Enhancing nerve regeneration in the peripheral nervous system using polymeric scaffolds, stem cell engineering and nanoparticle delivery system

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Enhancing nerve regeneration in the peripheral nervous system using polymeric scaffolds, stem cell engineering and nanoparticle delivery system

by

Anup Dutt Sharma

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

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CHAPTER 1: RESEARCH OBJECTIVES

1. Introduction

The main objective of this project was to enhance peripheral nerve regeneration following peripheral nerve injury (PNI) using interdisciplinary approaches involving polymeric scaffolds, stem cell therapies, cellular & tissue engineering and drug delivery. Biocompatible and biodegradable polymeric materials such as poly (lactic acid) (PLA) were used for engineering conduits with micropatterns capable of providing mechanical support and orientation to the regenerating axons and polyanhydrides for fabricating nano/microparticles for localized delivery of neurotrophic growth factors (NTFs) and cytokines at the site of injury. Transdifferentiated bone marrow stromal cells or mesenchymal stem cells (MSCs) were used as cellular replacements for degenerating Schwann cells (SCs) at the injured nerve tissue. MSCs that have been transdifferentiated into a Schwann cell like phenotype (tMSCs) were tested as substitute for the myelinating SCs. To further enhance the regeneration, nerve growth factor (NGF) and interleukin-4 (IL4) releasing polyanhydrides nano/microparticles were fabricated and transplanted along with tMSCs seeded conduits. Synergistic use of these proposed techniques were used for testing and developing efficient treatments in a peripheral nerve injury model.

When a peripheral nerve injury occurs, the nerve starts to degenerate; demyelination of the nerve occurs and causes a long-term loss of sensory and autonomic functions. Intervention in the form of treatment is required to help the nerve reestablish connections and to regain its normal function and avoid neurological deficits. Various types of treatments have been used in the past to treat nerve related injuries. All of them have certain advantages and disadvantages, and researchers are trying to improve upon or synergistically combine the existing methods to restore the normal function of damaged nerves.
Current medical treatments for peripheral nerve injury involve coaptation[1, 2], autologous nerve graft[3], allografts, and xenografts. Coaptation is a common surgical procedure in which the proximal and distal ends of a nerve are forcefully sutured with each other. Currently, autologous nerve graft is the customary and widely accepted clinical procedure for treatment for PNI. It is also considered as the Gold Standard [4-6] for regenerative processes as this is still the best treatment which is capable of improving the functional behavior more than any other clinical procedure. Autologous grafting is the technique in which part of a healthy nerve is obtained from the same person and is transplanted at the site of injury. By transplanting the tissue from the same person, this technique avoids ethical issues and also reduces chances of tissue rejection. There are many disadvantages associated with this technique such as donor site morbidity and multiple surgeries. Xenografts[7](transplantation of tissue from a different species) and allografts[8, 9](transplantation of tissue from different individual but same species) have also shown promising results but widespread use of these procedures raises ethical questions. Also, during these grafting procedures, patients are subjected to immunosuppression in order to decrease the possibility of foreign tissue rejection. This immunosuppression increases the possibilities of infections in the subject’s body and is not considered the most optimal way of doing surgeries.

A PNI usually divides a nerve into two segments. The part which is proximal is considered to be the one that remains directly attached to the cell bodies and continues to be healthy while the other part distal to the injury starts to degenerate, the myelin sheath surrounding the axons starts to degrade and macrophages with help of Schwann cells (SCs) starts to clean up the cellular debris [10, 11]. Figure 1 illustrates the various steps that a neuron undergoes after an injury. Schwann cells (SCs) in the PNS and oligodendrocytes in the CNS are responsible for formation
of the myelin sheath. While SCs help in the reinnervation of regenerating axons, oligodendrocytes do exactly opposite of that and inhibit the regrowth[12]. In CNS, demyelination can also occur because of diseases, infections or spinal cord injury and transplantation of SCs at the site of lesions has helped remyelination of the degenerating axons [13-15].

Figure 1. Schematic showing various steps that a neuron undergoes after a nerve injury.
(Josh Rastatter, Biomaterials for nerve regeneration.

Because of these advantages, Schwann cells [16, 17] are considered as an ideal candidate cell type as they help in reinnervation of the damaged nerve and myelination of regenerating axons. Myelination is important as it prevents the electric signals propagating inside axons from decaying down. Therefore, myelinating cells such as Schwann cells ensure the proper
physiological function after reinnervation of damaged nerves. However, the procedure of obtaining Schwann cells requires multiple surgeries and damage to a healthy nerve that can result in donor site morbidity. Extracting SCs is analogous to obtaining an autologous nerve graft for use in regeneration; a healthy nerve is sacrificed for filling in the gap created by nerve transection. Even after extraction, SCs are very difficult to harvest or grow for multiple passages and prolonged times. Because of such limitations, researchers are seeking alternative cells which can provide similar functionality as SCs and can be grown for long time periods in cell culture conditions.

Our hypothesis is that mesenchymal stem cells (MSCs) can be used as an alternative for Schwann cells. Mesenchymal stem cells [18] are multipotent cells which are capable of differentiating into multiple cells lineages such as osteocytes, chondrocytes and adipocytes. MSCs can be obtained from multiple sources such as bone marrow, adipose tissue and umbilical cord. MSCs can be extracted from bone marrow with an easy and less invasive surgical procedure compared to an autologous nerve grafts. MSCs, because of their high proliferation rate, self-renewing ability and differentiation capabilities into several lineages, have been suggested as an attractive cell type to be used for neural regeneration purposes. MSCs have also been shown to transdifferentiate [19, 20] into Schwann cell-like phenotypic cells when exposed to certain chemicals and growth factors. These transdifferentiated MSCs are similar to SCs in terms of morphology and expression of glial proteins such as S100β (Calcium binding protein), p75NTR (p75 Neurotrophin receptor protein).

The advantage of using MSCs is that the isolation procedure of MSCs from a patient’s bone marrow is a routine surgical procedure and also MSCs are much easier to cultivate in a cell culture environment as compared to Schwann cells. Once propagated in cell culture, even a very
small initial culture of MSCs is capable of yielding a large number of MSCs for transplantation. The idea is to collect, isolate and transdifferentiate MSCs obtained from the patient, into SC like-cells and then transplant them at the site of injury.

Another way in which MSCs can be used for transplantation is to genetically modify them to secrete neurotrophic factors such as BDNF (Brain derived neurotrophic factor), GDNF (Glial cell-derived neurotrophic factor), or both BDNF and GDNF and then seed them inside a conduit or at the site of damaged nerve. Neurotrophins are a family of proteins that induce the survival, development, and function of neurons. Neurotrophins GDNF [21, 22] and BDNF [23, 24] have been used in literature for promoting regeneration and provide neuroprotection against degenerative diseases. Before using these cells for in vivo studies they need to be characterized well to make sure that they are not very different from wild type MSCs. Enhanced neurotrophic factor secretion may result in faster myelination and recovery of injured nerves.

Because autologous nerve transplants and other surgical procedures have many disadvantages, currently researchers have started to focus on Neural Tissue Engineering (NTE)[25] to develop strategies and medical procedures to counter with deficits that occur after a nerve injury. NTE is a combination of the use of biomaterials, engineering, sciences and medicine together to create drug delivery vehicles and to support and orient the regenerating tissues using scaffolding and conduits. Before designing a conduit for neural regeneration, various functionalities[26] such as cell adhesion, migrations, proliferation, biocompatibility, biodegradability, release of non-toxic substances, orientation/directionality, nutrient transfer, local release of drugs and surrounding capable of mimicking extra cellular matrix(ECM) should be taken into account.
Artificial nerve conduits can be synthesized using a variety of artificial and natural polymeric substances[27]. Figure 2 illustrates functions of various conduit types engineered in different ways. A conduit can be engineered using conducting polymers for electrical stimulation, can have microporous lumens or aligned nanofibres and also can chronically release neurotrophic factors such as GDNF and BDNF. Both natural and artificial biomaterials have been used for making nerve conduits. Natural biomaterials are generally ECM components such as collagen, fibronectin, laminin, gelatin, chitosan and compounds formed by living organisms such as silk fibrins. On the other hand, artificial biomaterials such as polymers can be engineered in ways that enhance neural regeneration.

Figure 2. Schematic diagram demonstrates the properties of an ideal nerve guidance conduit. Various guidance cues can be incorporated into NGCs to mimic the ECM[28].

An important limitation with regenerating axons is their orientation. Regenerating axons are not able to grow in the correct direction, and instead of growing from the proximal to distal part of
the nerve injury site, after little regeneration, they start to form scars. Micropatterning [29, 30] is an effective way of increasing the directionality and orientation of growing axons and our rationale is that if we use micropatterning inside a conduit, it will help in improving the connections between segments of a severed nerve. Our focus is on using polymers approved by the Food and Drug Administration (FDA) for other applications for engineering the conduit. Properties such as biodegradability[31] will reduce the necessity of multiple surgeries while other physical properties such as porosity and micropatterning will help in transfer of the nutrients and gases from surrounding tissue and will give directionality to the growing axons. Polymers that we are focusing on are PLA (poly lactic acid) and polyanhydrides. Neurotrophins and cytokines can also be embedded inside the conduit material and as the conduit biodegrades, a continuous release of these factors will help in modulating the microenvironment at the site of injury and thus help in axons to grow more and reduce inflammation and swelling at the injury site.

Researchers have also used nanotechnology [32, 33] for delivering these therapeutic proteins to the regenerating axons. Nanotubes (NTs) and Nanofibers (NFs) have been used inside nerve conduits to help align and support the growing axons. Nano/microparticles (NPs) have been used for localized delivery of neurotrophic growth factors such as nerve growth factor (NGF) [34]. In this research, we will be fabricating NGF and IL4 releasing polyanhydride nano/microparticles for sustained release of proteins and then test them in vivo in a rat sciatic nerve injury model. In the literature, nanomaterials have been shown to improve as well as to inhibit [35, 36] neural regeneration across various research settings. Scientists have not yet been able to assess the drawbacks of nanotechnology and it is important to overcome the drawbacks before implementing them in clinical settings.
2. Summary

The main objectives of these research projects are:

1. Fabrication of polymer micropatterned films and conduits.

2. Transdifferentiation of MSCs in SC-like phenotypic cells on micropatterned polymeric substrates.

3. Characterization of NTFs secreting MSCs on various ECM substrates for application in neuroprotection and neuroregeneration.

4. Fabrication and testing of micro/nanoparticles to release neurotrophic factors.

5. These studies will lead to the transplantation of transdifferentiated MSCs seeded conduits in vivo in a sciatic nerve transection model with immunomodulation of macrophage phenotypes using Interleukin-4 releasing micro/nanoparticles.

3. Organization of thesis

Chapter 2 shows the literature review related to neural tissue engineering. Chapter 3 is adapted from a manuscript where we have published results related to transdifferentiation of MSCs on micropatterned substrates. Chapter 4 is adapted from manuscript in which we published the results related to the characterization of genetically modified MSCs using high content screening methodology. Chapter 5 and Chapter 6 are two manuscripts in preparation where we are going to publish results related to neurotrophic factor releasing polyanhydride nano/microparticles and proteomic analysis of transdifferentiated MSCs respectively. In chapter 7, I have assembled all the recently done preliminary work related to in vivo transplantation and cytokine releasing
In Chapter 8, I am summarizing the most important results and important conclusion drawn from this dissertation.

4. References

3. Millesi, H., Bridging defects: autologous nerve grafts
5. Dahlin, L., et al., Schwann cells, acutely dissociated from a predegenerated nerve trunk, can be applied into a matrix used to bridge nerve defects in rats
6. Hausner, T., et al., Nerve regeneration using tubular scaffolds from biodegradable Polyurethane


CHAPTER 2: NEURAL TISSUE ENGINEERING: A REVIEW

Nanomaterial section of this chapter is adapted from book chapter titled

Mustafa E. Marti, Anup D. Sharma, Donald S. Sakaguchi, Surya K. Mallapragada

1. Introduction

Nerve regeneration is a complex biological process. Nerves are intrinsic parts of the nervous system and are responsible for maintaining communication between the brain and other parts of body and vice versa. Nerves are very fragile and can easily get damaged when exposed to any external force. Depending on the extent of the force, a nerve can suffer three different types of injuries classified by Sir Herbert Seddon[1]

- Neuropraxia – This kind of injury is caused by mild compression of the nerve causing interruption in the flow of signals. Easily recoverable.

