modifiers. [134] Tjia have shown the benefits of adsorbed collagen onto a scaffold. [141] They have reported that, in the absence of any treatment, keratinocyte migration on PLGA substrates was poor. However, substrate conditioning with type I collagen can enhance the rate of migration significantly. Furthermore, the combined presence of substrate-adsorbed collagen in conjunction with a cell-derived protein (fibronectin) can activate keratinocyte migration onto PLGA substrates. [141] Additionally, He et al [142] have coated collagen on electrospun nanofibers and reported an enhanced spreading, attachment, and viability for human coronary artery endothelial cells.

Collagen grafting

Li et al have used electrospun nanofibers on which collagen had been immobilized for neural stem cell culture. [143] Collagen was grafted onto a copolymer of methyl methacrylate (MMA) and acrylic acid (AA) nanofiber surface via EDC/NHS activation. It was reported that the cell viability and cell proliferation were improved on the collagen-modified surfaces when compared to that on the unmodified surfaces. Ito et al [144] have immobilized collagen onto poly(methyl methacrylate) (PMMA) films, which has resulted in the flattening of cells during early cell adhesion but did not enhance cell growth significantly. [145]

2.9.4.2 Laminin

Laminin adsorption

Laminin plays an important role in cell migration, differentiation, and axonal growth. [146] It is one of the proteins present in the ECM and is continuously synthesized after nerve injury in the PNS. [147] Also, myelination in the PNS is not observed in the absence of laminin. [148, 149] Laminin adsorption onto scaffolds has been shown to promote neurite outgrowth through directional guidance. [150, 151]

Laminin grafting

Koh et al [152] have compared the addition of laminin to nanofibers using covalent binding, physical adsorption, and blended electrospinning. In general, they have shown that laminin modified nanofibers supported cell adhesion, cell viability and proliferation as well as neurite outgrowth when nerve growth factors were introduced. More laminin were found in the blended laminin-PLLA nanofibers than immobilized laminin and physically adsorbed laminin. This could be the reason why more neurite outgrowth of PC12 cells was observed on blended laminin-PLLA than on the other modified nanofibers. However, this technique was not efficient at achieving high cell viability. Laminin has a higher tendency to promote neurite outgrowth than to enhance the viability of nerve cells. [152, 153] Another drawback is that the approach undertaken in this study still required the use of nerve growth factors (NGF) in the medium. Some studies have shown that NGF plays a role in the maintenance of central neurons and in the control of laminin production. [153, 154]

2.9.4.3 EGF

EGF adsorption

EGF is known to be a functional target for the clinical treatment of damaged tissues. [155] As previously discussed, EGF plays an important role in cell proliferation, differentiation and migration, thus aiding in wound healing and tissue regeneration. Non-specific adsorption of the epidermal growth factor (EGF) onto a substrate has been shown to dramatically decrease its bioactivity. [156] This is most likely due to the inappropriate orientation of EGF or possible adsorption-induced conformational changes of EGF. [156] Some reports have also shown that physically adsorbed EGF on insoluble glass substrates showed no activity. [157] This could mean that receptor motility is important in the signaling for both the mitogenic and morphologic response or that the conformation of physically adsorbed EGF is unsuitable for stimulating the EGFR. [157] These results showed that the mere presence of EGF on the surface is insufficient to trigger a biological response. [157]

2.9.4.4 EGF grafting

When soluble EGF is used, it is difficult to control its local concentrations due to cellular uptake and diffusion. [158] For the cells to properly respond to growth factors, the time-span of their presence in the desired target tissues is important. [159] When a controlled release system is used, maintaining a signal at the cell surface is difficult due to endocytosis of growth factor-receptor complexes. [158] Therefore, new strategies are needed to overcome these obstacles. This is where the immobilization of growth factors comes into play; covalent immobilization of EGF onto a scaffold is non-endocytosible and non-diffusible, allowing cell surface events involving signal transduction to occur. Furthermore, immobilization can be used in order to spatially control EGF densities or concentrations.[158] To further support the use of tethered EGF, it has been reported that adsorbed EGF shows very low, if not non-existent biological activity. [157, 160] Furthermore, surface coating of proteins via adsorption may affect the topography of the scaffold and thus cell fate. [16] Grafting of proteins enable long-term delivery, allows proteins to remain stable and functional for several months, and have shown positive results in activating intracellular signaling pathways thus influencing cell fate. [161] Therefore, the purpose of this study is to optimize current neural tissue regeneration strategies by immobilizing EGF onto a PLLA scaffold.

2.10 Mechanical properties

Studies have shown that mechanical properties of the brain tissue affect neural stem cell behavior.[8, 162] Cells respond not only to biological and chemical properties but to the physical properties of the extracellular matrix, [163, 164] such as the stiffness of substrates. The stiffness of a substrate has an effect on cellular behavior in a cell-type specific manner.[165, 166] Mouse spinal cord neurons have less dendritic branches on stiffer bis-acrylamide cross-linked hydrogels (E ~ 1500 Pa) than on softer gels (E ~ 150 Pa) while glial cells do not grow on these gels at all.[166] The focal adhesions and motility of cells are affected by the substrate flexibility.[167] It is thus ideal for a substrate to have similar mechanical properties to its host tissue. Neural cells do not survive well in a biomaterial that is either too soft or too stiff. However, those that do survive at the lower stiffness tend towards a neuronal cell fate, whereas astrocytes develop more predominantly on stiffer materials. The young modulus of both white and grey matter of the human brain was reported to be 0.00015MPa-0.01MPa [168, 169]

2.11 Problem identification

Recent tissue engineering strategies exist that rely on the inclusion of growth factors (or other proteins) within appropriate structures made of biodegradable and biocompatible polymeric materials. However, current existing strategies have some limitations such as their use of scaffolds (i.e. PMMA) that are not biodegradable [144, 145] with collagen immobilization that did not enhance cell growth significantly. [144] Furthermore, current strategies using immobilized laminin did not enhance cell viability and still required the use of NGF in the medium (in addition to the immobilized laminin) to help promote cell proliferation and differentiation. [153] This study will optimize current existing tissue engineering strategies by immobilizing EGF (via aminolysis) that has been shown to promote cell proliferation, differentiation, etc onto the biodegradable, biocompatible electrospun PLLA that mimics the ECM.

2.12 General Objective

The general objective of this study is to establish the optimum topological features of nanofibrous mats for neuronal stem cells, to evaluate the effect of adding chemical functionalities via a surface modification strategy for the attachment of epidermal growth factors as well as study its impact on cell adhesion, proliferation, and viability.

2.12.1 Specific Objectives

In order to develop answers to the questions of the general objective, several specific objectives are defined:

The first specific objective is the development and amine functionalization of poly lactic acid (PLLA) nanofibers (with the desired characteristics such as average fiber diameter, fiber thickness, etc).

The second specific objective is the covalent immobilization of epidermal growth factors onto the modified structures in order to study the impact of the epidermal growth factor immobilization on biomaterial scaffolds by observing cell adhesion, proliferation and viability via cell culture assays.

CHAPITRE 3 FABRICATION AND SURFACE MODIFICATION OF NANOFIBERS

3.1 Presentation

Based on the literature review, electrospun PLLA biomaterials are the most ideal for our strategy. PLLA fibers with a selected diameter and with a high porosity were obtained. The specific topology (fibers diameter and alignment) were optimized (results summarized in the appendix) and used for the subsequent steps of the work. Surface modification of the electrospun PLLA via aminolysis and EGF grafting rendered the substrate more suitable for cell proliferation and spreading. These results were submitted in the Journal of Biomedical Materials Research - Part B and are presented below.

3.2 Article 1: Fabrication and Surface modification of poly L-lactic acid (PLLA) scaffolds for growth factor immobilization and neuronal stem cell delivery[1].

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[1] **Supporting Information** is available online from the Wiley Online Library or from the author.

ABSTRACT

Poly L-lactic acid (PLLA) is a polymer commonly used in neural tissue engineering. In this study, PLLA-based electrospun fibers destined to nervous system repair strategies were optimized by covalently grafting epidermal growth factor (EGF). Electrospun PLLA nanofibers were first functionalized with polyallylamine to introduce amine groups before EGF grafting via a bis-*N*-succinimidyl-pentaethylene glycol ester (PEG) linker. Engineered neural stem-like cells (NSLC) were then seeded onto the modified substrates and were shown to be viable up to 14 days, while proliferating and spreading.

KEYWORDS

Poly L-lactic acid, epidermal growth factor grafting, amine functionalization, cell proliferation, adhesion

3.2.1 Introduction

With 12,000 new cases occurring every year and over one million North Americans suffering from paralysis caused by spinal cord injury (SCI), SCI is a devastating condition for which no effective treatment exists. [170] Similarly, strokes, cancer, trauma and degenerative diseases affecting the brain (e.g. Parkinson's (PD) and Alzheimer's diseases (AD)) are also some of the many sources of damage of the central nervous system (CNS). They are costly to society and are the leading causes of death and injuries in North America. [1]

Intrinsic repair of the CNS is very limited, possibly due to some inhibitory factors present at the injury site associated with nearby glia, preventing functional regeneration. [4, 21] Of salient interest, recent progress within the field of stem cell therapy has provided researchers with promising repair strategies for CNS. The use of human neural stem/progenitor cells (NSPC) has attracted a lot of interest since these cells can be expanded easily, and since their neural lineage is inherently specified. They have given extremely promising results in animal models.[171-173]

Among the various strategies reported, the use of NSPC first requires their selective expansion, a process that involves the use of specific growth factors (i.e., the epidermal and fibroblast growth factors, EGF and FGF, respectively).[24, 174]

In many studies, the implantation of NSPC has also been accompanied with concomitant infusion of growth factors to promote cell survival, proliferation and differentiation. For example, mouse brain-derived NSPC differentiation into oligodendrocyte was promoted by the mixing of epidermal, fibroblast and platelet-derived growth factors (EGF, FGF and PDGF), ultimately resulting in improved locomotor function.[175, 176] Recently, the combination of these growth factors to a soluble dimeric version of the Nogo-66 receptor (to block the inhibitory effect of myelin-based inhibitors) was reported to increase survival of NSPC and augment axonal regeneration.[22] To retain growth factors (and other proteins) at the site of transplantation, the most recent experimental strategies rely on their inclusion within appropriate structures made of biodegradable and biocompatible polymeric materials. [21-23] This controls their spatiotemporal delivery to damaged regions of the CNS, while providing a scaffold to guide regeneration. For that purpose, electrospun poly(L-lactic acid) (PLLA) nanofibers have been studied for cell

differentiation and neurite outgrowth.[16, 88] Electrospun scaffolds are highly porous and their topography mimics the natural extracellular matrix (ECM), which make them good candidates for neural tissue engineering.[10, 89] Furthermore, PLLA is FDA-approved, biocompatible and biodegradable, making it a relevant polymer for these types of scaffolds.[16, 177] However, polyester surfaces are characterized by a suboptimal hydrophilicity for cell adhesion, which may limit their use unless functional groups or molecules promoting cell attachment are introduced. To improve cell adhesion and proliferation onto electrospun PLLA, previous studies reported the use of either laminin [152, 178] or other proteins, as they were shown to improve cell adhesion.[37, 130] The coating of adhesive molecules, however, is known to affect the surface topography of scaffolds.[16] To reduce this side effect, the incorporation of biomolecules onto the substrate via blends, electrospinning, co-electrospinning or subsequent covalent binding has been proposed.[108, 144, 152, 179-181] Surface modification such as the aminolysis technique has been widely studied in literature [124, 125]; the aminolysis reaction is based on the nucleophilic attack of an amine onto the ester bonds of a polymer chain via polymer-chain scission and is used for the addition of amine groups in order to covalently bind bioactive molecules. Studies have also reported the addition of amine groups using a small diamine, ethylenediamine (EtDA) [31, 126] as well as an aminated polymer, polyvinylamine (PVam).[127] The aminated PET films were followed with the functionalization of Succinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexanoate, LC-SPDP linker, an amino-reactive molecule. The LC-SPDP functionalization of substrates could be followed with the oriented immobilization of cysteine-tagged proteins.[128, 129]

In this study, PLLA nanofibers were fabricated by electrospinning to be used as neural tissue engineering scaffolds. The PLLA scaffolds were functionalized with amine groups via a novel aminolysis treatment using polyallylamine (PAAm). The prepared scaffolds were characterized by scanning electron microscopy (SEM) and amine moieties were quantified using the Orange II method.[182] Epidermal growth factor (EGF) was then tethered onto the PLLA electrospun scaffolds. EGF has been chosen because it is naturally present in the CNS and is known to promote the oligomerization and phosphorylation of cell surface EGF receptors (EGFRs) leading to cell proliferation.[24, 26-28, 174] The impact of our EGF-grafted PLLA nanofiber scaffolds upon neural stem like cells (NSLC) adhesion, survival and proliferation was

then evaluated, with the ultimate goal of developing an improved strategy for central nervous system repair.