- Axonotmesis – Synonymous to crushing injury. Damages to axon occur but the myelin covering around the nerve fiber remains intact. This causes paralysis and loss of sensory neuron function. Recovery is possible to some extent.

- Neurotmesis – Not only the nerve but the encapsulating tissue also undergoes damages. The nerve gets transected. There is complete loss of motor function and also there is very little chance of recovery.
When any of the aforementioned injuries occur, a cascade of cellular and molecular events occurs to overcome the effects of degeneration. After injuries, the nerve starts to degenerate; demyelination of the nerve occurs which causes a long term loss of sensory and motor functions. Degeneration mostly occurs in the distal part of the nerve stump while the proximal stump starts to grow and regenerate. Distal degeneration is also called Wallerian degeneration. The glial cells of the peripheral nervous system, Schwann cells (SCs), start to remove the cellular and myelin debris near the injured area along with macrophages which helps in process of phagocytosis (Figure 1). Though peripheral nerves have regeneration capabilities, regeneration is usually limited because of non-availability of mechanical support, orientation and absence of myelinating SCs. Depending on how big the nerve gap is after an injury, different treatment strategies are required to help the nerve regain its normal function and to avoid neurological deficits. Various types of treatments have been used in the past to treat nerve related injuries. All of them have certain advantages and disadvantages and there has not been a treatment so far which is capable of completely restoring the normal function of damaged nerve.
Figure 1. Process of nerve regeneration after an injury (adapted from Gaudet et. al, 2011 [2])
Various types of treatments that have been used for enhancing the nerve regeneration are described in the upcoming section of this chapter:

2. Surgical treatments

Current medical treatments for peripheral nerve injury involve coaptation[3, 4], autologous nerve grafts[5], allografts, xenografts. Coaptation is a common surgical procedure in which the proximal and distal end of a nerve is forcefully sutured with each other. But this type of treatment is effective only when the nerve gap is very small [6, 7]. Also, suturing should not enhance the nerve tension because that may result in inhibition of regeneration and increased nerve tension is one of the main reason why coaptation cannot be used as a treatment for addressing large nerve gaps. A nerve graft is mostly used in cases where injury creates a large gap in the peripheral nerves [8]. Currently, autologous nerve graft is the customary and widely accepted clinical procedure for treatment of PNI. It is also considered as the Gold Standard [9-11] for regenerative processes as this is still the best treatment capable of improving functional behavior, more than any other clinical procedure. Autologous grafting is the technique in which part of a healthy nerve is obtained from the patient and is transplanted at the site of injury. By transplanting the tissue from the same person, this technique avoids ethical issues and also reduces chances of tissue rejection. There are many disadvantages associated with this technique, such as donor site morbidity, limited length of graft material, multiple surgeries, and neuropathic pain [12, 13]. Also the success of autologous grafting always depends on the similarities between the organization of the donor and injured nerves[14]. Xenografts [15, 16](transplantation of tissue from a different species) and allografts [14, 17, 18](transplantation of tissue from different individuals from the same species) have also shown promising results, but widespread use of these procedures has given rise to ethical questions. Also, during these grafting procedures
patients are subjected to immunosuppression[19] in order to decrease the possibility of foreign tissue rejection. This immunosuppression increases the possibilities of infections in patient and can lead to other complications.

3. Nerve regeneration conduits: Biomaterial approaches

In order to avoid drawbacks associated with surgical treatments and to target even longer nerve gap injuries, researchers have come up with idea of polymeric scaffolds to facilitate and accelerate the tissue growth by mimicking the extra cellular environments around the injured area. These polymeric scaffold were fabricated in shape of conduits to connect the two severed ends of the nerve and thus they are also called nerve regeneration conduits (NRCs) (Figure 2). An ideal nerve regeneration conduit is expected to have various beneficial properties such as biodegradability, biocompatibility, non-immunogenicity, non-cytotoxicity, permeability, and be able to provide guidance cues, chemical cues, electrical cues, and trophic support to the regenerating axons and finally should be able to support the tissue growth for a sufficiently long period of time[20, 21]. Various types of natural and synthetic polymers have been used for fabrication of these NRCs. While natural polymers would be degradable and less cytotoxic, synthetic polymer properties can be altered to control the rate of degradation and release of trophic factors. It is of utmost importance to understand the intrinsic properties of these polymers and their compatibility with the biological materials before considering them for fabrication of scaffolds. NRCs have been modified internally to increase the surface area of interaction and also provide orientation/guidance to the regenerating axons. Various types of bio polymeric fillers have been used to provide additional support to the growing cells and tissues.
Figure 2. Schematic diagram demonstrates the properties of an ideal nerve guidance conduit. Various guidance cues can be incorporated into NGCs to mimic the ECM.[22]

Various types of natural polymers that have been used for fabrication of NRCs are collagen, chitosan, gelatin, hyaluronic acid (HA), silk fibroin, laminin, fibronectin, chitin, alginate, agarose, fibrin, etc.[21, 23]. Major disadvantages associated with natural polymers is that they can be immunogenic and also the lot-to-lot variance during their production that affects their structure and possibly their function too[24]. While there are certain disadvantages associated with natural polymers, the main reasons why researchers are interested in pursuing the polymers is because of their biodegradability, biocompatibility, stability, non-reactiveness, similarity to materials found in vivo, lower toxicity and the presence of specific binding sites which may impact the tissue regeneration in a positive fashion[24].
Collagen is the main fibrous protein in connective tissues and also the most abundant protein of the animal kingdom. There are 16 different types of collagen but the body usually consists of type I, II or III. Various FDA approved conduits such as NeuraGenTM, NeuroflexTM, NeuroMatrixTM, NeuroMendTM, NeuroWrapTM etc. are fabricated using type I collagen[25]. A porous three-dimensional crosslinked structure was obtained when chitosan was added to collagen by chemical modifications using glutaraldehyde. Freeze-drying steam-extrusion method was used for preparation of the nerve conduit scaffold[26]. Magnetically aligned collagen type I gel and control collagen gel was filled inside collagen nerve guides and they were implanted into 4 to 6mm nerve gaps in mice. It was shown that aligned collagen gel showed better regeneration as compared to the control collagen gel [27].

Chitosan is widely used for biomedical applications for agricultural and horticultural purposes, drug and gene delivery, tissue engineering scaffolds. It is also used for fabrication of nerve regeneration conduits because of its biodegradability, biocompatibility and excellent mechanical properties[28]. Chitosan conduits seeded with bone marrow stromal cell (BMSC)-derived Schwann cells were transplanted in 12 mm sciatic nerve gaps in adult rats and were shown to improve mid-shank circumference, nerve conduction velocity, average regenerated myelin area, and myelinated axon count as compared to phosphate buffered saline (PBS) filled conduits[29]. Laminin (LN)-modified chitosan conduits filled with BMSCs were successfully used for treating a 10mm nerve gap in Sprague dawley rats in terms of nerve regrowth, muscle mass of gastrocnemius, functional recovery and tract tracing[30]. Xiao et. al, 2013 used a use a collagen–chitosan (CCH) scaffold to mimic the bio-functional peripheral nerve and showed that it can support and guide the nerve regeneration after three months[31].
Gelatin is a natural polymer derived from collagen which is biodegradable, in vivo resorbable, and easy to modulate physical and chemical properties, which makes it a widely used polymer for biomedical applications[32]. Biodegradable gelatin conduits were used for bridging a 7 mm sciatic nerve gap in mice and shown to direct and enhance nerve regeneration[33]. A chitosan/gelatin nerve regeneration conduit constructed with collagenous matrices was used as a vehicle for Schwann cells and transforming growth factor-β1 to bridge a 10-mm gap of the sciatic nerve in rats. These cell seeded conduits enhanced nerve conduction velocity, average regenerated myelin area, and myelinated axon count similar to an autograft but significantly higher transplanted chitosan/gelatin nerve graft alone[34]. Different types of photocured gelatin conduits were implanted in 10mm sciatic nerve gap in adult male lewis rats for upto one year and were evaluated for their impact on functional recovery, electrophysiological responses, and tissue morphological regeneration[35].

Hyaluronic acid (HA) or Hyaluronan is a non-branched polysaccharide that is biodegradable, biocompatible, water soluble, viscoelastic, non-immunogenic and has derivatizable groups along its backbone, which makes it a suitable candidate for development of tissue engineering scaffolds and drug delivery systems[36, 37]. HA film sheaths were shown to reduce the scar formation in sciatic nerve end to end neurorrhaphy in male Sprague Dawley rats[38]. Freeform fabrication to fabricate micro-length scale features was used to fabricate a variety of scaffolds which can possible be used for nerve tissue engineering using HA. These scaffolds were further functionalized covalently using laminin to further enhance cell adhesion[39]. HA-Collagen
conduits seeded with neural stem cells (NSCs) were shown to facilitate facial nerve regeneration in rabbit facial nerve injury model observed using electrophysiology and histological analysis[40].

Synthetic polymers have also been used for fabrication of NRCs. Some of the advantages associated with synthetic polymers is their tailorable properties such as degradation rate. Another important advantage is the unlimited supply of these polymers and less lot-to-lot variance compared to natural polymers. Other important properties that are taken into account while copolymerizing these synthetic polymers are material strength, wettability, elasticity and thermal properties[21]. Synthetic polymers are divided into two categories: non-biodegradable and biodegradable. Common non-biodegradable polymers include silicone rubber, polydimethylsiloxane (PDMS), polyamide (PA), acrylic, polyethylene (PE), polysulfone (PSU), poly (acrylonitrile-co-vinyl chloride) (PAN-PVC), poly (2-hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA-MMA), polypyrrole (PPy), porous stainless steel [21, 41-43]. Common biodegradable polymers include poly(l-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic acid-ε-caprolactone), poly(l-lactide-co-glycolide), poly(1,3-trimethylenecarbonate-ε-caprolactone) and poly(caprolactone) (PCL), poly(3-hydroxybutyrate) (PHB), polyphosphoester (PPE), polytrimethylene carbonate-caprolactone (PTMC-CL), and polyurethane (PU) and other derivatives of these polymers[21].

Biodegradable synthetic polymers have been of most interest for researchers because of their unique property of biodegradation which can be further tailored by synthesizing derivatives of the polymers. Also because they biodegrade in vivo to release non-toxic by products, it
eliminates the need for a second surgery in order to remove the scaffold from the tissue. Because these polymers are usually elastic in nature, they also do not cause any additional stresses on the growing nervous tissue inside the conduit. Biodegradable polymers usually have ether, amide and ester groups on them and are usually synthesized by melt polycondensation reactions. Scaffolds made up of these polymers are designed in such a way that they do not lose their mechanical integrity till the end of the nerve regeneration process and support the regenerated tissue throughout the time period. Biodegradable polymers usually degrade by mechanism of hydrolysis, bulk erosion and surface erosion[44]. Most important properties of biodegradable polymers are their non-immunogenicity, accepted shelf life, degradation rate matching with regeneration process, appropriate mechanical strength to support the growing tissue, non-toxic by products, biocompatibility and permeability to biomolecules if needed[21, 45].

4. Cellular therapies

Depending on how big the nerve gap is, additional support to the regenerating axons is required to help them grow over longer distances. One of the most important strategies for enhancing regeneration is to provide a cellular alternative to the lost cells during nerve degeneration. A variety of cell types have been used as a cellular alternative to the native Schwann cells which includes autologous Schwann cells (SCs), mesenchymal stem cells, embryonic stem cells, neural stem cells, olfactory ensheathing cells etc.

Schwann cells (SCs) are the supporting cells of the peripheral nervous system and are responsible for performing a large variety of functions including ensheathing axons, providing trophic support and nutrition to the axonal tissue, production of myelin, removal of myelin debris in case of injury and extracellular matrix molecules production for the peripheral nerve
trunk[46]. Schwann cells isolated from a peripheral nerve can be expanded in cell culture with help of mitogens and then can be used for transplantation inside scaffolds at the site of injury[47]. Allogeneic SCs were proposed as an effective treatment as opposed to the time taken in expanding the autologous SCs [48]. Polyhydroxybutyrate conduits filled with alginate hydrogels and allogeneic or syngeneic genetically labeled SCs were used to treat a 10mm gap in rat sciatic nerve [48]. Both allogeneic and syngeneic SCs enhanced axonal regeneration but more axons were found in the case of transplanted syngeneic SCs. Allogeneic SCs were rejected after a period of 6 weeks. Autologous SCs (200,000 cells/µl) seeded in collagen conduit was transplanted at 13mm sciatic nerve gap in male fischer rats [49]. Number of myelinated axons at 16 weeks was found to be significantly higher than the tubes filled with only serum. Rutkowski et. al. 2004 used a porous poly(D,L-lactic acid) (PDLLA) conduit with inner micropatterned lumen seeded with SCs and showed that sciatic nerve regeneration was enhanced on basis of improved sciatic functional index and time of recovery in a 1cm sciatic nerve transection model in rats[50]. Acellular nerve sheaths repopulated ex vivo with Schwann cells (SCs) modified to express brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), or neurotrophin-3 (NT3) were used to repair 1cm defects in rat peroneal nerves and showed that different expression of aforementioned neurotrophic factors by genetically modified SCs had differential effects on graft morphology, the number and type of regenerating axons, myelination, and locomotor function[51].

Embryonic stem (ES) cells are pluripotent in nature which means they can differentiate into any other cell of the body including neural and glial lineages. Another important property of ES cells is to self-renew which helps in growing ES cells in culture for prolonged times and passages. As
a result ES cell transplantation is used as a therapy for curing nervous system disorders as well as nervous tissue repair[52]. Lee et. al. 2012 developed a strategy where they differentiated human ES cells into mesenchymal stem cells spheres (hESC-MSC) and then used these spheres for transplantation in a 2mm sciatic nerve gap in athymic nude mice. Nerve conduction tests in these mice showed that hESC-MSC sphere transplantation repaired the nerve significantly better than when the hESC-MSC were transplanted in the form of cell suspensions[53]. In another study, embryonic stem (ES) cell-derived neural progenitor cells (ES-NPCs) were implanted in a 1cm rat sciatic nerve transection model[54]. Transplantation enhanced the axonal regeneration and tissue repair compared to the culture medium controls.

Olfactory ensheathing cells (OECs) are the glial cells which ensheathe the axons of the olfactory receptor neurons[55]. OECs are capable of supporting regeneration not only in olfactory system but in central nervous system (CNS) as well. Also, they are capable of changing their states from non-myelinating to myelinating cells in vitro and have also intrigued researchers in doing further testing and using these cells for neural repair[56]. Purified olfactory ensheathing cells seeded inside silicone tube filled with laminin gel was found to be successful in regeneration of 15mm sciatic nerve gap in 50% of the animals[57]. The percentage of animals where regeneration occurred successfully was found to be even higher in case of 12mm gap. OECs were transplanted after rat sciatic nerve was cut using microsurgical procedure and was compared to the injection of control media[58]. eGFP expression showed that OECs survived and got incorporated inside the regenerated axons. Axons were also found to be myelinated by OECs and improvement in the conduction velocity in the transplanted group showed the effectiveness of OECs in treatment of peripheral nerve injuries. In another study, OECs were injected after section and resection of
2cm of sciatic nerve and was found to restore the contractile properties of the gastrocnemius muscle and complete repair of the SN[59]. Also RT-PCR showed that there was an increase in the presence of neurotrophic factors.

Neural stem cells (NSCs) or neural progenitor cells are also of great interest as a cellular replacement of SCs for treatment of PNIs. NSCs can be derived from ESCs and are also present in developing mammalian nervous system as well as the adult nervous system[60]. Because NSCs self-renew and are capable of differentiating into neurons and glial cells such as oligodendrocytes and astrocytes, they hold great promise in treating nervous system disorders[61]. Another advantage of using NSCs is that it is easier to manipulate the fate of differentiation of NSCs just by adding different type of trophic factors in vitro and in vivo [61]. Xu et. al. 2012 showed that silicone conduit seeded with brain derived neural stem cells when transplanted at a 10mm sciatic nerve gap, was able to enhance the peripheral nerve regeneration by measurement of sciatic functional index and sciatic nerve conduction velocity as compared to normal saline silicone conduits[62]. Co-transplantation of NSCs was done along with SCs in a hemisected adult rat spinal cord and it was shown that NSCs survive better when transplanted with SCs as compared to when NSCs are transplanted alone and also improves regeneration by measuring average number of myelinated axons in both treatment groups[63]. Fibroblast growth factor 1 (FGF1) immobilized microporous/micropatterned poly (D, L-lactic acid) (PLA) nerve conduits were seeded with neural stem cells and transplanted at 15mm sciatic nerve gap injury models in rat. Histology, walking track analysis and electrophysiology was used for testing the nerve regeneration and results showed that conduits seeded with NSCs enhanced the nerve regeneration equivalent to the autograft group[64]. Heine et. al 2004 transplanted GDNF
expressing NSCs in chronically denervated distal tibial nerve segments and by performing assays such as counting the number of axons and emergence of compound motor action potentials found that regeneration was better in animals transplanted with NSCs[65].

Mesenchymal stem cells (MSCs)[66] are multipotent cells which are capable of differentiating in multiple cells lineages such as osteocytes, chondrocytes and adipocytes. MSCs can be obtained from multiple sources such as bone marrow (BM), adipose tissue (AD) and umbilical cord (UC). MSCs can be extracted from bone marrow with quite an ease and less evasive surgical procedure as compared to autologous nerve graft. MSCs because of their high proliferation rate, self-renewing ability and differentiation capabilities in several lineages, have been suggested as an attractive cell type to be used for neural regeneration purposes. MSCs have also been shown to transdifferentiate [67, 68] into Schwann cell like phenotypic cells when exposed to certain chemicals and growth factors. An acellular nerve graft was affixed with BM-MSCs using fibrin glue and was used to bridge a 15mm sciatic nerve defect in Sprague dawley rats[69]. MSCs embedded in fibrin glue survived and were capable of producing neurotrophic growth factors and supported the nerve regeneration and functional recovery as measured using histological and functional testing of the rats transplanted with grafts. Costa et. al. 2013 compared undifferentiated BM-MSCs or Schwann-like cells that had differentiated from BM-MSCs transplanted at a rat facial nerve injury model using a polyglycolic acid tube. Both cell-containing groups had higher compound muscle action potential (CMAP) as compared to the control groups and the facial nerve with SC-like cell implantation had the highest mean axonal diameter[70]. Studies showed that both cell types enhanced nerve regeneration and the SC-like cells had superior effects. Allogeneic rat adipose derived stem cells (ADSCs) were transplanted
for evaluation in 15mm sciatic nerve gap by using a acellular nerve graft which helped in improving the walking behavior measured with footprint analysis, increased mass muscle ratio of gastrocnemius muscle, increased nerve conduction velocity and myelinated fibers within the graft[71]. A polycaprolactone tube filled with BM-MSCs was shown to improve nerve regeneration and motor neuron survival in 3mm sciatic nerve cut model in C57BL/6 mice by testing the animals for sciatic functional index, number of neurons in dorsal root ganglion and measuring weight and creatine phosphokinase enzyme activity in gastrocnemius muscle[72].

5. Regenerative cues

5.1. Topographical cues

Various types of guidance cues have been used in nerve regeneration conduits to orient the transplanted cells and regenerating axons which indirectly makes the nerve tissue to regrow for longer distances. Some of these topographical cues involve micropatterns/microgrooves, microporous luminal structures, aligned nanofibers and a gradient of ECM proteins in some cases [73]. Biodegradable PDLLA conduits with micropatterned inner lumen preseeded with Schwann cells was shown to enhance time of recovery and sciatic functional index in 1 cm sciatic nerve transection model in rats[50]. In another study, phase transition and micro printing was used to create a conduit with micropatterned inner lumen and also asymmetric pores through its wall using poly (D, L-lactide) (PLA) substrates [74]. After transplantation of the conduits in a 10mm sciatic nerve transection model in rats, asymmetric conduits with surface microgrooves had thicker myelin sheaths, improved myelination and a better functional recovery as compared to the symmetric conduits with microgrooves at end of 6 weeks. Aligned nanofibers have also been used by various groups for fabrication of nerve guidance conduits. A modified one step electrospinning technique was used for 3D aligned nanofibrous nerve conduits made of
collagen/PLGA which was then used for transplantation at a 13mm sciatic nerve gap in rats[75]. Such 3D conduits were found to provide better recovery than randomly aligned nanofibers conduits after testing the animals for motor function, nerve conduction, axonal and Schwann cell morphology, and marker expression. Yao. et. al, 2010 studied the effect of channel number on axonal regeneration by fabricating collagen conduits with varied number of channels (1, 2, 4 and 7) and found that 1-channel and 4-channel conduits showed better axonal regeneration in a 1cm sciatic nerve transection model[76]. Studies have shown that multiple channels inside conduit can limit the dispersion of axons without having any deleterious effects on nerve regeneration.

5.2. Electrical cues

Electrical stimulation has been shown to improve the nerve regeneration in various peripheral nerve injury models. There are various ways of providing electrical stimulations to the regenerating nerve such as Transcutaneous/percutaneous stimulation (TENS), Direct, brief, low-frequency electrical stimulation, Electrical stimulation via the synthetic nerve graft etc [77]. Electrical stimulation has been shown to affect the axonal regeneration, expression of neurotrophic factor and Schwann cell behavior[77]. Huang et. al 2013 investigated the effect of brief electrical stimulation (ES; 3 V, 20 Hz, 20 min) on delayed nerve injury repair. Sciatic nerve was transected and nerve repair was delayed for multiple time durations. Electrical stimulation (ES) increased the diameter and regeneration of regenerated axons, the thickness of myelin sheath, as well as the number of Fluoro-Gold retrograde-labeled motor neurons and sensory neurons showing that ES can be used as an effective way for treating delayed nerve injuries[78]. In another study, brief electrical stimulation increased the speed and accuracy of motor axonal regeneration. Brief ES reduced the period of staggered regeneration (from 10 to 3 weeks) and accelerated the preferential motor reinnervation [79]. Conductive polypyrrole/chitosan scaffold
and non-conductive chitosan scaffold were fabricated and was used to bridge a 15mm sciatic nerve gap in Sprague Dawley rats[80]. Intermittent ES (3 V, 20 Hz) was applied to the polypyrrole/chitosan to create an electrical environment at the site of nerve defect. In another study, various frequencies (1, 2, 20, or 200 Hz) of electrical stimulation was tested out to check frequency effect on nerve regeneration [81]. 10 mm sciatic nerve gap was created in sciatic nerve of rats and then proximal and distal end was sutured using a silicone rubber tube. Electrical stimulation at different frequencies was carried out every other day starting after first week of transplantation and up to 6 weeks. Histological testing and electrophysiology testing showed that higher frequency stimulation has less effect on regeneration as compared to lower frequency stimulation.

5.3. Biomolecular cues

Biomolecular cues refer to delivery of growth and trophic factors at the site of injury to promote the survival of degenerating cells and promote regeneration of growing axons. Neurotrophic factors are the ones which has interested the researchers the most. Neurotrophic factors (NTFs) are a family of proteins responsible for growth and survival of developing neurons. They also provide nutrition and trophic support to the mature neurons[82]. When tissues are healthy, neural tissues secrete these factors for a better viability of target cells. But post-injury, non-neuronal cells such as Schwann cells (SCs) secrete these factors to aid the outgrowth of regenerating axons. NTFs are divided into three major classes[82]: (1) Neurotrophins; (2) neurokines; and (3) the transforming growth factor beta (TGF)-β superfamily.

Neurotrophins consist of Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), Neurotrophin 3 (NT-3), Neurotrophin 3/4 (NT-3/4), Neurotrophin 6(NT-6) while neurokines
comprise of Ciliary neurotrophic factors (CNTFs) and leukemia inhibitory factor (LIF). TGF-β superfamily consists of TGF-β1, TGF-β2, TGF-β3 and glial cell line-derived factor (GDNF).

NRCs loaded with neurotrophic factor have been shown to enhance the axonal regeneration. Optimized dosage and a long term sustained delivery of the factors are two important parameters while fabricating such neuroregenerative conduits [83]. Usually, these growth factors are directly loaded into the lumen of the conduits. They fill the lumen, and are also bound to the inner circumference of the lumen using various biocompatible molecules called as matrix materials such as hydrogel-forming collagen, fibrin, laminin, alginate, heparin, and heparin sulfate [84]. Plasma treatment of the surface of the conduits can be used to attach larger quantities of protein, thereby, increasing bioactivity of the conduit.

The following section describes how different types of NTFs were loaded into NRCs.