3.2.2 Materials and methods

3.2.2.1 Materials

Commercial poly L-lactic acid (PLLA) pellets (Cat. # 4032D; 30% crystalline, L-content = 98%) were purchased from NatureWorks LLC (Blair, NE). Milli-Q quality water (18.2 MΩ cm; total organic compounds (TOC) = 4 ppb was produced from a Millipore Gradient A 10 purification system. Sodium hydroxide (NaOH, 99.3% purity), ethanol (EtOH, 99.9% purity), methanol (MeOH, 99.9% purity), dioxane (99+% purity), and hydrochloric acid (HCl, 37.7% v/v) were obtained from VWR International, Ltd. (Mont-Royal, QC). Orange II sodium salt (98% purity) and potassium chloride (KCl, 99+% purity) were obtained from Sigma-Aldrich Canada, Ltd. (Oakville, ON). Ethylenediamine (EtDA, 99+% purity) was obtained from Fisher Scientific, Ltd. (Nepean, ON). The linear aminated polymer polyvinylamine (PVAm) and polyallylamine (PAAm) were obtained from Polysciences, Inc. (Warrington, PA). Dulbecco's phosphate buffered saline (modified PBS, without calcium chloride and magnesium chloride), Tween 20, analytical grade dichloromethane (DCM) and trifluoroacetic acid (TFA) from Sigma-Aldrich (Oakville, ON) were used as received. Bis-*N*-succinimidyl-(pentaethylene glycol) ester (Bis(NHS)PEG_5) was purchased from Pierce Biotechnology (Rockford, IL). Streptavidin HRP enzyme (part # 890803), Substrate reagent pack (Cat. # DY999) containing Peroxide A (part # 895000) and Chromagen B (part # 895001) and the reagent diluent Bovine Serum Albumin (Cat. # DY995) were purchased from R&D systems (Minneapolis, MN, U.S.A). Ethanolamine (99+% purity) from Sigma-Aldrich (Oakville, ON) was used. Recombinant human epidermal growth factor (rhEGF, 97+% purity, Cat. # 236-EG) was from R&D systems (Minneapolis, MN, U.S.A).

3.2.2.2 Preparation of random PLLA nanofibers using electrospinning

The polymer solutions for the fabrication of the nanofibers were prepared by dissolving PLLA (2 g) into a mixture of DCM (7 mL) and TFA (6 mL) to yield a PLLA concentration of 15.4% w/v. The polymer solution was placed into a glass syringe (Cadence scientific, Staunton, VA; needle size 22 G) and the injection was controlled by a syringe pump (KDS100, KD Scientific Inc., Hollistin, MA) at a feeding rate of 0.5 mL h^{-1}. A rotating collector was used for

random nanofiber fabrication at a rotation speed of 175 rpm. A voltage of 23.75 kV was applied by a voltage regulated DC power supply (ES30P/0.2/230/M826, Gamma High Voltage Research, Ormond Beach, FL). The PLLA nanofibers were collected on non-stick aluminum foil at a distance of 10 cm from the needle.

3.2.2.3 Nanofiber amination and amine group quantification

Glassware was carefully cleansed by overnight immersion in a bath of KOH-saturated isopropyl alcohol, followed by intensive rinsing with Milli-Q water. The PLLA nanofibers were rinsed extensively in ethanol and dried in a laminar flow cabinet at room temperature (RT). Before immobilization of EGF on nanofibers, an aminolysis step was performed using three different procedures. After aminolysis, the PLLA nanofibers were extensively rinsed in ethanol and Milli-Q water and dried in a laminar flow cabinet at room temperature.

3.2.2.4 Polyallylamine (PAAm) functionalization

An alkaline solution of NaOH and KCl (1:1, 150 mM in Milli-Q water) as well as a solution of PAAm•HCl (85.5 g L^{-1} in Milli-Q water) were prepared. The aforementioned alkaline and PAAm solutions were then mixed with dioxane (67:26:7% v/v). Prior to dioxane addition, the pH was adjusted to pH 11.5 or 12.5 by adding few microliters of HCl (12.3 M). The solution was degassed under vacuum for 20 min while stirring. The PLLA fibers were then immersed in the solution and the reaction was carried out for 1 h at 50°C.

3.2.2.5 Polyvinylamine (PVam) functionalization

A solution of PVAm•HCl (120 g L^{-1} in Milli-Q water) was prepared. The PLLA fibers were immersed in a degassed mix of the alkaline solution previously described, the PVAm solution and dioxane (67:26:7% v/v) for 1 h at 50°C. The pH of the aqueous solution was set to pH 12.5 prior to dioxane addition.

3.2.2.6 Ethylenediamine (EtDA) functionalization

The PLLA fibers were immersed in a solution of EtDA/MeOH of varying concentrations (0.5, 1, 5, 20 and 30% v/v). The reaction was carried out at 50 °C for reaction time set to 10, 20, 30 and 40 min.

3.2.2.7 Amine group quantification

Following aminolysis, the density of amine groups was evaluated using a colorimetric assay (Orange II method).[182] Briefly, the substrates were immersed in 1.5 mL of dye solution (14 mg mL^{-1}) in acidic solution (Milli-Q water adjusted at pH 3 with 1 M HCl solution) for 30 min at 40°C. The samples were intensively rinsed 3 times using the acidic solution (pH 3) to remove unbound dye. The colored substrates were immersed in 1 mL of alkaline solution (Milli-Q water adjusted to pH 12 with a 1 M NaOH solution) once air-dried. The pH of the solution containing the desorbed dye was adjusted to pH 3 by adding 1% v/v of 12.3 M HCl. The absorbance of the solution was then measured at 484 nm.

3.2.2.8 Immobilization and quantification of epidermal growth factor (EGF) on nanofibers

EGF grafting on amine-functionalized nanofibers

Aminolysed nanofibers were reacted with a freshly prepared Bis-*N*-succinimidyl-(pentaethylene glycol) ester (Bis(NHS)PEG_5) linker, i.e. 2.5 mM Bis(NHS)PEG_5 in 10-mM phosphate buffer (PBS) containing 1% Dimethyl sulfoxide (DMSO) for 45 min at RT. The mats were intensively rinsed in PBS to remove any unbound linker. The PEG-covered nanofibers were then NHS-reactivated with a 50:50% v/v mixture of N-Hydroxysuccinimide (NHS) (0.1 M in Milli-Q water) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.4 M in Milli-Q water) for 15 min at RT. After rinsing with Milli-Q water, the mats were reacted with 100 nM EGF in PBS for 1 h at RT and rinsed with PBS. Unreacted NHS groups were blocked using 1 M ethanolamine (pH 8.5) followed by a rinsing step (PBS).

EGF density quantification via Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of covalently bound EGF was based on previously reported work.[183] Commercially available DuoSet ELISA kit was purchased from Cedarlane (Burlington, ON, Cat. # DY236). Circular mats (0.785 cm2) were cut and introduced in a 48-well plate. The mats were covered with 300 µL of bovine serum albumin (BSA) solution (1% BSA in PBS, PBS-BSA) for 15 min to prevent undesired non-specific interactions between the surface and the ELISA reagents. After rinsing with a wash solution containing 0.05% Tween 20 diluted in PBS (PBS-T),

300 µL of EGF detection antibody (50 ng mL-1 in PBS-BSA) was added to the wells followed by incubation for 1 h at RT. After a second wash with PBS-T, 300 µL of streptavidin-HRP (diluted 200 times with PBS-BSA) was added to the wells and the solution was incubated for 20 min at RT. Following a final wash with PBS-T, the reaction was revealed with 300 µL of substrate solution (50:50% v/v hydrogen peroxide/tetramethylbenzidine). After 30 min in the dark, the colorimetric reaction was stopped by adding 150 µL of 2 N H2SO4. The solutions were transferred to a 96-well plate and the optical density at 450 nm was measured using an ELISA plate reader (Victor 3V Multilabel Counter, PerkinElmer Inc.).

3.2.2.9 PLLA scaffolds characterization

Structural morphology of PLLA scaffolds

The topography and morphology of electrospun PLLA scaffolds were characterized using scanning electron microscopy (SEM) (JSM 5600, JEOL, Japan) with an accelerating voltage of 10 kV. The diameters of the fibers were measured from the SEM photographs using an image analysis software (ImageJ, National Institutes of Health, USA). Prior to characterization, the scaffolds were coated with gold (twice for 15 s) using a sputter coater (JEOL JFC-1200 fine coater, Japan).

Mechanical characterization of PLLA scaffolds

The PLLA fibers were tested according to ASTM D3822 using a 250 N load cell at a crosshead speed of 5 mm min^{-1} and a pressure of 20 psi. Small pneumatic clamps were used to grip the individual mats. The sample gauge length used was 1 mm. The Automatic Young's modulus, tensile stress at yield, tensile stress and tensile strain at maximum load were calculated using Bluehill software (Instron, Norwood, U.S). Prior to analysis, the mat dimensions were recorded and the mat thicknesses were measured using a micrometer. The analyses were performed on three to five samples per experimental condition.

Porosity calculations

Mat dimensions were measured along each sample and the average values were used for calculations. Weight was recorded for each sample. The porosity and the apparent density were evaluated according to:

$$\text{Porosity (\%)} = \left(1 - \frac{\text{apparent density (g/cm}^3\text{)}}{\text{bulk density (g/cm}^3\text{)}}\right) \times 100 \quad (1)$$

$$\text{Apparent density } \left(\frac{\text{g}}{\text{cm}^3}\right) = \frac{\text{mat mass (g)}}{\text{mat thickness (cm)} \times \text{mat area (cm}^2\text{)}} \quad (2)$$

where the bulk density of pure PLLA is 1.25 g/cm3. [184]

Cell culture

Engineered Neural Stem-Like Cells (NSLC) provided by NewWorld Laboratories Inc. (Laval, QC) were cultured in a medium prepared by mixing neural progenitor basal medium (NPBM, Cedarlane, Cat. # 3210) with neural progenitor supplement singlequot kit (Cedarlane, Cat. # 4242) and neural progenitor maintenance medium (NPMM) singlequot kit. The NPMM kit contained recombinant human epidermal growth factor rhEGF and human fibroblast growth factor rhFGF (Cedarlane, Cat. # 4241); 0.1 ml of rhEGF and rhFGF were used for cell seeding medium. The cell culture medium was supplied as a sterile liquid (containing dextrose); rhEGF and rhFGF were supplied in a sterile buffered BSA saline solution. Laminin (Sigma-Aldrich Canada Ltd, Cat. # L2020-1MG) was also added as supplement (5 mg L-1) for cell culture. The cells were seeded in flasks (Corning CellBIND, Cat. # 3337) and cultivated in a standard humidified incubator at 37 °C with 5% CO2 in complete maintenance medium. The cells were dissociated with 0.01% trypsin/10 mM EDTA (Lonza, Cat. # 5012) centrifuged and re-suspended in medium prior to cell seeding on nanofibers or subculturing. Cells were always used between passage 10 and 14.

Prior to cell seeding, the nanofiber mats were cut into 1-cm diameter disks with a round hole-arch punch, dipped in ethanol (70% in Milli-Q water) and exposed to UV for 30 min for sterilization. The samples were then placed into polystyrene tissue culture 48-well plates (VWR, Cat. # 82050-888) under a sterile laminar hood. Autoclaved glass cylinders (8 mm inner diameter) were placed onto the samples. When mentioned, samples were coated with laminin (20 μg mL-1, Cat. # L2020- 1MG) in PBS for 1 h and then gently washed three times with PBS. Cell suspension (1 mL) containing 1.6×104 cells was then added to each well to inoculate the cells on nanofibers. The medium (2 mL) was replenished every two days. The cells were incubated within either growth factor-free medium (referred to as ‘basal medium’), basal medium + soluble FGF

(referred to ‘EGF-free medium’), or basal medium + soluble FGF + soluble EGF (referred to ‘full medium’). Note that the pH of the culture medium was an uncontrolled variable.

Proliferation assays

Cell counts were performed at different time points, i.e. 2, 6 and 10 days to assess adhesion and growth. The cell-seeded samples were first washed with PBS three times and fixed in a formaldehyde solution (3.75% w/v in PBS) for 30 min in an incubator. After three additional PBS washes, the cells on the nanofibers were stained with SYTOX Green Nucleic Acid Stain (1 μM in PBS; 300 μL per well; Invitrogen, Burlington, ON, Cat. # S7020) for 15 min in the dark then washed again with PBS three times. The stained samples were visualized under a fluorescent microscope (Axio Observer Z1, Zeiss Inc.) and images of complete samples were captured using the MosaiX acquisition from the AxioVision imaging software (AxioVision, Zeiss Inc.). Cells were counted on SYTOX stained images with the software ImageJ, for four different samples per group. Note that fluorescence staining was required for these studies due to the opacity of the fibers, which prevented normal light observation.