5.3.1. Nerve growth factor (NGF)

NGF is the most commonly used neurotrophic factor for sciatic nerve regeneration. Kim et. al. used NGF immobilized porous polycaprolactone (PCL)/Pluronic F127 as a biological stimulator for the growing axons [85]. They found that NGF loaded conduit had a positive effect on neural regeneration viz. thicker neural tissues than the control nerves). Wang et. al. immobilized NGF on a nerve conduit made of chitosan using genipin crosslinking and observed the conduit to have nerve function comparable to that of an autologous nerve graft [86].

5.3.2. Glial cell-derived neurotrophic factor (GDNF)

GDNF has been shown to enhance the survival of both sensory and motor neurons. An affinity based delivery system [87] of GDNF using a fibrin matrix inside a silicone conduit improved the sciatic nerve regeneration in a 13mm rat sciatic nerve defect model [88]. A considerably high number of large diameter fibers showed the increase in the mature neural content using GDNF
based delivery system [88]. NRCs were also fabricated using collagen and silk fibrion to reduce the initial burst of GDNF and a long term (28 days) sustained delivery [89]. Moore et. al. showed that GDNF from a fibrin-based delivery system enhanced motor nerve regeneration at a level similar to an isograft in the femoral motor nerve model. This level was observed in terms of total nerve fibers, percentage of neural tissue, and nerve density [90].

5.3.3. *Glial growth factor (GGF)/Neuregulin*

GGF-containing conduits have been shown to improve the sustained axonal regeneration and the muscle innervation when used with a poly-3-hydroxybutyrate (PHB) conduit [91]. PHB-GGF conduits were found to have much more myelinated fibers as compared to the control PHB conduits. Also, GGF has been shown to facilitate nerve regeneration by speeding up the migration rate of Schwann cells [92].

5.3.4. *Fibroblast growth factor (FGFs)*

Use of immobilized FGF1 has been proposed as an effective way to increase the bioactivity of NRCs. This is believed to speed up the regeneration rate for severely damaged nerves [64]. bFGF (basic FGF) has also been shown to promote the extension of early regenerating axons by directly influencing them, via a mechanism that excludes Schwann cells or angiogenesis [93]. bFGF embedded in the inner layer of poly(D,L-Lactide) (PDLLA) conduits have been proven to significantly improve the regeneration and nerve function in a transected 15-mm sciatic nerve of Sprague Dawley rats [94]. bFGF released from such conduits also enhanced the sprouting of nerve fibers from dorsal root ganglia and the proliferation of Schwann cells.

5.3.5. *Ciliary neurotrophic factor (CNTF)*

CNTF is considered as one of the important neurotrophic factor which can stimulate neurite outgrowth in sensory and motor neurons [95, 96]. CNTF along with chitosan was coated on a
PLGA conduit after treatment with pulsed plasma. This was shown to promote nerve regeneration in 25mm long canine tibial nerve defect [97]. The PLGA/chitosan conduits-CNTF conduits group performed better than PLGA/chitosan conduits alone and were found to be similar to the autologous nerve grafts group. Cao et. al. used a different approach wherein they cross linked linear ordered collagen scaffold (LOCS) fibers with laminin followed by fusion of N-terminal of CNTF to the laminin binding domain in order to retain CNTF on the LOCS [98]. They found that functional biomaterial retained more CNTF and promoted the functional recovery and enhanced nerve regeneration.

Another way of delivering neurotrophic factors is to encapsulate NTF secreting cells into microcapsules and transplant them at site of injury. Cell encapsulation technology is widely used for immobilizing cells inside a semi-permeable polymeric membrane which allows bidirectional flow of nutrients and therapeutic proteins. Oxygen and other essential nutrients diffuse from outside the capsule into the cells and therapeutic proteins released from the cells permeate outwards to support healing of targeted injured tissue[99]. Also cells inside the capsule remain protected from the immune response of the host as well as antibiotics [100, 101]. This has been attributed to the pore size of the capsule membrane being impermeable to host cells. Various types of biopolymers such as cellulose sulphate, collagen, chitosan, gelatin and agarose have been used for encapsulating cells.

6. Nanomaterials
Nanotechnology is the use of engineered materials on a nanometer (nm) scale. The sizes of these materials are generally between 1- and ~100 nm, and they are compatible with biological systems including regenerative neural tissue therapy. Nano-engineered structures, especially nanotubes (NTs) and nanofibers (NFs) imitate the structure of the extracellular matrix (ECM) and can mimic the environment of the nervous system. Recent and remarkable improvements in nanotechnology offer promising alternatives for neural regeneration studies. Nanotechnology can be used to engineer scaffolds with oriented NFs, NTs and other materials that are functionalized to serve as cell-binding domains or for the release of trophic- or growth factors. These scaffolds also facilitate nutrient- and oxygen diffusion, and provide topographical cues. Recent advances in nanotechnology, biomaterials and tissue engineering have improved the effectiveness of NGCs and enabled their use with a control at nano-scale to obtain enhanced and oriented (Figure 3) neuroregeneration [102].

In the next few sections, the use of nanotechnology combined with other guidance cues (e.g., biomaterials, support cells, chemical factors, etc.) in neural tissue engineering strategies is reviewed. Nano-structure fabrication techniques and nano-materials in NTE are presented. Applications and recent advances in research are also discussed.
Figure 3 The schematic diagram shows growth and orientation of nerve cell fibers (A: Axons stained with anti-tau-protein, B: the nerve cell bodies stained with anti-MAP2 protein). Confocal microscope images of elongated axons (C: tubulin, D: phosphorylated neurofilament E: tau protein) (Reproduced with permission from [103] and [104]).

6.1. Nano-scaffold design techniques

The structure and geometry of the nano-structural scaffolds are very important to promote enhanced cell activity for neural regeneration. Thus, graft technology to produce these materials is critical to obtain optimum mechanical strength and surface properties for axon regeneration. Nano-structures made of several biomaterials have been shown to support axonal outgrowth. Several methods have been used to fabricate nano-structural scaffolds such as electrospinning, self-assembly and phase separation [105].

6.1.1. Electrospinning

Electrospinning is a technique for producing NFs in micro-/nano-scale from natural and synthetic polymers or polymer composites. A solution of the starting material in an appropriate solvent is
charged using a spinneret and a high voltage supply. This is done at a polymer concentration above the critical entanglement concentration, below which NFs are not produced. Under the influence of an electrical field, the surface tension of the polymer is overcome at the tip of the spinneret and the charged polymer jet is directed at a target, resulting in formation of a Taylor cone. Nanofibers emerging from spinneret are then collected in a parallel orientation using a rotating collector (Figure 4). The latter can be a drum or a disk as well as a plate. The use of a stationary collector may result in randomly oriented NFs.

Figure 4. This schematic diagram illustrates the experimental set-up for the production of aligned nanofibers using electrospinning. Reproduced with permission from [106].

By electrospinning, fibers of nano- to micrometer size dimensions can be fabricated. Solution properties (i.e. concentration, conductivity, viscosity and elasticity) and process parameters (i.e. voltage, distance from target, needle identity, and temperature) can be altered to fabricate various types of fibers for various applications. The mechanical properties of the NFs (e.g., thickness,
composition, surface area and porosity) are adjusted by controlling the solution and process parameters to optimize the alignment, stability and morphology [105, 107]. For different applications, the collection schemes can be varied as single ground, rotating single ground, horizontal ring, and in vitro onto cells [108]. To overcome the disadvantages associated with the fiber thickness and random orientation, blends of different biomaterials can be used. Thus, various types of biological materials, synthetic biopolymers and composites have been used to manufacture NFs by electrospinning.

For neural regeneration, the features of the scaffolds are extremely critical to mimic the ECM and achieve successful repair. For example, electric fields used during fabrication enhance control of fiber alignment, which is significant for neural regeneration. Electrospun NFs have been shown to enhance and accelerate cellular activities such as proliferation, axon regeneration, growth and migration [109, 110] and have been used for neural tissue engineering applications [106, 111]. Research to produce nano-structural scaffolds with desired mechanical- and surface properties for nerve conduits using electrospinning is continuing.

6.1.2. Self-Assembly

Self-assembly (SA) is the reversible and spontaneous formation of a stable organized/ordered structure, from disordered elements through various weak interactions, e.g. non-covalent bonds and hydrophobic interactions. SA can occur at the molecular level and in the nano- and micro-scale. In nature, numerous types of SA processes are carried out. Amino acids, fatty acids, and other molecules come together to form self-assembled nano- or larger structures [112].

The SA process has been shown to occur with polypeptide sequences, and di- and triblock peptide amphiphilic copolymers. Porous NFs of 5-10 nm diameter fabricated by self-assembly from various biodegradable/biocompatible materials have the potential to mimic the 3-D
microenvironment of the ECM and promote neural regeneration by enhancing cell attachment, proliferation, differentiation and migration. Amphiphilic oligopeptides composed of repeating units of hydrophilic and hydrophobic amino acids formed stable \( \beta \)-sheet structures in water and have been used to make scaffolds for neuronal regeneration [113]. Zhang et al. reported that with the addition of salts, these oligopeptides self-assembled into stable macroscopic structures of ordered filaments with porous enclosures, and supported cell attachment and synapse formation. Holmes et al. demonstrated the enhancement of neurite outgrowth with self-assembled peptide hydrogels [114]. Various macromolecules such as peptides have the ability to self-assemble and form ordered structures and nanomaterials due to several interactions between the components [115]. Sur et al. designed a hybrid matrix consisting of collagen type I and a peptide amphiphile (PA). They produced homogenous NFs of 20-30 nm in diameter having the structural properties of the former and epitope-presenting ability of the latter components by self-assembly [116]. The cellular density was shown to be manipulated by epitope concentration, which could be changed by PA concentration. Besides, controllable axon and dendrite growth were carried out as well as neuronal survival and maturation with epitope concentration adjustment. Furthermore, controllable axon and dendrite growth were carried out, as well as enhanced neuronal survival and maturation with epitope concentration adjustment. In another recent study, Angeloni et al. used biodegradable bundles of PA-NFs (Figure 4), which were fabricated by self-assembly and incorporated sonic hedgehog (SHH) protein molecules, for controlled delivery of the latter, which has an important role in cavernous nerve integrity [117].
Figure 5 Structure of peptide-amphiphile (PA) used to form mono-domain noodle gels (a), molecular model of the PA molecule (b), nanofiber formation through self-assembly (c), nanofiber bundles assembled in a longitudinal alignment after noodle formation. Reproduced with permission from [117].

SA is viewed as an approach to change and control the functions of the scaffolds for neural repair [114, 118]. However, self-assembled nanomaterials have limited mechanical strength and unfavorable degradation rates. Moreover, fabrication of these structures using this technique for large neural gaps is not very practical [115].

6.1.3. Phase Separation

Another technique for NF production is phase separation (PS). The rationale for phase separation is the physical incompatibility or immiscibility of the polymer and the solvent. Initially, the polymer is dissolved in a solvent and the resulting solution is kept at the gelation temperature to promote gel formation of the polymer phase. After this, the solvent can be extracted from the gel and a porous nanofibrous skeleton is recovered. There are various types of PS methods for preparing NFs, such as nonsolvent-, chemically- and thermally-induced phase separation (TIPS).
In TIPS, polymer-rich and solvent-rich phases are produced by reducing the temperature of the polymer solution. The solvent-rich phase is removed from the solution by extraction or simple evaporation, leaving behind a solid, skeletal-like polymer matrix with pores. The morphology of the pores depends on various factors like concentration of the polymer solution, solvent properties, PS temperature and type of solvent removal techniques. TIPS is classified into different methods depending on solvent freezing- and phase separation temperatures. If the former is lower than the phase separation temperature, then PS occurs when the solution is cooled to between the lower- and upper critical solution temperatures i.e. in the unstable region of the binary phase diagram. At this temperature, a stable morphological pore network forms in the polymer foam. This is called the liquid-liquid phase separation (LLPS) process. Several synthetic biodegradable polymer scaffolds have been formed using this method in solvents like tetrahydrofuran and dioxane/water etc. [119-121].

In the solid-liquid phase separation (SLPS) process, the solvent freezing point is higher than the phase separation temperature of the solution, and the solvent freezes when temperature is reduced below this point. The polymer comes out of solution with a pore structure corresponding to the crystallization front of the solvent. Similar to LLPS, some of the synthetic biodegradable scaffolds have been produced using this method in solvent mixtures of dioxane and water [122]. Microporous membranes of polypropylene, polyethylene etc. have been prepared using TIPS and SLPS [123].

6.2. Nano-structures

Nanomaterials of different morphologies, produced by electrospinning, phase separation and self-assembly, can enhance axonal outgrowth and facilitate neural regeneration. Most important
of these nanomaterials are nanogels (NGs), nanoparticles (NPs) and, in particular, nanotubes (NTs) and nanofibers (NFs), as these can mimic the tubular structures and within cells and tissues [124].

6.2.1. Nanotubes

Nanotubes are cylindrical structures with diameters of ~1-100 nm [125-128]. They are capable of simulating various intracellular structures such as microtubules and axons, etc. and also can penetrate cell membranes. They have a very high tensile strength (i.e. high strength to volume ratio) [129, 130]. With respect to neural regeneration, NGCs with bundles of NTs may provide higher surface area compared to that of conduits alone [131]. Moreover, elongation in 1-D can provide a better support and orientation to axon regeneration [132, 133]. Carbon NTs (CNTs) were tested and recommended for use as NGCs because of their favorable mechanical, elastic, electrical and rheological properties [134-136]. Graphene, the main constituent of CNTs, has been shown to promote neurite sprouting and outgrowth compared to tissue culture polystyrene substrates [137]. Various functional groups (e.g., carboxylic acid, ethylenediamine etc.) can be attached to CNTs to modify their chemical and electrical properties to promote controlled neuronal regeneration [138]. It was reported that human embryonic stem cells differentiated into neural lineages more efficiently on silk-CNT scaffolds with higher expression levels of β-III tubulin and nestin [139]. CNTs were also shown to promote formation of synaptic contacts and modulation of the cells’ plasticity [140].

6.2.2. Nanofibers

Nanofibers or nanowires are similar to NTs in structure and functionality. They are porous cylinders oriented in 1-D with a high interconnectivity and high specific surface area. They can
mimic the tubular structure associated with ECM like NTs. Various biomaterials have been used to make NFs using electrospinning [109, 141, 142]. NFs made of polyhydroxyalkanoate (PHA) were observed to promote neural stem cell attachment, synapse formations (synaptogenesis) and central nervous system regeneration [16]. Chitosan NF mesh tubes immobilized with electrically-charged β-tricalcium phosphate (β-TCP) particles improved nerve regeneration by increasing axon density and area [143]. Mammadov et al. used a synthetic peptide nanofiber to mimic the activity of laminin and heparan sulfate proteoglycans to enhance axonal growth and induce neuritogenesis, respectively. These self-assembled NF scaffolds were shown to be effective for neurite outgrowth as well as surmounting the inhibitory effect of chondroitin sulfate proteoglycans [144]. Electrospun-collagen NFs impregnated with neurotrophin-3 (NT-3) and chondroitinase ABC (ChABC) supported neuronal culture and neurite outgrowth for a longer period than bolus delivery of NT-3. These hold promise for nerve regeneration following spinal cord related injuries, as they provide topographical and biochemical cues and suppress the inhibitory activity during axonal regrowth in the CNS [110].

6.2.3. Nanoparticles

Nanoparticles are tiny particles having at least one of dimension under 100 nm. NPs with lower melting points [145], higher solar absorption capabilities [146], and super paramagnetism [147] etc., are typically different from bulk materials. Due to this, NPs have been used in various industrial applications, e.g., electronics, biomedical engineering, optics etc. NPs have very high surface area-to-volume ratios resulting in high diffusion gradients [148] and enhanced released of substances such as drugs, proteins, peptides etc., from NPs to the surrounding environments.
Chitosan-heparin NPs impregnated with nerve growth factors were shown to improve nerve regeneration in mice and to function as a robust NP drug delivery system to the sciatic nerve [149]. Super-paramagnetic NPs functionalized with TrkB receptor antibodies were shown to be endocytosed into signaling endosomes by neurons which in turn promoted neurite outgrowth and activated TrkB dependent signaling [150]. Nano-silver embedded into collagen scaffolds coated with laminin and fibronectin were shown to increase axonal regeneration and number of the recovered nerves were comparable to an autologous nerve graft [151]. Nerve regeneration was improved using microstructured polymer filaments in the form of nerve implants containing chitosan/siRNA NPs. These stable were rapidly internalized by cells and did not affect cell viability [152]. A nanostructured 2-D substrate comprising gold NPs attached to the surface of a cover glass via an adsorption system has been shown to improve neurite outgrowth of PC12 cells in the presence of electrical stimuli [153]. Such gold NPs can be used as suitable tools for nerve regeneration from neuronal cells.

6.2.4. Nanogels

Nanogels are among the more recent products to be used in nanotechnology. They are nano-scale hydrophilic cross-linked networks of biocompatible polymers and are mostly used for encapsulating drugs and as efficient drug delivery vehicles [154, 155]. Nanogels are prepared using polymerization techniques such as free radical crosslinking [156], free radical precipitation [157], nano-emulsion polymerization [158], etc. Porous scaffolds have been prepared from protein NGs [159] which may be useful for NTE purposes as well. These gels have also been suggested as a promising system for delivering of drugs like oligonucleotides to the brain [160].
7. Conclusions and future directions

The cellular and chemical mechanisms essential for functionality of the nervous system are extremely complex and still not entirely understood. This in itself presents formidable challenges to neural tissue engineering efforts. Research to discover and implement neural regeneration strategies have been underway for several decades. Substantial improvements in neuroregeneration with functional recovery have been obtained using NRC implantation. However, technology for NRCs has not sufficiently advanced to allow them to completely replace grafts for their effectiveness, especially in cases of severe injuries and longer gaps. Advances in nanotechnology along with other neural tissue engineering strategies have provided alternatives and implementation of guidance cues to facilitate healing of neural lesions.

Several scaffold types have been used to make nano-NRCs from blends of natural or synthetic materials with the desired physical and chemical properties compatible with extracellular matrices. Their use has improved cell survival, adhesion, growth, proliferation, orientation and differentiation. Composites fabricated using the latest techniques in entubulization and bioengineering have produced encouraging results. Guidance cues are also critical to success, among them the use of specific cell types to maximize reinnervation and promote axonal growth to bridge nerve gaps. Growth factors have been combined with other guidance cues to facilitate nerve regeneration. Recent advances in tissue engineering have enabled the use of cells genetically modified to secrete specific growth factors. Thus, NRCs’ function is not only as guidance cues, but also as platforms for controlled release of therapeutic agents such as trophic factors and cellular constituents such as ECM molecules. Incorporation of these guidance cues may mimic the molecular and cellular components of the microenvironment.
The use of nanotechnology offers many significant and attractive advantages; however, at the molecular level, it poses a number of challenges. Nano-engineered structures can be functionalized for specific use in neural regeneration and other applications. For example, nanoparticles are used in designing devices for the controlled release of medicines, while NFs and NTs can mimic the tubular structures of the ECM, a desirable feature in designing NRCs for the repair of neural lesions. However, improvements in specificity are still needed to enhance and expand their therapeutic potential for clinical treatments [161]. In the case of NRCs, the use of nano-structures for improved control of guidance cues offers potential for successful neuroregeneration therapies.

Neurotrophic factor releasing systems are also expected to play a pivotal role in the design NRCs. Current limitations of such devices are lower encapsulation efficiency, degradation products, sustained release, faster fabrication procedures and cost effectiveness. Combining variety of NTFs in required amounts in one single device is anticipated to be one of the challenges for developing such optimized systems. Another challenge is the time dependent release of proteins corresponding to the various stages of the neural regeneration. Additionally, reducing the cytotoxicity caused by these devices and reduction of negative impact on tissue sections other than the targeted areas should also be considered.

In spite of the progress made to date, more research on scaffold design and evaluation in vivo is still needed. Using single regenerative cue at a time has shown to improve the nerve regeneration but still there are various limitations associated with them such as incomplete recovery and no functional benefits. Autologous nerve graft, also considered as the gold standard of nerve repair strategies comes with many drawbacks such as donor site morbidity, multiple surgeries and
cannot be used for larger nerve injuries. In our approach, we are trying a combinatorial approach to combat such limitations. We are combining various regenerative cues together to enhance the peripheral nerve regeneration. Topographical cues in form of micropatterned conduit can help the axons to grow larger distances. Biological cues by using transdifferentiated patient specific bone marrow derived MSCs may help in replacing the native Schwann cells of the peripheral nervous system. Chemical cues in form of NGF releasing nano/microparticles will provide the additional growth and trophic factors to the growing axons. In turn, combining these topographical, biological and chemical cues may result in an important strategy to overcome the limitations and to further enhance the peripheral nerve regeneration. Neural tissue engineering is a challenging undertaking, especially given the complexities of the nervous system. The ability to duplicate the microenvironment of the ECM with guidance cues must be evaluated in models that mimic the human body. Trials with animals larger than rodents will provide a better understanding of how regenerative therapies will perform in humans. The contributions of nanotechnology should be used for NRCs after overcoming the drawbacks and possible health risks. In spite of the complex challenges, nanotechnology, along with improved biomaterials, drug delivery capability and tissue engineering will provide alternative therapies with successful clinical applications.

8. References


5. Millesi, H., *Bridging defects: autologous nerve grafts*


10. Dahlin, L., et al., *Schwann cells, acutely dissociated from a predegenerated nerve trunk, can be applied into a matrix used to bridge nerve defects in rats*


11. Hausner, T., et al., *Nerve regeneration using tubular scaffolds from biodegradable Polyurethane*


CHAPTER 3: ORIENTED GROWTH AND TRANSDIFFERENTIATION OF MESENCHYMAL STEM CELLS TOWARDS A SCHWANN CELL FATE ON MICROPATTERNED SUBSTRATES

Adapted from manuscript:


1. Abstract

While Schwann cells (SCs) have a significant role in peripheral nerve regeneration, their use in treatments has been limited because of lack of a readily available source. To address this issue, this study focused on the effect of guidance cues by employing micropatterned polymeric films to influence the alignment, morphology and transdifferentiation of bone marrow-derived rat mesenchymal stem cells (MSCs) towards a Schwann cell (SC) - like fate. Two different types of polymers, biocompatible polystyrene (PS) and biodegradable poly (lactic acid) (PLA) were used to fabricate patterned films. Percentages of transdifferentiated MSCs (tMSCs) immunolabeled with SC markers (α-S100β and α-p75NTR) were found to be similar on patterned versus smooth PS and PLA substrates. However, patterning had a significant effect on the alignment and elongation of the tMSCs. More than 80% of the tMSCs were oriented in the direction of microgrooves (0°-20°), while cells on the smooth substrates were randomly oriented. The aspect ratio (AR) of the tMSCs on patterned substrates had a value of
approximately five, as compared to cells on smooth substrates where the AR was one. Understanding responses to these cues in vitro helps us in understanding the behavior and interaction of the cells with the 3D environment of the scaffolds, facilitating the application of these concepts to designing effective nerve guidance conduits for peripheral nerve regeneration.

2. Keywords
Micropatterning, Polymeric films, Mesenchymal stem cells, Transdifferentiation, Schwann cells.

3. Introduction
Schwann cells are peripheral glia that form myelin sheaths, and are known to play significant roles during peripheral nerve regeneration[1]. Nerve injury in the peripheral nervous system (PNS) is followed by Wallerian degeneration, which continues at the distal stump. Schwann cells clear the myelin debris while proliferating and forming bands of Büngner that promote axonal regeneration through the distal nerve stump. In addition, SCs secrete trophic and growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF), all of which promote neural regeneration [2, 3]. It has been shown that implantation of SCs supports axonal elongation and regeneration in vitro and in vivo [4, 5]. Furthermore, SCs were also reported to overcome the inhibitory effect of central nervous system (CNS) components following injury [6]. These properties enable SCs to be one of the most attractive cell based therapies for PNS and CNS regeneration.

However, despite their enormous potential, harvesting SCs requires multiple surgeries and sacrifice of a donor nerve that may result in donor site morbidity. These drawbacks have led to alternative approaches, such as transdifferentiation of more readily available cells into SC-like
phenotypes. Multipotent MSCs are emerging as an important and versatile cell source for neural repair strategies. It has been shown that upon proper treatment, MSCs can be transdifferentiated into SC-like phenotypes [7]. These cells resemble typical SCs morphologically and molecularly [5]. Due to self-renewal ability, high proliferation rate, and multi-lineage differentiation abilities, MSCs are an attractive cell type to be used in neuroregenerative strategies [8]. MSCs can be harvested from several sources, i.e. bone marrow, adipose tissue, and umbilical cord and can differentiate into multiple mesodermal cell lineages (chondrocytes, osteocytes, and adipocytes)[9]; and several neuronal and glial phenotypes [10, 11] under certain conditions and stimulations.