Phenotype analysis

To assess cell phenotype, samples were rinsed three times with 1 mL PBS and fixed in 1 mL formaldehyde (3.7%) during 30 min. Fixed cells were permeabilized using Triton (0.5%, 20 min). To avoid non-specific adsorption of primary antibodies on PLLA nanofibers, a blocking step was performed using normal donkey serum (NDS) in PBS (Sigma-Aldrich, Cat. # D9663-10ML) for 1h at 5% and 2h at 10% prior to nestin and TUJ-1 immunostaining, respectively. Samples were immunostained at 4°C overnight with different primary antibodies: goat anti-nestin (Santa Cruz Biotech, Dallas, TX, Cat. # sc-21248) or mouse anti-TUJ-1 (Neuromics, Edina, MN, Cat. # MO15013). Primary antibodies were dissolved in 0.1% Triton, 1% NDS in PBS, to reach a concentration of 3 μg mL-1. After 3 additional washes, samples were incubated with secondary antibodies: donkey anti-goat or goat anti-mouse (Santa Cruz Biotech, Dallas, TX, Cat. # sc-362265 and sc-362257, respectively) in 1% NDS for nestin stained samples and in 8% NDS for TUJ-1 stained samples. After a 1-h incubation, cells were washed in 0.1% Tween/ PBS 3 times for 5 min each. At the end, SYTOX green was also added during 15 min at 1 μM to stain nuclei. The stained samples were visualized under fluorescent microscope (Axio Observer Z1, Zeiss

Inc.) and representative images were captured using the imaging software (AxioVision, Zeiss Inc.).

Statistical analysis

All data are expressed as means ± standard deviations of experiments carried out at least in triplicate. Statistical analysis was performed with a two-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (student t-tests and Bonferroni). Data sets were considered significant for p values below 0.05.

3.2.3 Results

3.2.3.1 Preparation and properties of random PLLA nanofibers

Many factors influence the diameter and morphology of electrospun nanofibers such as polymer concentration, molecular weight of polymer, the applied voltage, flow rate, humidity and temperature of the surrounding environment. Some of these conditions were explored to generate the nanofibrous mats used in this study (manuscript in preparation). Electrospinning of PLA nanofibers mats according to the condition described in the materials and methods section allowed for the creation of randomly oriented 3D scaffolds with interconnected pores (see Scanning Electron Microscope (SEM) images of pristine mats in Figure 3-2A). The average fiber diameter of the nanofibers was determined to be 408 ± 77nm with a porosity of 86.8 ± 1.2 %.

3.2.3.2 Aminolysis of PLLA nanofibers

Amine functionalization of the electrospun PLLA nanofibers was performed using two aminated polymers, polyallylamine (PAAm, 15 kDa) and polyvinylamine (PVAm, 25 kDa) as well as a short diamine molecule, i.e., ethylenediamine (EtDA). The chemical reaction involves a nucleophilic acyl substitution where the carbonyl group of PLLA could accept the electron pair from the nucleophilic amine functionalities. As a result, covalent amide bonds were formed between the PLLA and the amine-containing (macro)molecules, as presented in Figure 3-1.

Quantification of surface-exposed amine groups was performed using the Orange II dye (Table 1), as previously reported.[182] Amine groups were successfully added to the PLLA fibers using PAAm (Table 1, conditions B-F). A significant increase in amine density was indeed observed when compared to the control samples (condition H: pristine samples immersed in

polymer-free solution mixture, i.e. water and dioxane only) for which the surface concentration of amine groups was almost zero (0.3 ± 0.1 μmol g-^{1}). PAAm-based aminolysis was performed on the PLLA samples for 1, 3, and 20 h at 60 °C and pH 12.5 (condition B, C and D, respectively; Table 1 and Figure 3-2). Although the average fiber diameter (AFD) remained intact throughout at 448 ± 78 nm (as previously reported for poly(caprolactone) electrospun nanofibers after aminolysis [185]), the nanofibers had noticeably degraded after 20 h of reaction time as shown in Figure 3-2D. It should be noted that a complete dissolution of the PLLA nanofibers was observed for a reaction time of 24 h (data not shown). For reaction time ranging from 1 to 3 h, no significant change in amine group density was noticed, i.e. 109.8 ± 23.7 and 120.3 ± 26.3 μmol g^{-1} (p = 0.54). PAAm-based aminolysis was also performed at a temperature lower than the *Tg* of the PLLA (ca. 57 °C).[186] Decreasing the reaction temperature from 60 to 50 °C (conditions B and F, respectively) had no influence on the surface amine group quantity (p = 0.20) as well as the AFD (p = 0.20). However, when the pH of the reaction was adjusted to 11.5 (condition E), a significant decrease in amine group density was observed compared to nanofibers reacting at pH 12.5 (condition F), i.e. 88.6 ± 22.7 and 133.2 ± 16.3 μmol g^{-1} (p < 0.05), respectively. Of interest, the density of exposed amine groups on PLLA nanofibers via a polymer-based aminolysis appeared to be mainly guided by the pH of the reaction mixture, as previously observed for PET films [182] and PET fibers.[187] No significant difference in weight was observed before and after aminolysis (p = 0.69 and p = 0.34) for PAAm and PVAm respectively at 1 h (data not shown).

In conclusion, the optimal reaction condition for PAAm-based aminolysis providing the highest amino group density (133.2 ± 16.3 μmol g^{-1}) with a minimal impact on AFD (p = 0.1) and porosity (+ 2.5 %) compared to the pristine mat (condition A) was the condition F, i.e. reaction performed at 50 °C and pH 12.5 for 1 h. Furthermore, mechanical testing of these mats revealed that, although the Young's modulus of the pristine mats decreased after they had been placed in wet conditions (PBS or water and dioxane only), no significant difference was observed between the samples after exposure in wet conditions and after aminolysis (data not shown). Therefore, the decrease in Young's modulus was probably due to hydration as water is known to act as a plasticizer.[188, 189] This decrease in young modulus brings the fiber closer to the young modulus in the CNS. [168, 169] A more complete study on the evolution of mat morphology with process and material parameters will be published elsewhere (manuscript in preparation).

For the sake of comparison, aminolysis was also performed using PVAm and EtDA. Under the optimized conditions for PAAm, the PVAm (condition G) was found to be less efficient at introducing amino groups to the PLLA nanofibers, giving rise to a density of amino group of 76.68 ± 9.84 μmol g^{-1} without affecting the AFD (p = 0.9) compared to pristine mat (condition A). In contrast, when the small EtDA molecule was reacted with PLLA mats (conditions J-L), the resulting amino group density remained low (ca. 0.4 ± 0.1 μmol g^{-1}) for reaction time varying from 10 to 30 min and was on the same order of magnitude as the negative control (condition M: pristine samples immersed in MeOH only, 0.2 ± 0.1 μmol g^{-1}). It should be noted that a large degradation of the PLLA mats was observed after EtDA-based aminolysis starting from 10 min, as revealed by very poor mechanical properties (data not shown). This observation was in agreement with what had been previously observed for PET fibers. [187]

3.2.3.3 EGF grafting on aminated PLLA mats

NHS ester functionalized homobifunctional PEG linker (Bis(NHS)PEG_5) was used to chemically graft human EGF on PAAm-aminolysed mats. EGF grafting was evaluated via direct enzyme-linked immunosorbent assay (ELISA, Figure 3-3), as previously proposed.[183] NHS-reactive functionality of the PEG linker was first reacted with the surface-exposed amine groups to create stable amide bonds. Remaining NHS groups were then used to couple human EGF via its available amino groups (displayed on lysine residues or at the N-terminus of the proteins). After EGF grafting, an ethanolamine-mediated NHS deactivation was applied to prevent undesired attachment of other molecules containing amino groups such as the secondary antibodies used in the ELISA method. 100 nM of EGF dissolved in 10 mM phosphate buffer (pH 7.4) was found to be the optimal condition to obtain a dense grafted layer on PEG-functionalized mat (data not shown), in agreement with previous work.[156] The resulting EGF layer was characterized by an ELISA optical density (O.D.) of 0.774 ± 0.062. As negative control, human EGF was incubated on ethanolamine-deactivated PEG (O.D. value of 0.238 ± 0.027). Minor variations in O.D. values were noticed between the deactivated PEG layers with or without exposure to EGF (-0.023). Furthermore, another negative control was performed with aminated mats only. An even lower value (p = 0.033 ± 0.0009) was observed compared to the ethanolamine-deactivated PEG layers. This was probably due to the high density of exposed amine groups on the PLLA surface being protein-repellent.[80, 190-193] Altogether, these results

indicated that EGF was mainly covalently attached thanks to the linker layer and that EGF non-specific adsorption was negligible.

3.2.3.4 Cell proliferation assays

To evaluate the influence of EGF grafting on NSLC proliferation, cells were seeded and cultivated on different treated PLLA scaffolds for ten days, while changing the media every two days as mentioned in the material and methods section. EGF-grafted PLLA mats were compared with their negative controls, namely pristine and aminated mats. To differentiate the bioactivity of grafted EGF from that of soluble growth factors present in the medium supplements (i.e., soluble EGF and FGF), different media formulations were used: growth factor-free medium (referred to as 'basal'), EGF-free medium (with soluble FGF) and full medium (with both soluble FGF and EGF). As positive control, NSLC proliferation was also characterized on laminin-coated mats in EGF-free medium.

When grown in basal medium, the number of NSLC was significantly higher ($p < 0.05$) on EGF-grafted mats than on aminated scaffolds or pristine scaffolds for all three time points (i.e., day 2, 6 and 10, see Figure 3-4). These results indicated that randomly grafted EGF remained at least partly bioactive, in spite of already documented negative impacts of the random amine coupling strategy [156] and still showed its well-known effect on proliferation.[27, 174] Similarly, when grown in EGF-free medium, NSLC proliferation was also significantly higher ($p < 0.05$) on EGF-grafted mats than on aminated scaffolds (Figure 3-4).

Interestingly, NSLC were able to proliferate similarly on EGF-grafted mats in basal medium as well as in EGF-free medium, i.e., up to 18447 ± 3480 and 16546 ± 3065 cells at day 10. Note that NSLC remained very few on aminated mats even at day 10, regardless of the presence of growth factors. No significant differences were observed between the EGF-grafted mats in EGF-free medium, basal medium and the positive control (laminin-coated mats in EGF-free medium), $p = 0.78$ and $p = 0.34$ respectively.

Representative fluorescent images taken at day 10 confirmed the high cell density observed on EGF-grafted mats, regardless of the medium (Figure 3-5, E to G). An abundance of cell clusters was observed on pristine and aminated mats in full media (Figure 3-5B and 5D), suggesting that untreated PLLA scaffolds were not optimal to grow NSLC. In stark contrast, cell

distribution appeared quite homogeneous on EGF-treated mats in basal media and EG-free medium, although a few small clusters were noticeable when cultured in full media (Figure 3-5G, arrows). Furthermore, even the positive control, i.e., laminin-coated mat, appeared to have cells less spread out compared to EGF-grafted mats.

Immunostaining was performed on NSLC grown on EGF-grafted scaffolds as well as on pristine scaffolds as control, in medium supplemented with FGF only (Figure 3-6). β-III-tubulin staining (TUJ-1) showed some neurite outgrowth, which indicated that the cells remained viable for up to 14 days of culture on both EGF-grafted and pristine mats, although large clusters could be observed on pristine mats. NSLC were positive to nestin staining. The small neurite extensions visible for both experimental conditions as well as the lack of large cellular network indicated that the cells remained pluripotent.

3.2.4 Discussion

PLLA nanofiber mats were produced by electrospinning, as biodegradable and highly porous (86.8 ± 1.2 %) scaffolds suitable for nerve regeneration. To improve NSLC growth onto these scaffolds, surface functionalization (amination) was performed. Our strategy was to bring reactive moieties onto PLLA electrospun mats to subsequently graft relevant cell-signaling molecules, thereby tailoring our PLLA scaffold. To control the identity and quantity of added functional groups, wet chemistry aminolysis was used. This technique allows for the addition of amine functional groups while limiting the degradation of PLLA. We have already reported a non-damaging method for amination of polyethylene terephthalate (PET) materials, based on the use of polyvinylamine (PVAm), which could be applied to create reactive amino groups at the surface of PLLA nanofibers.[187] Due to the degradable nature of PLLA, it can be quite difficult to preserve its topography and bulk integrity while functionalizing it, even via wet chemistry aminolysis. The aforementioned chemical treatment was therefore adapted to our highly degradable polyester by decreasing the initial reaction time and temperature (i.e. 24 h at 70°C) to 1 h at 50°C as well as degassing the solution prior to experiment, illustrating the differences in degradability of PET and PLLA. As previously reported, the pH was found to be a critical parameter as basic conditions are needed for the aminolysis to occur, via the unprotonated primary amines of the PVAm.[187] In our study, polyallylamine (PAAm) was used instead of PVAm because of their strong similarities (one additional carbon on the side chains of PAAm)

and the lower cost of PAAm. Compared to the commonly used ethylenediamine (EtDA) the polymer-based aminolysis allowed us to mostly preserve the physical properties of the electrospun mats, i.e. porosity and fiber diameter, without any noticeable change on SEM images.

Epidermal growth factor (EGF), that is known to promote cell proliferation, was then grafted onto the available amino groups of PAAm-functionalized PLLA mats. A PEG molecule was used as both spacer and chemical linker between PAAm and EGF amino groups. This grafting strategy proved to be both efficient and specific as negligible non-specific adsorption of EGF was observed.

To evaluate the bioactivity of our EGF-grafted mats, an extensive proliferation study was carried out on various modified PLLA mats in various media. When soluble growth factors were present in the medium, NSLC were able to proliferate on pristine PLLA mats, however, they tended to form cell clusters (Figure 3-5B and supplementary data Figure S1), an indication of their low affinity for the hydrophobic substrate. Their high cell proliferation rate and density were most likely due to the many cell clusters that had started to form at day 6 (Supplementary data Figure S1-C).[194-197] EGF-immobilization on PLLA highly supported cell proliferation compared to pristine PLLA mats. Our functionalization of PLLA mats with EGF not only provided the cells with a substrate harboring proliferative cues but may have also modified the surface properties of the PLLA mat to make it more suitable for cell adhesion and growth without greatly affecting its surface topography. As the first step towards EGF grafting, PLLA mats were modified with PAAm aminated polymer, thereby switching surface properties from hydrophobic to hydrophilic. However, aminated PLLA scaffolds showed very low NLSC adhesion and proliferation, with a slower growth than that observed on pristine PLLA mats (Figure 3-4). Such a behavior has been previously reported on surfaces coated with cationic polymers [190], suggesting that PAAm coating most probably provided the mats with inadequate surface properties such as a high positive charge and a very high hydrophilicity. Indeed, surfaces with moderate wettability (neither too hydrophobic nor too hydrophilic) are desirable for good cell adhesion and positive cell response. [80, 181, 190-192] Coatings that were too hydrophilic were shown to prevent proliferation as well as adhesion of both proteins and cells, such is the case for dense PEG coatings.[191, 198, 199] That hypothesis is supported by our data for the first time point of our proliferation study (Figure 3-4, day 2), which was mostly representative of adhesion, considering that the NSLC have a doubling time of 37 hours. On aminated mats, NSLC adhesion

levels were indeed quite low, regardless of the presence of growth factors in the media, that is in basal medium, EGF-free medium and full medium (6340 ± 1403, 5469 ± 1068 and 4721 ± 400 cells, respectively). Cell adhesion being mostly driven by protein adsorption, our results suggest that PAAm-modified mats were indeed protein-repellent, unlike pristine mats onto which cells adhered better in full medium (9718 ± 264 cells) than in basal medium (3491 ± 1259 cells). Another report stated that PAAm may not prevent cell growth per se but could hinder cell spreading and consequently proliferation.[193]

The amino groups available on the PAAm-coated mats were used to graft EGF via a PEG linker. That full functionalization proved to be beneficial right from cell adhesion, be it in basal medium, EGF-free media or full media (11327 ± 1233, 9696 ± 1436 and 9187 ± 1037 cells, respectively, at day 2, see Figure 3-4). That additional layer of molecules most probably hid part of the PAAm hydrophilic background, thus promoting cell adhesion in a similar fashion to adsorbed proteins, in addition to providing the cells with a pro-proliferative cue, i.e., grafted EGF. The bioactivity of grafted EGF per se was also demonstrated since NSLC were able to proliferate on EGF-modified scaffolds at rates similar to our positive control (Figure 3-4), and remained viable up to day 14 by maintaining multipotent NSC in a proliferative state (as evidenced by the nestin expression) (Figure 3-6). Of interest, grafted EGF promoted NSLC growth in the absence of soluble growth factors as well as in synergy with soluble FGF. This shows that immobilization of EGF improved the poor ability of PLLA to support cell adhesion and proliferation. The increased number of cells results from the accelerated cell growth and can also be attributed to the high mitogenic activity related to the presence of EGF. Therefore, culturing NLSC on EGF-grafted nanofibers could be used to maintain the self-renewal and stemness of these cells. It has been reported that tethered EGF enhance cell proliferation when compared to its soluble version, more likely because this mode of presentation increases EGF density at the cell surface [144], which in turn may prolong receptor phosphorylation and signaling pathway activation.[30]

Of salient interest, when the medium was supplemented with soluble EGF in combination to grafted EGF, cells formed clusters at day 10 (Figure 3-5G). These clusters were also observed on aminated mats in full medium at days 6 and 10 (Supplementary data Figure S1- B & E) as well as for pristine mats in full medium (Supplementary data Figure S1- C & F). Previous studies have shown that A431 cell clusters could form as concentration of EGF in medium is increased.

[200] Therefore, although our EGF-grafted scaffolds were able to promote significant NLSC proliferation, a grafted-EGF density-dependent study should be carried out to further optimize our strategy.

Randomly oriented covalent immobilization of EGF through its reactive amine groups present at the N-terminus or on its lysine chain are the most common method used [30] as was performed in this study. This method, however, may not be the most optimal strategy as it can negatively impact protein conformation, relative molecular orientation, and spatial homogeneity, leading to diminished bioactivity.[30, 156, 157, 201, 202] Therefore, for improved bioactivity, EGF may be tethered to our PLLA scaffold in an oriented fashion, e.g. as described elsewhere. [24, 156, 203, 204] Furthermore, the progenitor cells may be promoted to differentiate to specific and appropriate lineages. Binan *et al* have reported the differentiation of neural stem cells into motor neurons using electrospun PLLA/gelatin scaffold.[205] In their approach, NSLC were seeded onto scaffolds releasing, retinoic acid and purmorphamine. These scaffolds were shown to enhance the differentiation of NSLC into motor neuronal lineage as well as promote neurite outgrowth. The combination of both approaches onto a structure releasing retinoic acid and purmorphamine while displaying tethered EGF may thus be promising.

3.2.5 Conclusion

In this study, we report the successful surface functionalization of Poly L-lactic acid (PLLA) electrospun nanofibers with amine groups while maintaining the overall mechanical and structural properties of the scaffold without any changes in average fiber diameter (AFD). Further scaffold modifications allowed to covalently immobilize EGF onto PLLA fibers in a two-step process: surface aminolysis followed by conjugation of EGF via a PEG linker. Cell culture studies clearly demonstrated that EGF enhanced cell viability by maintaining multipotent cells in a proliferative state, which was supported by fluorescence microscopic images. With this information, EGF immobilization on nanofibers could be a promising avenue to design scaffolds for neural stem cell-based regenerative medicine applications.

3.2.6 Acknowledgement

This work was supported by a Research and Development Collaborative grant from the National Sciences and Engineering Research Council of Canada (NSERC) and New World Laboratories Inc., as well as by the Canada Research Chair on Protein-enhanced Biomaterials (G.D.C.). Authors would also like to thank Loïc Binan for help during this work.

3.2.7 References

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3.2.8 Figures

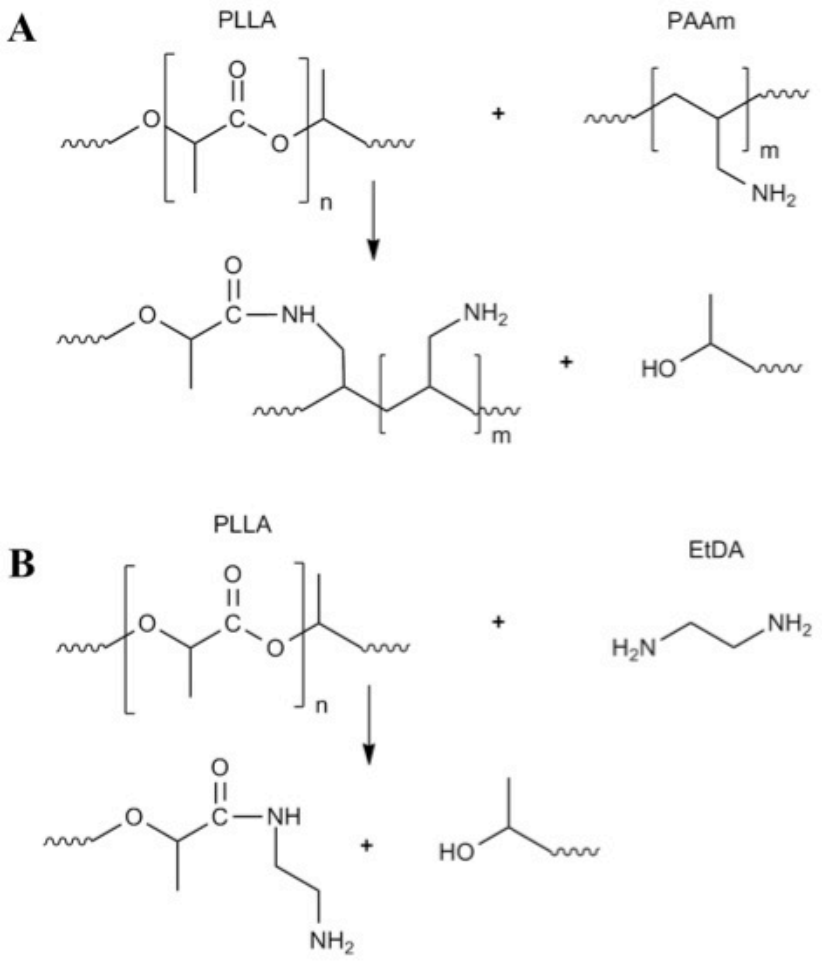


Figure 3-1: PLLA aminolysis reaction involving (A) polyallylamine (PAAm) and (B) ethylenediamine (EtDA).

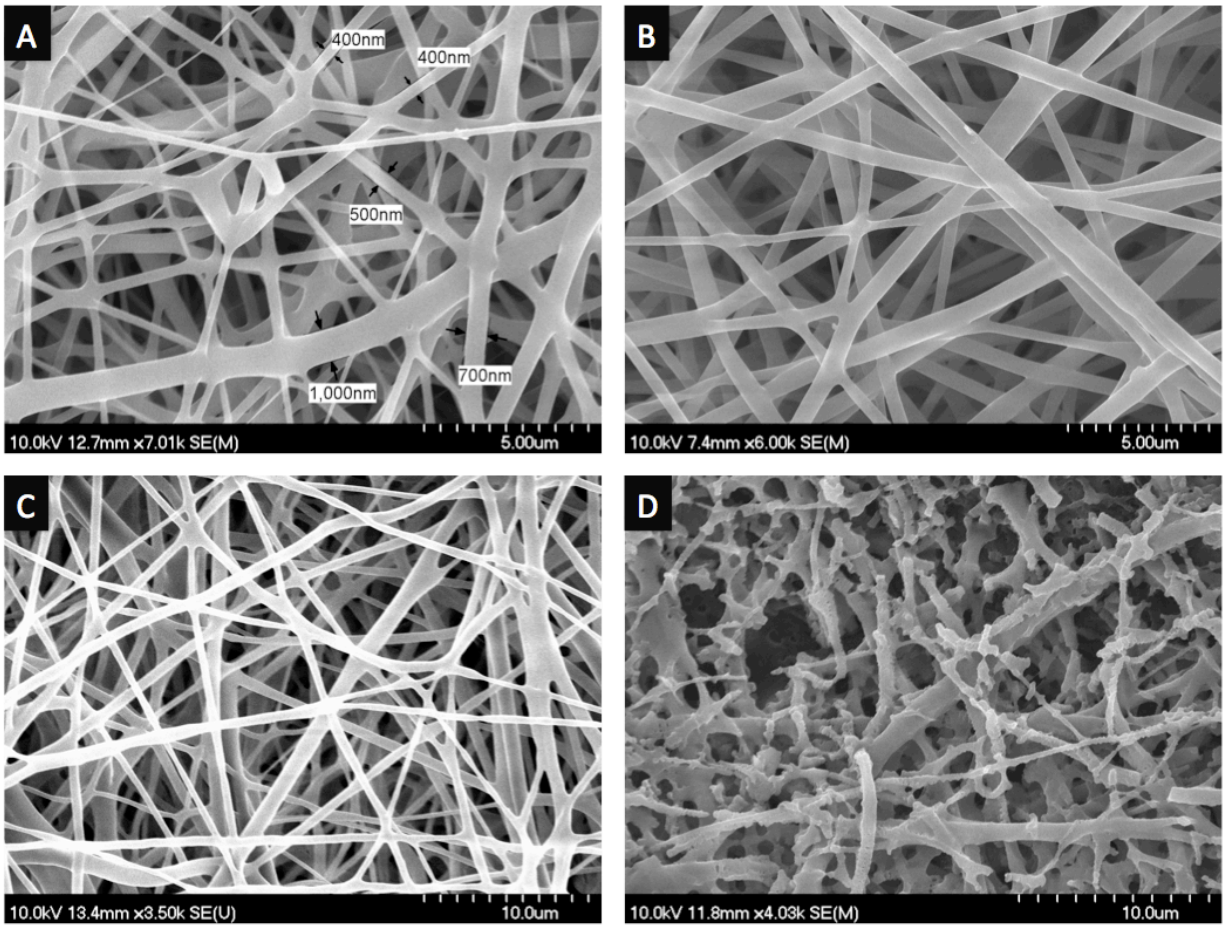


Figure 3-2: SEM imaging of (A) pristine and (B-D) PAAm-treated PLLA nanofibers. PAAm grafting was carried out at pH 12.5 and 60 deg. C for (B) 1 h, (C) 3 h, and (D) 20 h.