The morphological and phenotypic changes in the transdifferentiated MSCs (tMSCs) were demonstrated using SC markers by immunocytochemistry (ICC), enzyme-linked immunosorbent assay (ELISA) and Western blotting analyses [5, 7, 12]. These phenotypes were shown to enhance axonal growth [13] and remyelination [14] as well as promote functional outcomes [7, 14-16].

Enhanced neuronal regeneration should be supported with directionality. An oriented axonal regrowth or cell alignment will be superior compared to randomized cellular therapies for nerve regeneration. Specific microarchitectures and topographical cues such as grooves, pores etc., have been shown to promote axonal growth and direct cellular orientation [17, 18]. Micropatterned biodegradable substrates in porous conduits, and pre-seeded with SCs have been shown to promote neurite outgrowth, axonal regrowth and nerve regeneration [19-21]. Micropatterning of the polymer scaffold enables building of specific biomaterial architectures and can also influence stem cell differentiation. Previously our lab has reported enhancement in neuronal differentiation and 75% alignment along micropatterned substrates for adult rat
hippocampal progenitor cells (AHPCs) [22]. In another study, the synergistic effects of micropatterned substrates, astrocyte-derived soluble factors and co-culturing with astrocytes, resulted in enhanced neuronal differentiation of AHPCs [23]. Qi et.al [24] demonstrated that the processes of neural stem cells (NSCs) were oriented parallel to the microgrooves, while those on the non-patterned surfaces were randomly positioned in vitro.

Therefore, in the present study, the transdifferentiation of MSCs seeded on micropatterned substrates to SC-like phenotypes was investigated, due to the ease of harvesting bone marrow stem cells and for their potential use in conduits for nerve regeneration. Rat MSCs from bone marrows of Brown Norway rats were used. Several MSC types have been tested for their transdifferentiation ability; however, Brown Norway rat MSCs have not been tested yet, to our knowledge. They were favored due to the ease of harvesting bone marrow stem cells. Biocompatible polymers polystyrene (PS) and biodegradable poly (lactic acid) (PLA) were used for fabricating the micropatterned films to investigate the effect of topographical cues on transdifferentiation, alignment and morphology of the MSCs. In the present study, the transdifferentiation ability of Brown-Norway rat MSCs to a SC-like phenotype was tested quantitatively as well as qualitatively on smooth and micropatterned polymer substrates.

4. Materials and methods

4.1. Fabrication of micropatterned polymeric films

Two different types of polymers were chosen for polymeric film fabrication – polystyrene (PS) (M.W. 125,000-250,000) (Cat #00575, Polysciences, Inc. Warrington, PA) and poly (lactic acid) (PLA). PS is a widely used biocompatible polymer in tissue culture applications, while PLA is a biodegradable polymer and is frequently used for making transplantable scaffolds for in vivo studies. Previously in our group, we have developed a method [22, 25] of producing
micropatterned PS and PLA films using micropatterned silicon wafers. Micropatterned silicon wafers were fabricated using conventional lithographic techniques and polymer films were fabricated using solvent casting [22] and detached from the wafers. These films have been shown to support growth of dorsal root ganglion cells, SCs, astrocytes and neural progenitor cells (NPCs) [20, 22, 25, 26].

For PS films, 6% PS solution in toluene was poured on the micropatterned silicon wafer and dried for approximately 4 hours. For PLA films, 10% PLA solution was poured onto a silicon wafer spinning at a speed of 75 rpm using a spin coater (Model PWM32-PS-R790, Headway Research, Inc. Garland, TX). The PLA solution on the wafer was left to dry for 10 minutes on the spin coater before transferring to a chemical hood for an additional 6 hours. Films were carefully peeled off using forceps after submerging the wafer and the dried films in deionized water. Dimensions of the micropatterns obtained on the polymeric films were measured using scanning electron microscopy (SEM, Quant FEG 250, Hillsboro, OR) and were found to be 11-13 µm, 16-18 µm and 3.5-4.5 µm in groove, mesa and depth, respectively for both PS and PLA films. The dried square films were cut to the size of 0.71 cm² with a razor blade, with half of the film being patterned and the other half smooth. Silastic medical adhesive (Dow Corning Corp., CITY, STATE) was used to attach the square film with a side length of 8.4 mm to a detergent-cleaned 12 mm glass coverslip. The glass coverslips with attached films were sterilized with 70% ethanol and UV light, and coated with poly-L-lysine (PLL, Sigma Aldrich, St. Louis, MO) at a dilution of 1:10 before cell plating.

4.2. Isolation of Brown Norway rat MSCs

All animals were acquired and cared for in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals and all procedures adhered to the principles
presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience and had the approval of the Iowa State University Institutional Animal Care and Use Committee, and were performed in accordance with committee guidelines. Brown Norway rats were obtained from Charles River Labs, and at 6 weeks of age were used for the isolation of bone marrow. The animals were kept in a constant environment (temperature: 22°C; humidity: 20%; 14/10-hour light-dark cycle) with food and water provided ad libitum until the bone marrow was isolated. The rats were allowed to acclimate after arrival for at least 7 days before harvesting bone marrow.

The rats were euthanized with isoflurane before the dissection of the femora and tibiae. The bones were placed in 50 mL conical tubes with ice-cold maintenance media (MM) composed of alpha minimum essential media (αMEM, Gibco BRL, Gaithersburg, MD), supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 4 mM L-glutamine (Gibco BRL, Gaithersburg, MD), and antibiotic-antimycotic (Invitrogen, Carlsbad, CA). Using a syringe and a 23-gauge needle filled with 3 mL MM, the marrow was forced from the Epiphysis of each bone, into a culture dish. The marrow suspension was dispersed through a 70 µm nylon cell strainer (BD Falcon, Bedford MA), dampened with MM and transferred into a T75 flask with 20 mL MM. The cell suspension was maintained in a culture incubator (37°C, 5% CO2/95% humidified air atmosphere). After 48 hours, post-harvest, spindle-shaped cells adhered to the bottom of the flask. The cells were washed with phosphate buffered saline (PBS; Gibco BRL, Gaithersburg, MD) and the media was replaced with fresh MM (Ye et al. 2014, in preparation).

4.3. Culturing of Brown Norway rat MSCs

Brown Norway rat MSCs were plated in T75 flasks in MM, consisting of α minimum essential medium (αMEM, Gibco BRL, Gaithersburg, MD), 20% fetal bovine serum (FBS; Atlanta Bio.),
4 mM L-glutamine (Gibco), and antibiotic-antimycotic (Invitrogen, Carlsbad, CA) and incubated at 37°C/ 5% CO2/ 95% humidified air atm. Adherence to the flask was observed from flat fibroblast-like cells. When the MSCs were ~80% confluent, media was aspirated and cells were washed with PBS to remove serum before trypsinizing the cells. Cells were collected transferred into 15 ml conical tubes and centrifuged at 500 rpm for 5 minutes. Following centrifugation, the cell pellets were resuspended in MM and the cell suspensions plated out onto various polymeric films following Trypan blue viable cell counts.

4.4. In vitro transdifferentiation of rat MSCs into SC-like phenotypes

In vitro chemical induction of the rat MSCs into SC-like phenotypes was performed in three steps following the protocol established by Dezawa and coworkers [7]. Rat MSCs were kept in MM for 1-2 days in T75 flasks at 37°C/ 5% CO2. After reaching 30-40% confluence, the MM media was replaced with the transdifferentiation media-1 (TDM-1) containing αMEM supplemented with 1 mM β-mercaptoethanol (BME; Sigma-Aldrich, St. Louis, MO) and incubated for one day. After a PBS wash, TDM-1 was replaced with the TDM-2 containing αMEM, 10% fetal bovine serum (FBS) and 35 ng/ml all-trans-retinoic acid (ATRA; Sigma, St. Louis, MO). The cells were incubated in TDM-2 for the following 3 days at 37°C/ 5% CO2. Finally, the cells were incubated in TDM-3 which contained αMEM, 10% FBS, 14 μL forskolin (FSK; EMD Millipore, Billerica, MA), 5 ng/mL platelet derived growth factor (PDGF; Sigma), 10 ng/mL basic fibroblast growth factor (bFGF, Promega Corporation, Madison, WI) and 200 ng/mL heregulin β1 (HRG; Calbiochem, EMD Millipore, Billerica, MA ) for 8 days in vitro (DIV). Prior to the media changes, cells were always washed with PBS. At the transition to TDM-3, the cells were plated onto glass coverslips or half patterned-half smooth polymeric substrates attached to glass coverslips. The polymer film substrates were coated with poly-L-
lysine (PLL) and placed in a 35 mm petri dish etched into quadrants with a soldering iron. Control or undifferentiated MSCs (uMSCs; MSCs cultured in MM) were always grown in parallel to the MSCs undergoing transdifferentiation (tMSCs; SC like-cells). Undifferentiated and transdifferentiated MSCs grew at different rates in MM and different TDMs respectively. In order to obtain a comparable cell number after a period of 14 DIV, initial cell plating density was altered. Undifferentiated MSCs were plated at a density of 1000 cells/cm² while tMSCs were plated at a density of 3000 cells/cm². MSCs from passages 4 to 7 were used for replicate studies of the transdifferentiation experiment. Immunocytochemical procedures were used to characterize the uMSCs and tMSCs (see below). The transdifferentiation and cell morphology on the patterned half of each substrate was compared with that of the smooth half on the same substrate.

4.5. Immunocytochemistry (ICC)

After 8 DIV, the cells in culture dishes were rinsed with 0.1 M PO4 buffer and fixed with 4% paraformaldehyde prepared in 0.1 M PO4 buffer. The cells were then rinsed with filtered phosphate buffered saline (PBS; Fisher-Scientific) for 3 times every 7 minutes and incubated in blocking solution of PBS supplemented with 5% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA), 0.4% bovine serum albumin (BSA; Sigma) and 0.2% Triton X-100 (Fisher Scientific).

Several antibodies were used for ICC analysis for characterization of undifferentiated MSCs (uMSCs) (Table 1) and transdifferentiated MSCs (tMSCs) (Table 2). The primary antibodies were diluted with blocking solution. Samples were incubated with primary antibodies at 4°C overnight. On the following day, the cells were rinsed with PBS for 4 times every 8 minutes, and subsequently incubated in appropriate secondary antibodies diluted in blocking solution. The
following secondary antibodies were used: Donkey-α-Mouse-Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA), Donkey-α-Rabbit-Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA). In addition, MSCs were stained with Alexa Fluor 488 Phalloidin (1:1000, Molecular Probes-Life Technologies, Carlsbad, CA), and DAPI (4',6-diamidino-2-phenylindole) (1:2000, Invitrogen, Carlsbad, CA), all diluted in the same blocking solution used for secondary antibody staining. The cells were incubated for 60-90 minutes in the dark at room temperature and then rinsed with PBS for 3 times every 7 minutes. Immunolabeling control samples were incubated without primary antibodies, and with secondary antibodies. The glass coverslips were mounted cell side down on microscope slides with Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and clear nail polish was used for creating an air-tight seal. The sample was mounted with careful removal of the polymeric film from the cover slip with a forceps. The film was slid into the middle of a drop of Vectashield, cell side up, on a microscope slide. Cover glass was placed on top of the film and Vectashield and an air tight seal was formed with nail polish. Preparations were examined and images captured using an upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital camera controlled by QCapture software (QImaging, Surrey, BC, Canada). For image analysis, a minimum of 8 microscope fields from each substrate were taken randomly using a 20X objective. Each field represents 0.17 mm² (476 µm x 357 µm). To calculate the percentage of immunoreactive cells on each substrate, the number of cells immunoreactive for each antibody was divided by the total number of cells (DAPI-stained nuclei). Images were uploaded to MetaXpress software (Molecular Devices, Sunnyvale, CA) and with help of multiwavelength cell scoring module; number of cells stained with different antibodies/wavelengths were obtained.
Table 1. Undifferentiated MSCs were characterized using various positive and negative MSC antibody markers.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Specific Protein</th>
<th>Protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse α CD54</td>
<td>1:100</td>
<td>Intracellular adhesion molecules-1 (ICAM 1) Proteins</td>
<td>Positive</td>
</tr>
<tr>
<td>Rabbit α CD29</td>
<td>1:1000</td>
<td>Integran β1</td>
<td>Positive</td>
</tr>
<tr>
<td>Rabbit α Collagen</td>
<td>1:200</td>
<td>Collagen</td>
<td>Positive</td>
</tr>
<tr>
<td>Rabbit α Fibronectin</td>
<td>1:1000</td>
<td>Fibronectin</td>
<td>Positive</td>
</tr>
<tr>
<td>Mouse α CD14</td>
<td>1:100</td>
<td>Hematopoietic cell surface markers - present on leukocytes</td>
<td>Negative</td>
</tr>
<tr>
<td>Mouse α CD45</td>
<td>1:200</td>
<td>Hematopoietic cell surface markers - present on monocytes and macrophages</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 2. Antibody markers used for immunostaining transdifferentiated MSCs.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Specific Protein</th>
<th>Positive Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse α S100β</td>
<td>1:1000</td>
<td>Calcium Binding Protein</td>
<td>Glial Cells</td>
</tr>
<tr>
<td>Mouse α GFAP</td>
<td>1:1000</td>
<td>Glial Fibrillary Acidic Protein</td>
<td>Glial Cells</td>
</tr>
<tr>
<td>Rabbit α p75NTR</td>
<td>1:1000</td>
<td>Neurotrophic Receptor</td>
<td>Glial Cells</td>
</tr>
</tbody>
</table>

4.6. Treatment with bromodeoxyuridine (BrdU)

MSCs were cultured for 3 DIV and 12 hours before fixation, the cells were incubated with MM supplemented with BrdU to a final concentration of 5 µM. After fixation, the cells were treated with 2N HCl at 37°C for 15 minutes and then replaced with 0.1 M borate neutralization buffer
for 15 minutes. The neutralizer was removed and the cells were rinsed with PBS for 3 times every 7 minutes. The rest of the procedure was carried out in accordance with the ICC stated above, starting with the one hour incubation in blocking solution. Primary antibody used was Rat α BrdU (1:200, Abcam, Cambridge, MA).