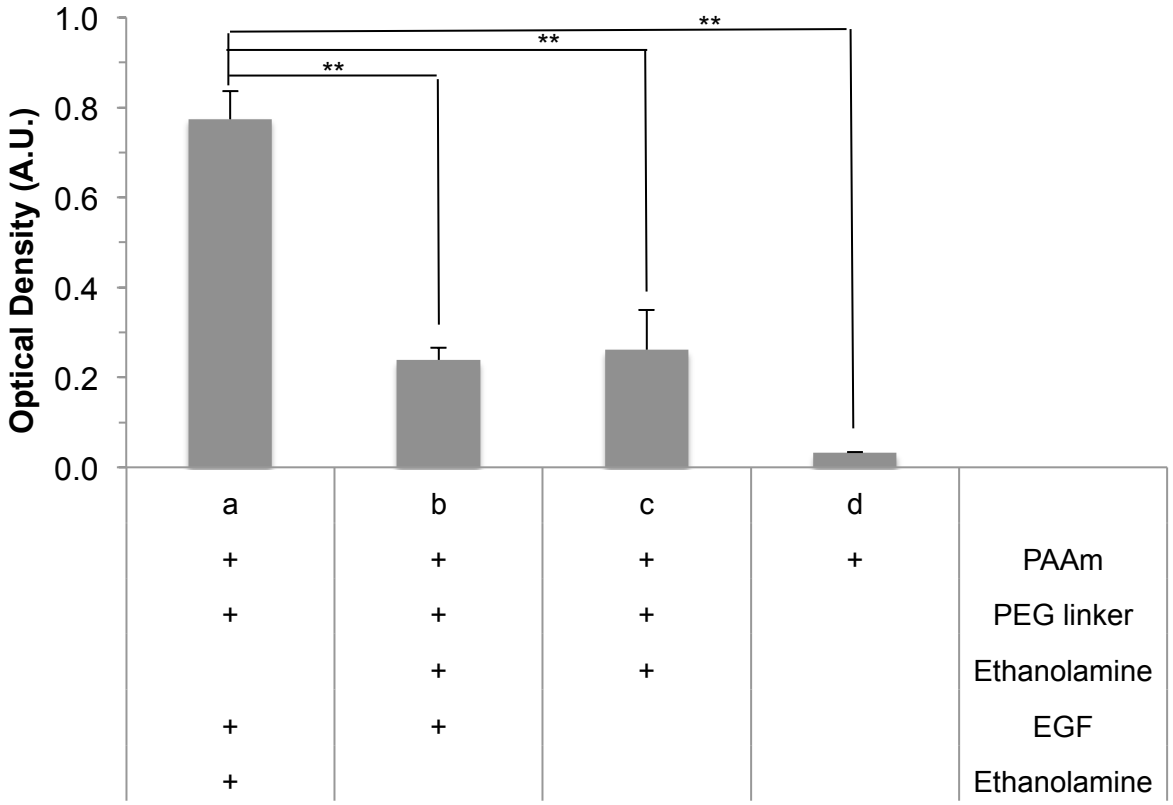


Figure 3-3: Characterization of the EGF grafting on PLLA nanofibers by direct ELISA. Optical densities (O.D.) corresponding to PAAm-covered mats treated with (a) PEG linker, EGF and ethanolamine (for deactivation of unreacted PEG). Mats covered with PEG linkers that were deactivated before EGF incubation (c) or without any EGF incubation (d) were used as negative controls. As reference, the O.D. value obtained on (d) unmodified PAAm-covered mats is presented. Statistical differences are noted ** ($p < 0.01$). Experiments were performed using four samples ($n = 4$).

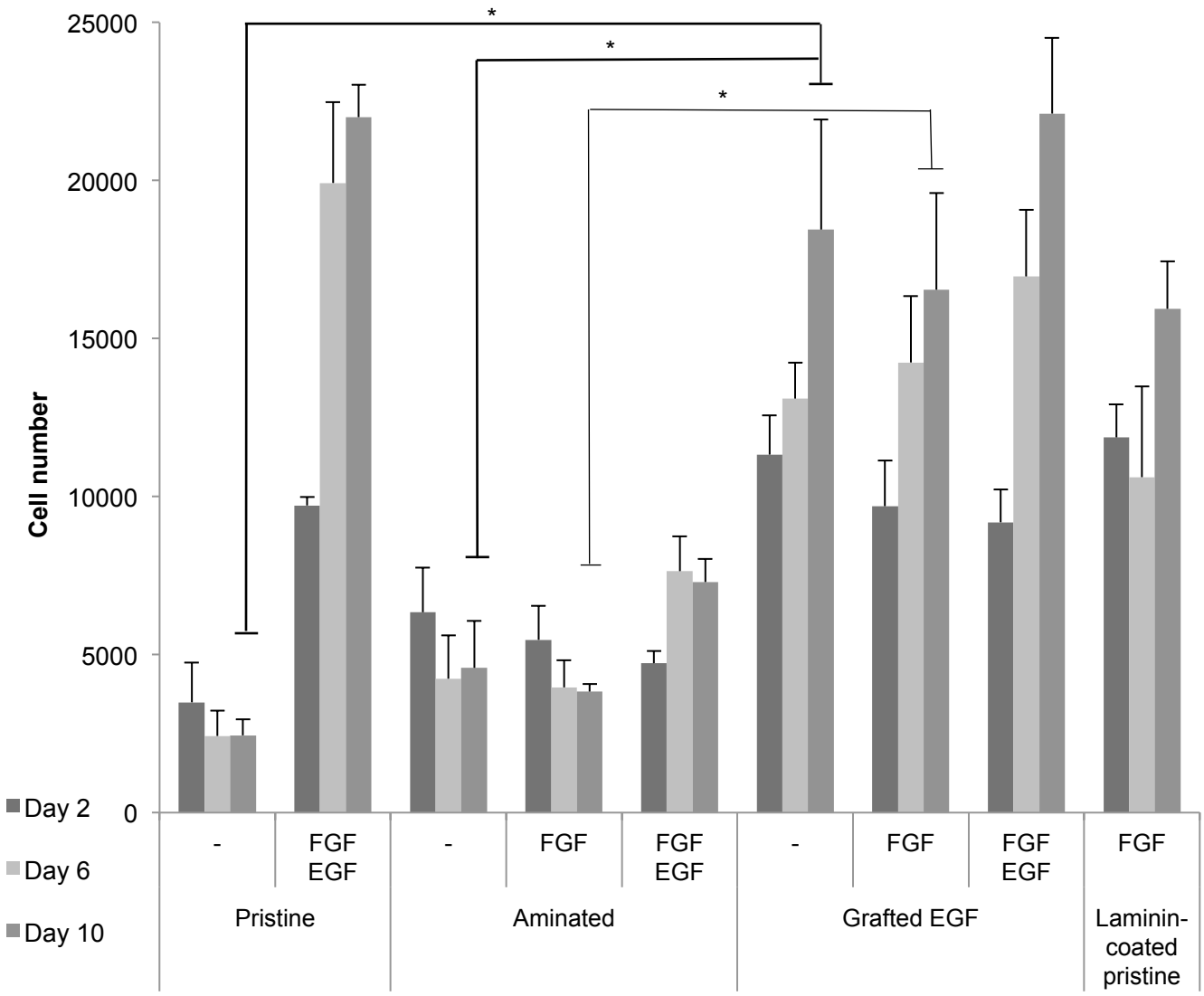


Figure 3-4: NSLC proliferation on pristine, PAAm-grafted, EGF-grafted and laminin-coated PLLA mats. Cells were exposed to basal medium (denoted '-') or basal medium supplemented with FGF only or a mix of FGF and EGF. Statistical differences noted * correspond to $p < 0.05$ ($n = 3$).

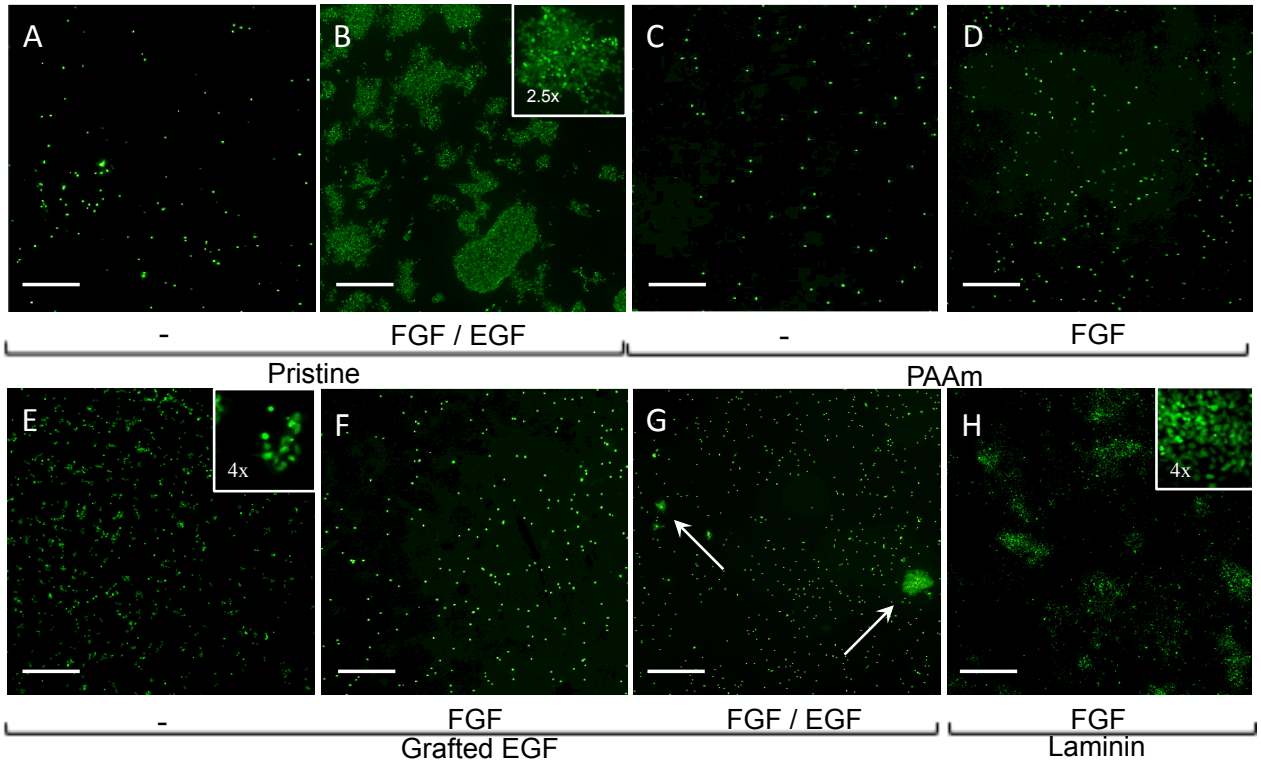


Figure 3-5: Fluorescence microscopy images of NSLC after 10 days of cell culture onto various surfaces. Scale bars correspond to 400 µm.