4.7. Data and statistical analysis

Aspect ratio (AR) of an object is defined as the ratio of object’s width to its height, when the width of the object is longer than its height. Aspect ratio of MSCs have been shown to affect their lineage commitment [27]. Objects such as regular polygons or circular objects have AR equal to one, which means that there is no difference in height and width of the object. Images were binarized by determining minimum threshold intensity to differentiate between the background and cells stained with phalloidin. Integrated morphometric analysis module of MetaXpress software was used for measuring various morphological parameters such as width, height, cellular area and orientation of the cellular objects obtained after thresholding. Orientation of the cells was measured between -90 to +90 degrees from the horizontal where all the images captured had micropatterns parallel to the horizontal. Thirty cells per condition (PLA pattern, PLA smooth, PS pattern, PS smooth) per experiment were analyzed to obtain the data. Three independent experiments were analyzed, giving 90 cells per condition. GraphPad prism v6 software (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis. One way ANOVA with Tukey post-hoc test was used to compare the means across the various conditions. A p-value ≤ 0.05 was considered significant during mean comparisons. Error bars in graphs represent standard error of the mean.
5. Results

Patterned PS and PLA films were successfully fabricated by solvent casting using micropatterned silicon wafers. Immunocytochemistry and cell staining procedures were used to examine changes in MSCs’ transdifferentiation, morphology and alignment. We have demonstrated that MSCs align themselves along the direction of microgrooves when grown on micropatterned substrates. In contrast, MSCs growing on a smooth, non-patterned surface were randomly oriented. Patterning did not affect transdifferentiation of MSCs towards SC-like phenotypic cells as compared to smooth polymeric substrates. Results indicate that substrate topography strongly influences the morphology and growth of the MSCs.

5.1. Characterization of uMSCs

Undifferentiated MSCs isolated from bone marrows of Brown Norway rats were characterized by immunostaining for various positive and negative rat MSC markers. MSCs were found to be positively immunostained against Fibronectin, Collagen, CD54 (ICAM1, Intracellular adhesion molecule 1), and CD29 (Integrin β1). Negative rat MSC markers such as CD14 and CD45 did not stain any of these cells. Also MSCs were successfully differentiated to form osteoblasts and adipocytes showing that they are multipotent in nature (Ye et al. 2014, in preparation).

5.2. Fabrication of Polystyrene and Poly (lactic acid) films

PS and PLA films were successfully fabricated by solvent casting, and scanning electron microscopy (SEM) was used to measure the dimensions of micropatterns. Dimensions of the micropatterns obtained on the polymeric films were found to be 11-13 µm, 16-18 µm and 3.5-4.5 µm in groove, mesa and depth, respectively for both PS and PLA films. PS films obtained were similar to the ones fabricated by Recknor et. al. [22, 25]. Figure 1 shows SEM images of
micropatterned PS and PLA films at different magnifications. PLA films were found to have a small taper at the side of mesas as compared to PS films.

Figure 1. Scanning Electron Microscopy (SEM) images of micropatterned Polystyrene (PS) and poly (lactic acid) (PLA) films. Dimensions of the micropatterns obtained on the polymeric films were found to be 11-13 µm, 16-18 µm and 3.5-4.5 µm in groove, mesa and depth, respectively for both PS and PLA films. (A) PS film at 300X magnification. (B) PS film at 1500X magnification. (C) PLA film at 300X magnification. (D) PLA film at 3000X magnification.
5.3. Effect of guidance cues on alignment and morphology of MSCs

Undifferentiated MSCs (uMSCs) were grown on poly-L-Lysine coated half patterned – half smooth polymeric substrates in their normal maintenance medium (MM) for 3DIV (days in vitro). While the cells on smooth substrates were found to be fibroblastic in morphology, cells on patterned substrates were found to be elongated and growing in parallel to the direction of the grooves. Cells were found to be growing both within the grooves as well as on the top of the mesas. In order to determine the orientation and morphological changes associated with guidance cues, cells were then stained with Alexa Fluor 488 Phalloidin and DAPI, which stains actin filaments and nuclei, respectively.

Angle of the longest chord through each cell relative to the horizontal axis (i.e. parallel with the direction of the micropatterns) was used for the orientation data and was compared to the cells grown on the smooth part of the substrate. Number of cells was grouped between 10o sectors between 0o to 90o. More than 80% of the cells were found to be oriented in the direction of micropatterns (0o to 20o) (Fig. 2A for PS substrates and 2B for PLA substrates).
Figure 2. The distribution of uMSCs alignment on micropatterned and smooth PS and PLA substrates. It was demonstrated that an oriented cell alignment was obtained with micropatterning for uMSCs. The data was grouped in 10º sectors from 0º to 90º. Over 80% of
the cells were found to be in range of 0°-20° for patterned substrates while cells were found to be randomly oriented on smooth substrates. Thirty cells per experiment (Total 90 cells/condition) were analyzed. (A) uMSCs alignment on Polystyrene films. (B) uMSCs alignment on PLA films. N=3 independent experiments and error bars represents standard error of the mean.

Here, in this study we also determined the AR of the cells grown on patterned substrate and compared to the AR of the cells grown on smooth substrate. Figure 3A showed that cells on patterned substrates were elongated with an AR closer to 5, while cells on the smooth substrates had an AR close to 1, showing the inclination of the cells to have a circular shape in the absence of patterns. Cells grown on PS Pattern and PLA Pattern were found to have AR of 5.03±1.14 and 4.99±1.72 respectively as compared to values of 1.08±0.06 and 1.28±0.19 for PS Smooth and PLA Smooth, respectively. Cellular area was also compared between the cells grown on smooth versus patterned substrates. While the uMSCs seem to be elongated on patterned substrates, the average area of the cells on pattern substrates was found to be less as compared to cells grown on smooth substrates (Fig. 3B). Cellular area for cells grown on Pattern PS, Pattern PLA, Smooth PS and Smooth PLA was found to be 821.10±194.02, 594.29±73.34, 1196.26±147.60 and 957.43±67.56 µm², respectively.
Figure 3. The comparison of aspect ratios and cellular areas of uMSCs grown on micropatterned vs. smooth PS and PLA substrates. (A) Aspect ratio of uMSCs on various substrates. Aspect ratio is ratio of width to the height of the cell. Aspect ratios greater than one implies elongation of the object in one particular direction. (B) Cellular area of uMSCs on various substrates. Aspect ratios were found to be significantly different while cellular areas were not.
Aspect ratio of the cells growing on patterned PS (PS Pa) and patterned PLA (PLA Pa) was found to be significantly higher than smooth polystyrene (PS Sm) and smooth PLA (PLA Sm) substrates at p ≤ 0.05. Cellular area was found to be greater for the cells growing on smooth substrates. However, no significant differences were observed between cellular areas of the cells growing on patterned and smooth parts of the same polymer film at p ≤ 0.05.

5.4. Effect of guidance cues on proliferation of MSCs
In addition to quantifying the morphological changes of the cells associated with micropatterning, we also investigated whether patterning affects the proliferation of uMSCs as compared to the smooth control part of the polymeric film. BrdU was added to the cells 12 hours prior (at 2.5DIV) to fixation of the cells (3DIV). No significant differences were observed in the proliferation of uMSCs on the basis of BrdU immunostained cells at p ≤ 0.05 across all 4 conditions (PS Pa, PS Sm, PLA Pa and PLA Sm). Percentage of BrdU-stained uMSCs grown on PS Pa, PS Sm, PLA Pa and PLA Sm was found to be 33.62±8.01, 39.24±8.62, 42.44±14.87 and 36.53±10.24% respectively (Figure 4). Proliferation was found to be slightly higher on PLA substrates as compared to PS substrates, suggesting that the biodegradability of substrates may have some influence on proliferation of uMSCs. Proliferation was found to be least for glass substrates (data not shown). Glass substrates were used for initial characterization and were no longer used for comparison for their inability to support cellular proliferation. Culturing of the cells on micropatterned substrates seems to cause no obvious reduction in proliferation. Supplemental Figure 3 shows percentage BrdU immunostained uMSCs data for both smooth and pattern PS and PLA substrates.
Figure 4. Graph showing percentage of BrdU-labeled uMSCs for various polymeric substrate conditions. Error bar represents standard error of the mean. N=3 independent experiments. No significant differences were observed between any of the substrate types and patterning does not seem to influence proliferation of uMSCs.

These results show that micropatterning of polymeric substrates can be used as a strong tool to influence the morphology and alignment of the cells without causing any unexpected or deleterious changes in the elementary natural characteristics of the MSCs.

5.5. Effect of guidance cues on transdifferentiation of MSCs toward a SC-like phenotype.
Identification of the transdifferentiated MSCs (tMSCs) was based upon immunolabeling with antibody markers used to identify SCs, anti-S100β and anti-p75NTR [28].
The cells were plated on the polymeric films attached to a glass coverslip. MSCs subjected to transdifferentiation conditions grew at a slower rate compared to uMSCs. Based on an initial study, the control undifferentiated MSCs (uMSCs) were plated at 1000 cells/cm² and the MSCs subjected to the transdifferentiation conditions at 3000 cells/cm². The results, obtained by fluorescent microscopy, revealed that both anti-S100β (Fig. 5 and 6) and anti-p75NTR (Supplemental Fig. 1 and 2) antibodies immunolabeled very few uMSCs compared to tMSCs. While no anti-S100β immunostaining was detected for the uMSCs, anti-p75NTR sometimes stained the nuclei of the uMSCs. However, anti-p75NTR staining for tMSCs was found to be cytoplasmic as opposed to occasional nuclear staining of uMSCs.
Figure 5. Transdifferentiated MSCs (tMSCs) on micropatterned PLA and PS films have a higher percentage of cells labeling with anti-S100β antibody than uMSCs. A, A’ and A’’ represents the corresponding anti-S100B, actin filaments (phalloidin) staining and merged image of tMSCs on patterned PLA substrate. B, B’ and B’’ represents the corresponding anti-S100B, actin filaments (phalloidin) staining and merged image of uMSCs on patterned PLA substrate. C, C’ and C’’ represents the corresponding anti-S100B, actin filaments (phalloidin) staining and merged image of tMSCs on patterned PS substrate. D, D’ and D’’ represents the corresponding anti-S100B,
actin filaments (phalloidin) staining and merged image of uMSCs on patterned PS substrate. Abbreviations: uMSCs, undifferentiated mesenchymal stem cells; tMSCs, transdifferentiated mesenchymal stem cells; S100β, anti-S100β antibody; PLA, poly(lactic acid); PS, polystyrene; Merged, merged image of S100β, Alexa-488 Phalloidin, DAPI and DIC (Differential interference contrast). Scale bar = 100µm
Figure 6. Transdifferentiated MSCs (tMSCs) on non-patterned (smooth) PLA and PS films have a higher percentage of cells labeling with anti-S100β antibody than uMSCs. A, A’ and A” represents the corresponding anti-S100B, actin filaments (phalloidin) staining and merged image of tMSCs on smooth PLA substrate. B, B’ and B” represents the corresponding anti-S100B, actin filaments (phalloidin) staining and merged image of uMSCs on smooth PLA substrate. C, C’ and C” represents the corresponding anti-S100B, actin filaments (phalloidin) staining and merged image of tMSCs on smooth PS substrate. D, D’ and D” represents the corresponding
anti-S100B, actin filaments (phalloidin) staining and merged image of uMSCs on smooth PS substrate. Abbreviations: uMSCs, undifferentiated mesenchymal stem cells; tMSCs, transdifferentiated mesenchymal stem cells; S100β, anti- S100β antibody; PLA, poly(lactic acid); PS, polystyrene; Merged, merged image of S100β, Alexa-488 Phalloidin, DAPI and DIC (Differential interference contrast). Scale bar = 100µm