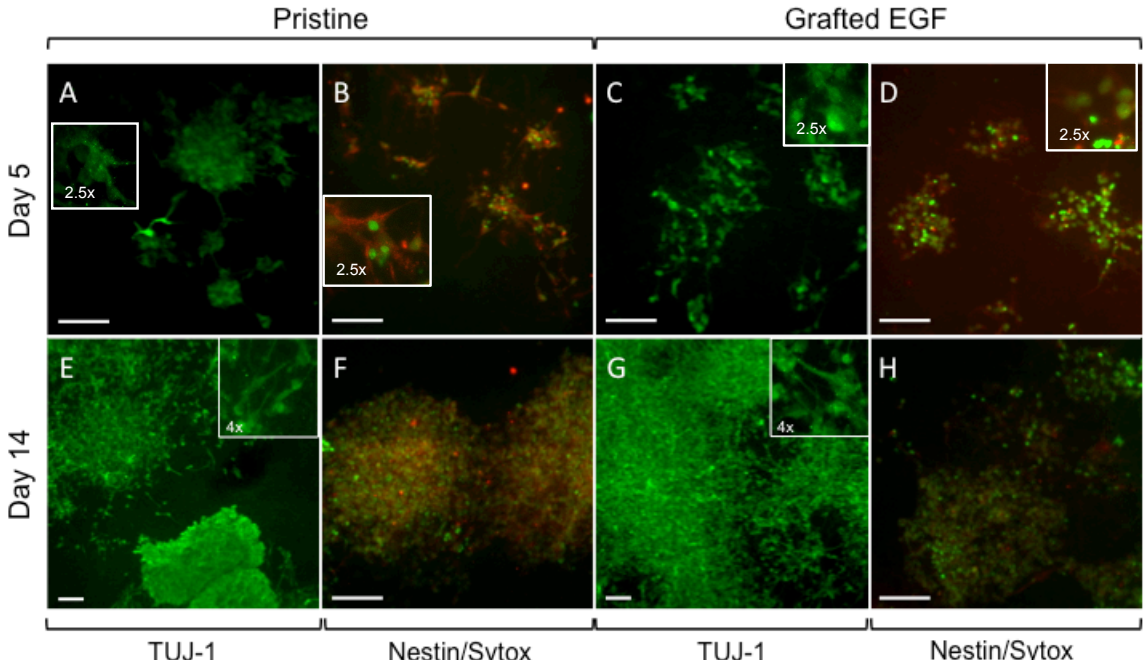


Figure 3-6: Fluorescence microscopy images of NSLC at days 5 (A-D) and day 14 (E-H) in culture on nanofibers with grafted EGF or pristine nanofibers. Scale bars correspond to 100 μm.

3.2.9 Table

Table 1. Properties of pristine and aminolysed PLLA nanofibers.

	Treatment	pH	Reaction time	Reaction temperature (°C)	Average Fiber Diameter (nm)	Porosity	Amine group density (μmol g^{-1})
A	Pristine				408 ± 77	86.8 ± 1.2	
B	PAAm	12.5	1 h	60	407 ± 62		109.8 ± 23.7
C		12.5	3 h	60	474 ± 69		120.3 ± 26.3
D		12.5	20 h	60	461 ± 94		N/A
E		11.5	1 h	50	465 ± 114	88.5 ± 1.0	88.6 ± 22.7
F		12.5	1 h	50	472 ± 59	89.4 ± 0.9	133.2 ± 16.3
G	PVAm	12.5	1 h	50	414 ± 119	88.0 ± 0.9	76.7 ± 9.8
H	Control *(water + dioxane)*		1 h	60	428 ± 68		0.3 ± 0.1
I	Control *(water + dioxane)*		1 h	50	420 ± 85	84.6 ± 1.2	0.4 ± 0.1
J	EtDA		10 min	50			0.3 ± 0.1
K			20 min	50			0.4 ± 0.1
L			30 min	50			0.3 ± 0.1
M	Control *(MeOH only)*		20 min	50			0.2 ± 0.1

CHAPITRE 4 GENERAL DISCUSSION

The fabrication and surface modification of poly L-lactic acid via aminolysis and EGF grafting were developed in this work. This optimization step was then followed by the investigation of its effect on NSLC adhesion, proliferation, and cell viability.

The desired topology include high porosity (higher than 80%) [85], and nano-metric fiber diameters. [16] An ideal fiber thickness would be lower than 150-200 μm [121, 123] and mechanical strength closer to the human brain (0.00015MPa-0.01MPa)[168, 169]. Yang et al [16] have shown that the rate of NSC differentiation was higher for PLLA nanofibers than microfibers. Also, studies have shown that smaller fiber diameters, high porosity, and suitable mechanical properties of the substrates enhanced cell proliferation effectively. [206] Nanometric-scale fibers have also shown to enhance cell proliferation and spreading. [16, 37] A high porosity of a scaffold increases the water adsorption of the scaffold and enhances the cell attachment, proliferation, and differentiation of cells. [207, 208] Our own results, presented in the appendix, also showed that the optimal topology is an aligned oriented nanofiber; they demonstrate superiority in cell adhesion and cell proliferation.

In this study some experimental challenges were faced; challenges during the electrospinning process included high ambient humidity and temperature as well as dust (i.e. from dessicants). The high ambient humidity causes beads as shown in Appendix C (Figure C3). To maintain the relative humidity between 20-30%, dessicants (Drierite ®) were placed inside the electrospinning experimental set-up. The formation of dust on the fibers (Figures C1&2) should be decreased as much as possible as they could pose potential problems on cell culture assays (even after intensive sterilization procedures) such as the interference of cell attachment on the fibers. Therefore, on top of placing samples directly in dessicators at all times, dessicant dust should be avoided by transferring under the fume-wood while allowing dust to settle completely prior to placement in electrospinning set-up.

Other experimental challenges included a large amount of solution dripping from the needle tip, resulting in a low amount of polymer solution jet reaching and forming fibers on the collector. Therefore, the process was optimized, by varying the electrospinning conditions to achieve the desired AFD and fiber thickness. Notably, after various trial conditions, the tip-

collector distance was decreased from 15 to 10 cm and the voltage increased from 15kV to 23.75 kV. Some solution dripping was still observed but was greatly decreased.

Therefore, the fabrication of PLLA fibers using electrospinning with high porosity (86.8 ± 1.2 %) in the nanometric range (408 ± 77 nm) and fiber thickness in the range of 97 ± 20 μm was used for the random nanofibers was used in this study.

The rationale for our technological choices is as follows: The electrospinning technique was used because it creates nanofibers that resemble the extracellular matrix of the host tissue in terms of porosity, structure, topography, and interconnecting pores. [10, 89]

An aminolysis reaction (for amine functionalization on PET films) reported by Noel et al [31] was optimized and utilized in order to attach amine moieties onto the PLLA substrate. The aminolysis reaction was adapted from the afore-mentioned protocol due to the fragility of electrospun PLLA. The sonication process had degraded the PLLA nanofiber while the presence of bubbles on the fiber's surface still remained. Therefore, in order to remove the formation of bubbles on the fiber's surface, the solution mixture was degassed under vacuum for 20 mins while stirring, prior addition to PLLA. Furthermore, the experimental temperature was decreased from the initial reaction time and temperature (i.e. 24 h at 70°C) to 1 h at 50°C.

In conclusion, the substrate remained physically intact, the AFD and porosity remained unchanged following successful amine functionalization. This is in stark contrast with amination protocols relying on plasma treatment that has been reported to degrade PLA [15] or aminolysis using the small molecule ethylenediamine. A PEG linker was then used to covalently couple EGF via its free amines to the amines introduced onto the PLLA structure via aminolysis.

Cell culture assays revealed that cells grown on substrates grafted with EGF were still viable up to 14 days. No differences were observed for neural stem character, with and without EGF surface modification. Furthermore, cell adhesion and proliferation was improved when substrates were grafted with EGF, when compared to substrates that were only aminated. In this work, the functionalization of PLLA mats with EGF not only provided the cells with a substrate harboring proliferative cues but may have also modified the surface properties of the PLLA mat to make it more suitable for cell adhesion and growth. As positive control, NSLC proliferation was also characterized on laminin-coated mats in EGF-free medium where no significant differences in cell proliferation were observed between the EGF-grafted substrates and the

positive control. Note that it was not possible to determine which cells in the histology were in contact with PLLA fibers (with or without EGF modification) due to technical limitations in the ability to visualize the scaffold nanofibers by optical or epifluorescence microscopy. Therefore, this new functionalized and EGF-grafted biomaterial has achieved efficient cell adhesion, proliferation as well as cell viability up to 14 days and has promising use in stem cell regeneration therapy.

CONCLUSION

Electrospun PLLA nanofibers have promising potential in neural tissue engineering. This work proposes an efficient method of manufacturing a graft structure for implantation and subsequent regeneration of damaged cells in the CNS. This work describes an optimized method and efficiency of the surface modified structure on cell adhesion, proliferation and viability without the need of direct injection of growth factors or stem cells and without the need of multiple surgeries. Firstly, the aminolysis method was optimized to sufficiently add amine moieties without affecting the average fiber diameter, porosity while maintaining the nanofiber physically intact. Secondly, EGF was successfully immobilized via the PEG linker and showed improved cluster-free cell adhesion and proliferation compared to the negative controls (pristine and aminated fibers). No significant difference was observed for cell adhesion and proliferation between the positive controls (laminin coated fibers) and EGF-grafted fibers in EGF-free and basal medium, however, the cells were less spread out on the positive controls compared to the EGF-grafted fibers. Therefore, we can conclude that EGF-grafted fibers are a successful optimized strategy for neural tissue regeneration.

PROPOSED IMPROVEMENTS - FUTURE WORK DIRECTIONS

Future developments could include i) a grafted-EGF density-dependent study, ii) tethering EGF to the PLLA scaffold in an oriented fashion for improved bioactivity, and iii) the study of the differentiation of the progenitor cells to specific and appropriate lineages.

i) Grafted-EGF density-dependent study:

The cell proliferation results showed that cells formed clusters at day 10 when seeded onto PLLA grafted with EGF in the presence of medium supplemented with soluble EGF as well as FGF (full medium). Clusters were also observed on aminated mats as well as pristine mats in full medium at days 6 and 10. Previous studies have shown that A431 cell clusters could form as concentration of EGF in the medium is increased.[200] Therefore, although our EGF-grafted scaffolds were able to promote significant NLSC proliferation, a grafted-EGF density-dependent study should be carried out to further optimize our strategy. This can be done by increasing or decreasing the amount of grafted EGF and observing its effect on cell culture assays.

ii) Tethering EGF to the PLLA scaffold in an oriented fashion for improved bioactivity:

Randomly oriented covalent immobilization of EGF through its reactive amine groups present at the N-terminus or on its lysine chain is the most common method used [30] as was performed in this study. This method, however, may not be the most optimal strategy as it has been shown to negatively impact protein conformation, relative molecular orientation, and spatial homogeneity, leading to diminished bioactivity. [30, 156, 209] Boucher et al [210] have shown that epidermal growth factor receptor (EGFR) phosphorylation was more intense with coiled-coil oriented EGF with higher initial A-431 cell adhesion compared to randomly coupled EGF. Lequoy et al [183] have also shown that higher EGF surface densities were obtained when oriented tethering of EGF was used compared to covalent EGf tethering, while using much lower concentrations of EGF during incubation. For improved bioactivity, EGF may be tethered to our PLLA scaffold in an oriented fashion using the k/e coil peptide strategy. The PLLA nanofibers can be attached to a k-coil peptide via a PEG linker (specifically, NHS-PEG_4-Maleimide cross-linker). The cysteine-tagged k-coil can then link to e-coil via the cysteine as previously reported. [156] Finally, e-coil peptide can be attached to EGF via a glycine linker. [156]

iii) Differentiation of the progenitor cells to specific and appropriate lineages:

Furthermore, the progenitor cells may be promoted to differentiate to specific and appropriate lineages. Binan *et al* have reported the differentiation of neuronal stem cells into motor neurons using electrospun PLLA/gelatin scaffold. [205] In their approach, NSLC were seeded onto scaffolds releasing retinoic acid and purmorphamine. These scaffolds were shown to enhance the differentiation of NSLC into motor neuronal lineage as well as promote neurite outgrowth. The combination of both approaches onto a structure releasing retinoic acid and purmorphamine while displaying tethered EGF may thus be promising.

APPENDIX A – TOPOLOGY OPTIMIZATION OF PLLA SCAFFOLDS: FIBER ORIENTATION AND BEAD PRESENCE

Effect of fiber topology on cell proliferation:

Previous research has been performed on aligned and random fibers where aligned fibers demonstrated superiority at mediating interaction between the NSLC and the scaffolds. Neurite outgrowth and NSC differentiation are also greatly improved on aligned nanofibers compared to other electrospun fibers such as microfibers and random nanofibers. [211]

There are various contradicting studies on whether NSLC show improved alignment on aligned or random cells. Some studies have shown that neurites extended along the axis of fiber when cultured on aligned nanofibers whereas no specific directionality was observed when cultured on random nanofibers. [212] In contrast, some studies reported neurites growing in a perpendicular manner to the main direction of fiber alignment, with poor penetration of neurites inside the structure. [213] Neural stem cells were also tested on aligned and random collagen nanofiber scaffolds, where cell proliferation was found to be higher on the aligned fibers. [214] Zhong et al [215] have compared aligned and random collagen scaffolds that had been crosslinked with glutaraldehyde on cell culture assays of rabbit conjunctiva fibroblasts. Results showed that although aligned scaffolds demonstrated lower cell adhesion, higher cell proliferation were observed compared to random scaffolds. Wang et al [216] have also shown that spinal cord derived neural progenitor cells (NPCs) had expanded faster on aligned nanofibers compared to random nanofibers. Other studies, on the other hand, have shown higher cell proliferation on random scaffolds compared to aligned scaffolds. Gupta et al [217] have compared aligned and random nanofibrous substrates for the in vitro culture of Schwann cells for neural tissue engineering. Their results have shown an increase of Schwann cell proliferation on random PCL/gelatin nanofibrous scaffolds compared to aligned PCL/gelatin nanofibrous scaffolds. Furthermore, Jahani et al [218] have demonstrated higher cell proliferation of mesenchymal stem cells on plasma treated and randomly oriented poly-C-caprolactone nanofiber scaffolds than on aligned orientation of the same scaffold.

Corey et al [219] have also demonstrated that aligned electrospun fibers provide a main direction for the growth of neurites from spinal root ganglia to neurons. This property would allow axons to align to reproduce the nerve structure. In addition to aligning neurites, the neurite

extension are also 20% longer on highly aligned fibers and 16% longer on partially aligned fibers, compared to random fibers.

In this project, aligned, random as well as beaded nanofibers were fabricated and their effect on cell proliferation compared.

Methodology

Sample preparation for cell seeding

The nanofiber mats were cut into 1.5 cm diameter disks with a round hole-arch punch, dipped in ethanol (70% in Milli-Q water), and further sterilized by UV exposure for 30 mins. The samples were then immersed in polystyrene tissue culture 24-well plates (VWR, Cat. # 82050-888) in quintuplicate under a sterile laminar hood. Autoclaved Glass cylinders (1.3/1.5 cm in inner/outer diameters) were placed onto the samples. The samples were coated with laminin (20 μg/ml) in PBS for one hour and then gently washed three times with PBS.

Cell seeding

Cells were diluted to 10,000 cells /ml and added to each well (1ml) (VWR, Cat.# 82050-888). As control, the cells were also cultured directly on laminin-coated Tissue Culture Polystyrene TCPS (Corning® CellBIND® 24 -Well Product #3337). An additional 1ml of culture medium were added to all wells. The test plates were incubated for 2, 5, and 10 days and the culture medium was replenished (2ml) every 48 hours.

Results

Different structures of electrospun nanofibrous scaffolds were fabricated under different parameters, characterized to have average fiber diameters of 350 ± 60 nm, and mat thickness 60 ± 9 μm before being compared in cell cultures. Porous, nano-scaled fibrous structures were revealed for the random, aligned, and beaded nanofibers as shown in the SEM images below.

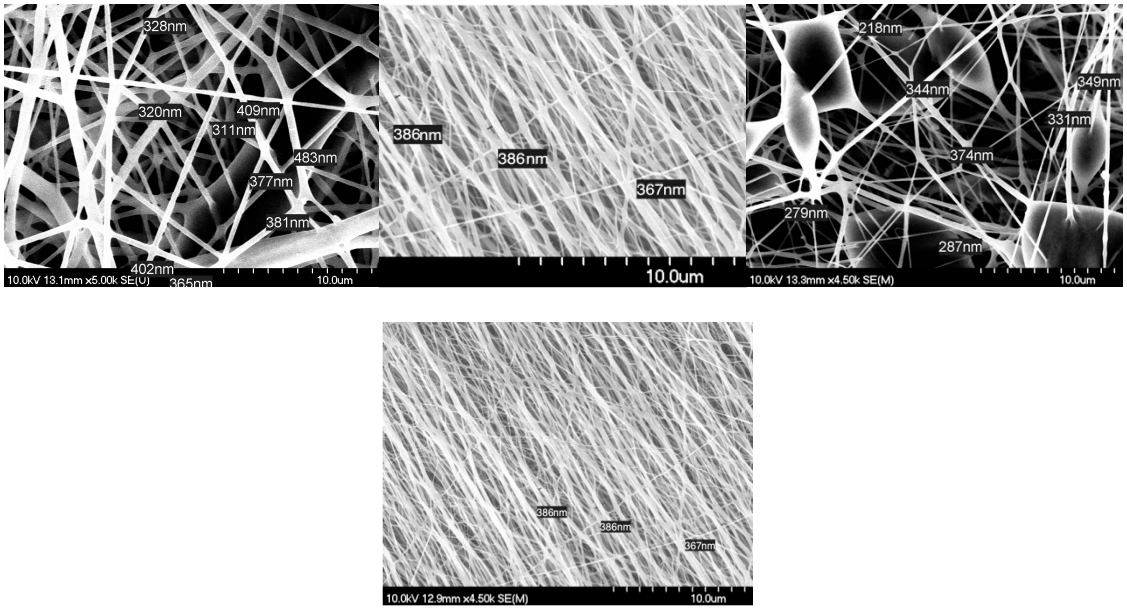


Figure A1: SEM images of different electrospun nanofiber structures from top left to right: random, aligned (image expanded), and beaded. Bottom image: aligned nanofibers.

Table 2: Average fiber diameter (AFD) and porosity for aligned, random, and beaded nanofibers

Nanofiber	**AFD (nm)**	**Porosity (%)**
Aligned	323.2 ± 51.8	85.2 ± 2.5
Random	380.1 ± 60.7	83.8 ± 0.6
Beaded	320.5 ± 81.4	83.0 ± 0.6

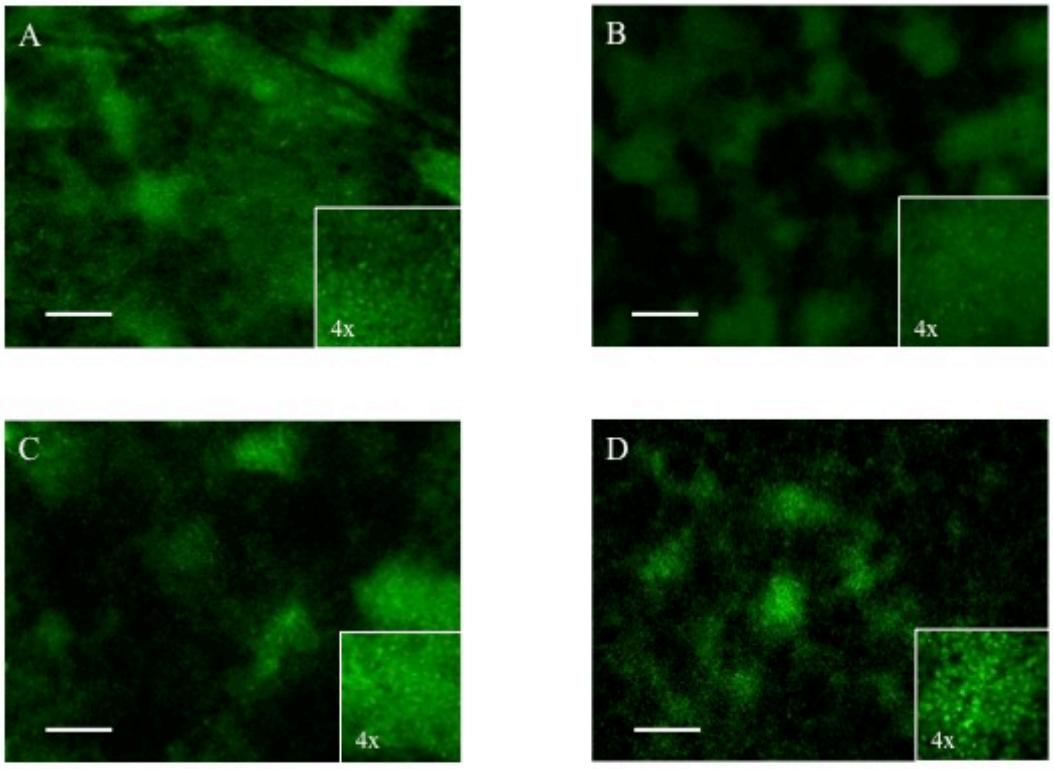


Figure A2: Representative images of cell proliferation assays at day 10 for laminin-coated aligned (A), random (B), beaded (C) nanofibers and TCPS (D)

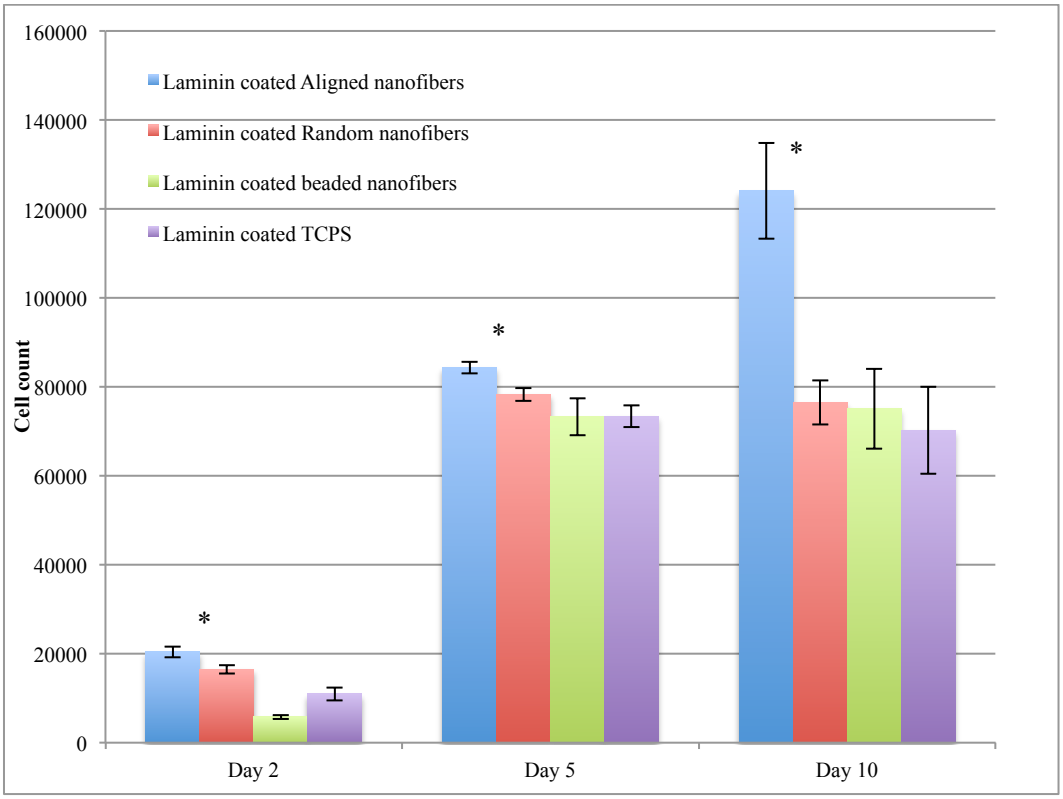


Figure A3: Impact of nanofiber structures on cell proliferation

The different nanofiber structures shown in figure A1 were tested for their effects on NSLC proliferation. All of the samples were coated with laminin and as can be seen in figure A3, higher cell proliferation was observed on the aligned fibers; the cells are also more spread out compared to the other nanostructures as well as the laminin-coated TCPS control group. The cells cultured in the laminin-coated control wells (Cellbind) proliferated at the same rate as the random and beaded nanofibers due to the 2D surface, however, a higher number (and larger aggregates) formed in the control wells compared to the aligned nanofibers due to poor surface contact and biocompatibility. Cell adhesion was also higher for aligned nanofibers as shown at day 2. Furthermore, figure A4 shows that when coated with laminin, cell adhesion is improved; cells are also more spread out and less likely to form clusters.

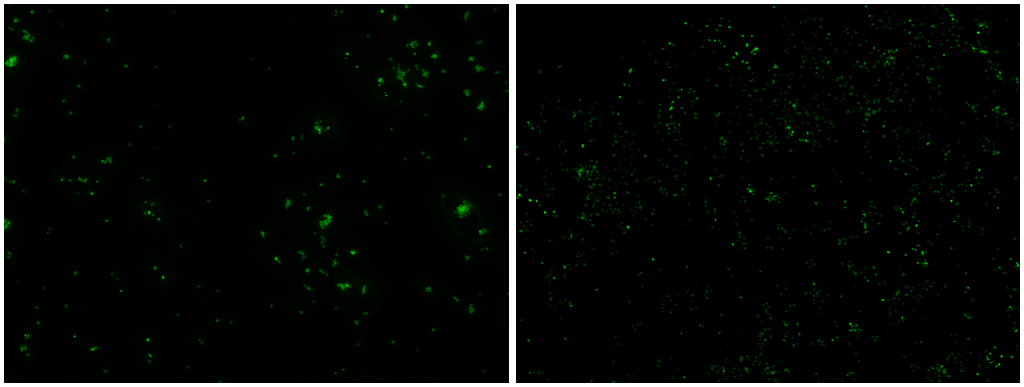


Figure A4: Un-coated and laminin-coated random nanofibers at day 1

Discussion

In this paper, aligned, random, and beaded fibers were compared where NSLC proliferation on aligned nanofibers were found to be higher ($P<0.05$) than random and beaded fibers as well as the control group (with less cell clusters as well).

Cell culture protocol used in this study was optimized from previous protocols as experimental challenges were faced. Firstly, cell death was observed; in order to avoid cell death in future experiments, 2 ml of media was used in the 24-well plates and changed every 2 days (as opposed to 1 ml of media changed every 2 days).

Secondly, the NSCs were proliferating at an alarming rate where cells had already reached their maximum confluence on the nanofibers at day 5, rendering comparisons very difficult. The amount of growth factors in media was decreased (from 0.4 ml to 0.1 ml); the number of cells seeded was also decreased from 50,000 to eventually 10,000 cells per well. Laminin concentration was also increased from 10 to 20 μg/ml in order to prevent clusters that had formed at 10 μg/ml.

The results of this study demonstrated that NSCs indeed prefer aligned fibers, consistent with the general literature. It has been shown in literature that some filament-like structures extend out from the NSLC cell body and neurites to attach to the nanofibers whereas no filament-like structure of NSLC had been observed on random fibers. It has been postulated that the fiber alignment may have an effect on the interaction between NSLC and scaffolds. [16] These are referred to focal adhesions (structurally defined adhesion sites) between the cells and the ECM. [89] If cells have superior neurite outgrowth and interaction with scaffolds of aligned nanofibers,

then it can be postulated they would induce higher proliferation as well, as was shown in this paper.

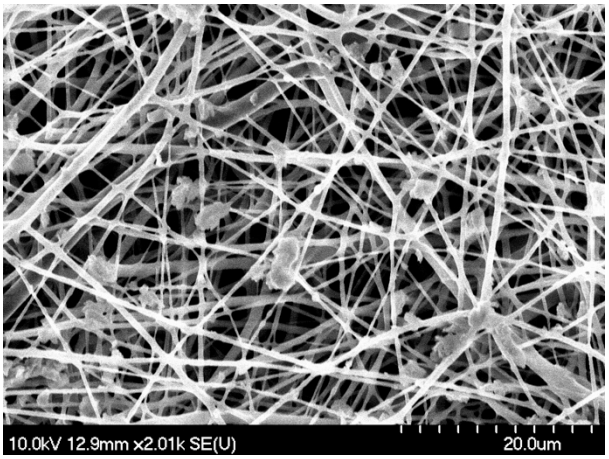
APPENDIX B-SUMMARY OF GROWTH FACTORS

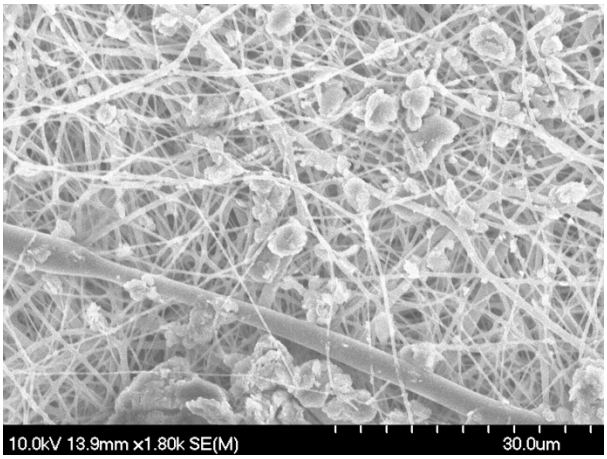
Table 3: Summary of some growth factors in neural tissue engineering

Growth factor	Effect	Phenotype or Pathway	Delivery technique	Species	Ref
BDNF	- Modulates brain elasticity and coping of environmental stimuli - Promotes proliferation and migration of pluripotent brain cells - Increases number of neurons in the adult olfactory bulb, striatum, septum, thalamus, and hypothalamus - Prevents degeneration of the red nucleus cell body in the brainstem	N/A	Intraventricular administration	Rat	[48] [51] [18]
	- Enhances myelination - Inhibits migration	- p75NTR pathway for cell death - RhoA/ Rho kinase pathway	Injection	Rat	[220]
CNTF	Prevents degeneration and supports survival of motoneurons	Embyronic motoneuron	Injection	Rat	[48] [221]
GDNF	Increases number and survival of motoneurons, regulates proliferation and differentiation	Embyronic motoneuron Ras-MAP and PI3 kinase pathyways	N/A	Chick	[52]
EGF	Promotes proliferation, differentiation and migration of NSCs	Neural stem cell	Tethered on scaffold	Mouse	[24] [29] [30]
FGF-2	Maintains cell viability, increases cell proliferation	N/A	Soluble (addition to medium, in vitro	Rat	[45] [37]

Growth factor	Effect	Phenotype or Pathway	Delivery technique	Species	Ref
NGF	Promotes choline acetyltransferase in forebrain	Motoneuron	Injection	human	[18]
NT-3	- Neuronal survival and differentiation - Increase synaptic activity at the amphibian neuromuscular junction, increases frequency of action potentials, synchronizes excitatory synaptic activities in neurons - Inhibits γ-aminobutyratergic synaptic transmission	Cortical neurons (Embryonic rat somato-sensory cortex cells)	N/A	Rat	[222]
NT-4/5	- Prevents atrophy of neurons - Stimulates GAP-43 and Talpha1-tubulin mRNA expression	Rat rubrospinal neuron	N/A	Rat	[51]
VEGF	- Supports neuronal migration - Promotes neurogenesis - Has trophic effects on neurons and glia - Essential for neuroprotection & neural patterning	N/A	Intraventricular pump implantation	Mouse	[223]

APPENDIX C - SEM IMAGES





Figures C1 & 2: An (extreme) example of a fiber covered with dust

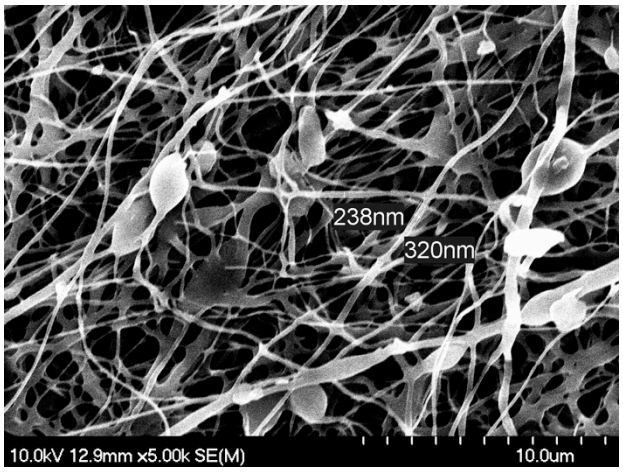


Figure C3: Formation of unwanted beads and thinning of fibers due to high ambient humidity

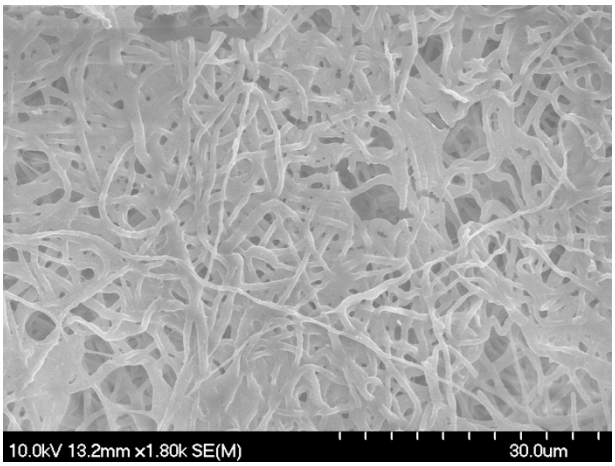


Figure C4: Fusion of fibers due to high ambient humidity

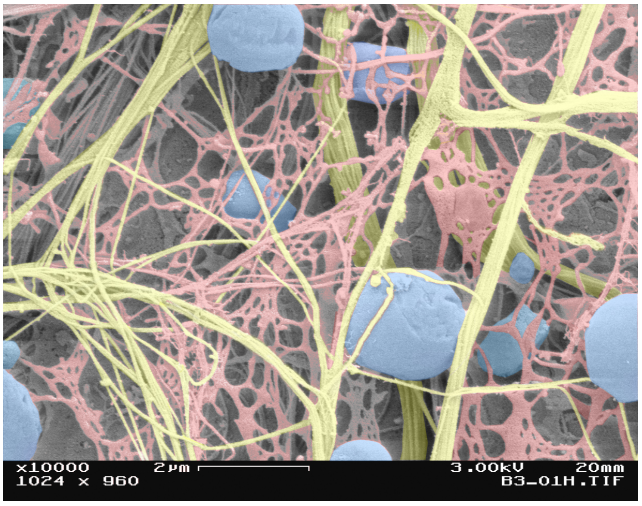


Figure C5: Extracellular matrix (red), nerves and nerve bundles (yellow), and ganglion cells (blue). [224]

Supplementary Data:

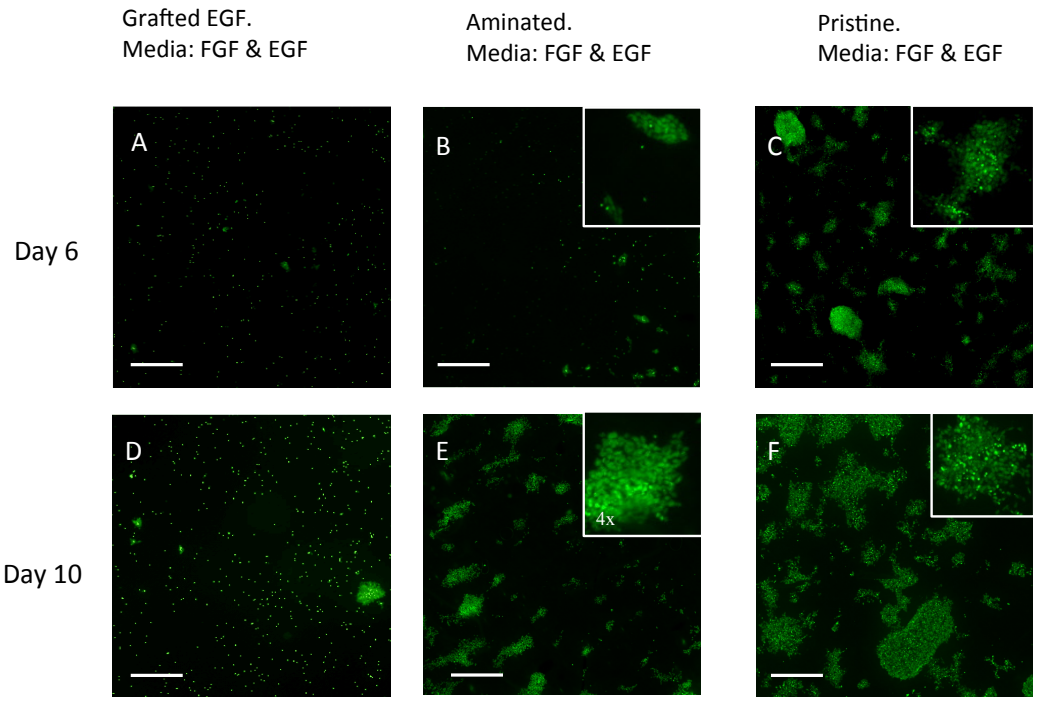


Figure S1: Fluorescence microscopy images of grafted EGF, aminated and pristine mats in growth factors at day 6 and 10. Scale bars correspond to 400 μm.

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