During data quantification, tMSCs and uMSCs were counted only when cytoplasmic staining of anti-p75NTR was observed. Figures 7A and 7B graphically show the comparison of data across various substrate conditions for immunostaining of anti-S100β and anti-p75NTR, respectively for both tMSCs and uMSCs. These results demonstrated that a greater proportion of MSCs subjected to the transdifferentiation protocol were anti-S100β and anti-p75NTR immunoreactive in comparison to the MSCs cultured under control conditions (cultured in maintenance media (MM)). It was also observed that MSCs transdifferentiated on polymeric (PS and PLA) substrates have a higher percentage of cells immunoreactive for S100β and p75NTR compared to glass coverslips (data not shown). Neurotrophin receptor p75NTR immunostaining also followed a similar trend as S100β, where very few uMSCs were found to be immunolabeled for p75NTR as compared to tMSCs where more than 90% cells showed presence of p75NTR protein. For both PS and PLA patterned and smooth substrates, the percentage of S100β and p75NTR immunolabeled cells were found to be significantly higher in cells subjected to the transdifferentiation protocol (tMSCs) as compared to cells grown just in MM (control cells: uMSCs) at p ≤ 0.05.
Figure 7. Graphs showing percentage of S100β and p75NTR labeled cells for various polymeric substrate conditions. (A) S100β labeling of the tMSCs were found to significantly different ($p \leq 0.05$) than the uMSCs for all substrate types. (B) p75NTR labeling of the tMSCs was significantly greater ($p \leq 0.05$) than the uMSCs for all substrate. Patterning caused no deleterious
effect on the transdifferentiation of MSCs into SC-like phenotypic cells. Both polymers were found to have more S100β immunostaining as compared to glass coverslips (data not shown). Error bar represents standard error of the mean. N=3 independent transdifferentiation experiments were carried out. *Significantly different from smooth substrates at p ≤ 0.05.

As shown before for uMSCs, more than 80% of tMSCs were found to be oriented in the direction of the micropatterns for both PS (Figure 8A) and PLA (Figure 8B) substrates. These results illustrate that micropatterning did not cause any decrease in the level of transdifferentiation, and yet facilitated an oriented cell alignment, which is likely to be advantageous for enhanced neural regeneration. Aspect ratio (Figure 9A) was found to be significantly higher on patterned polymeric substrates as compared to their smooth counterpart at p ≤ 0.05. Average cellular area (Figure 9B) was found to be greater on smooth substrates as compared to pattern substrates, but no significant differences were observed at p ≤ 0.05.
Figure 8. The distribution of tMSCs alignment on micropatterned and smooth PS and PLA substrates. It was demonstrated that an oriented cell alignment was obtained with micropatterning for tMSCs. The data was grouped in 10° sectors from 0° to 90°. Over 80% of the
cells were found to be in range of 0°-20° for patterned substrates while cells were found to be random]

ly oriented on smooth substrates. Thirty cells per experiment (Total 90 cells) were analyzed. (A) tMSCs alignmen
t on Polystyrene films. (B) tMSCs alignment on PLA films. N=3 independent experiments and error bars represent standard error of the mean.
Figure 9. The comparison of aspect ratio and cellular area of tMSCs grown on micropatterned vs. smooth PS and PLA substrates. (A) Aspect ratio of tMSCs on various substrates. Aspect ratio is ratio of width to the height of the cell. Aspect ratio of more than one means elongation of the
object in one particular direction. (B) Cellular area of tMSCs on various substrates. Aspect ratios were found to be significantly different while cellular areas were not.

Thirty cells per experiment (Total 90 cells) were analyzed. N = 3 independent experiments and error bars represents standard error of the mean. Abbreviations: PS, polystyrene; PLA, poly(lactic acid), Pa, patterned substrate; Sm, smooth substrate. *Significantly different from smooth substrate at p ≤ 0.05.

6. Discussion

In this study, we have successfully shown that micropatterned polymeric substrates act as very useful tools in providing orientation to the growing uMSCs and tMSCs. Mesenchymal stem cells grown on patterned substrates grew in the direction of the microgrooves, were highly oriented and showed elongation along the grooves. More than 80% of the cells were oriented parallel with the grooves on both PS and PLA patterned substrates. Cells were profoundly elongated (AR > 4) in the direction of the patterned substrates compared to their smooth counterparts (AR ~ 1). On the other hand, cells on the smooth substrates were found to have higher average cellular areas than cells on micropatterned substrates, but no significant differences were observed. While we were able to control the morphological characteristics of the cells with the help of these guidance cues, we found no obvious deleterious effect of patterning on the proliferation and transdifferentiation of the cells. Biodegradable PLA substrates supported the growth and directionality of the cells in a similar fashion to control biocompatible PS substrates. Transdifferentiation seemed to occur more on polymeric substrates as opposed to glass coverslips. Transdifferentiation of MSCs towards a SC fate on the smooth substrates were found to have a variety of morphologies from flat, fibroblast to multipolar in nature as opposed to
mostly flat, fibroblast looking shape of undifferentiated control MSCs. The transdifferentiated cells also immunolabeled for SC-specific protein markers such as α-S100β and α-p75NTR. Micropatterned substrates did not affect transdifferentiation of cells in any way as compared to smooth substrates and tMSCs on patterned substrates were elongated, oriented along direction of microgrooves and expressed SC marker proteins.

6.1. Transdifferentiation of MSCs

Autologous nerve grafts are considered as gold standards for nerve regeneration. But because of various disadvantages associated with autografts such as donor site morbidity and limited length of graft tissue, researchers have refocused on neural regeneration conduits/scaffolds (NRCs). Along with NRCs, SCs have been the foremost choice for cellular transplantation at the site of nerve injuries. However, the only source of SCs is to sacrifice a healthy nerve (analogous to an autograft) and SCs do not seem to proliferate under cell culture environments [29, 30]. Researchers have started looking into alternative cell types that can replace SCs. A variety of cell types have been investigated as cellular replacements of SCs for nerve regeneration strategies such as olfactory ensheathing cells [31-34], neural stem cells [35-38] and MSCs derived from various sources (bone marrow [39, 40], adipose derived [39, 41], umbilical cord derived [42], dental pulp [43] etc.). Both bone marrow and adipose derived MSCs are of considerable interest since their potential to transdifferentiate into SC-like cells has been documented [5, 7, 8, 10, 13, 16, 44]. In the present study, we have used bone marrow derived MSCs because, in a clinical setting, they can easily be isolated by performing a routine surgical procedure under general anesthesia. MSCs are multipotent cells capable of differentiating into multiple cell types such as adipocytes, osteoblasts and chondrocytes. MSCs grown in large numbers under cell culture conditions and then transdifferentiated into SC-like cells (tMSCs), can be used as a great
alternative source of SCs. These tMSCs were characterized and compared with undifferentiated MSCs (uMSCs) by immunostaining using glial markers such as α-S100β, α-p75NTR and α-GFAP [13, 44, 45]. Ladak et.al.[31] and Zaminy et.al.[45] have reported the S100β and p75NTR expression in the tMSCs to be about 50% and 75% respectively. In our experiments, on polymeric substrates, we found that S100β expression was about 70-80% and p75NTR about 90-95% in the transdifferentiated cells. Also, we did not see any expression of GFAP protein on tMSCs unlike some of the previous transdifferentiation studies. Possible reasons for this difference in percentages could be attributed to the fact that we used MSCs from a different rodent model (Brown Norway rat) and we performed transdifferentiation on biodegradable and biocompatible polymeric films unlike the previous studies conducted on glass coverslips. In the experiments where we transdifferentiated the cells on glass coverslips in parallel to polymeric films, the percentage of glial marker-stained cells was found to be lower on glass coverslips as compared to polymeric films (data not shown). One of the differences between the uMSCs and tMSCs is the change in the morphology of the cells. Transdifferentiated cells have been shown to express a bipolar, spindle shape [5, 7] while uMSCs showed a flat-fibroblast–like morphology. In our experiments, the tMSCs population was a heterogeneous one and consisted of multipolar, spindle as well as fibroblastic cells (on the smooth substrates) while uMSCs were found to be mostly flat-fibroblast like cells. Also, tMSCs were found to be proliferating more slowly than uMSCs. Different concentrations of FBS in transdifferentiation media (10%) and maintenance media (20%) may have produced the difference in proliferation rates. In order to ensure a similar number of cells at the end of experiment, we plated uMSCs (1000 cells/cm2) and tMSCs (3000 cells/cm2) at different initial seeding densities.
6.2. Scaffolding and MSCs

Controlling the behavior and morphology of the cells using biomaterial scaffolds has become an important area of research in the past 20 years for tissue regeneration purposes [46-48]. Both natural and synthetic biomaterials have been used for fabrication of these scaffolds. Here we have used two commonly used synthetic polymers – biocompatible polystyrene (PS) and biodegradable poly (lactic acid) (PLA) for fabrication of micropatterned films using solvent casting. Our group has previously produced patterned PS films combining novel patterning techniques via reactive ion etching and solvent casting to study the effect of physical guidance cues on alignment and differentiation of glial cells [20, 22, 23] and neural stem cells [25, 26]. These micropatterned films significantly enhanced the alignment of the cells in all the previous studies conducted using these polymeric films. We are able to achieve similar results while we grew bone marrow derived MSCs on these films. Both PS and PLA films supported the growth of MSCs on both smooth and patterned regions of the substrates. MSCs were successfully transdifferentiated on these films and no significant differences were observed by percentages of \( \alpha \)-S100\( \beta \) and \( \alpha \)-p75NTR immunostained tMSCs (>70%) between smooth and patterned substrates. Undifferentiated MSCs grown in parallel to tMSCs on these smooth and patterned substrates had a lower percentage of cells immunostained with the glial markers (<10%), showing that only transdifferentiation reagents were responsible for the transdifferentiation of the MSCs and patterning has no effect on transdifferentiation. Also no significant differences in the percentage of glial marker-immunolabeled cells between smooth and patterned substrates shows that patterning does not cause any deleterious effects on the differentiation potential of MSCs. Physical guidance provided by the microgrooves greatly increased the alignment of the cells in the direction of the patterns for both PS and PLA substrates. More than 80% of the
uMSCs as well as tMSCs were found to be aligned in the direction of the micropatterns (0°-20°) while the cells growing on smooth substrates were found to be randomly oriented. In order to quantify the morphological changes of uMSCs and tMSCs associated with micropatterns, we calculated the AR, a parameter which takes into account both the length (in direction of microgrooves) and breadth (in direction perpendicular to microgrooves) of the cells into account. Transdifferentiated MSCs and uMSCs growing on patterned PS and PLA substrates were found to have AR of about 5 showing that cells were highly elongated while growing on the patterned substrates. Both tMSCs and uMSCs had an AR of about 1 when they were grown on smooth polymeric films displaying that cells were, in general, circular in shape (same length and width) or they do not have inclination to elongate in one particular direction. Changes in AR of cells on patterned substrates were significantly different from the cells on smooth substrates at p≤0.05. Also when we compared the cellular areas of the tMSCs and uMSCs between the smooth and patterned substrate we found that cells growing on smooth substrates had a somewhat larger surface area than cells growing on patterned substrates but no significant differences were observed at p≤0.05. Differences in surface area of the cells may be due to the 3D nature of the patterned substrates, as the method we used for calculating the surface area gives us a 2D projection of the surface area and does not take into account the surface area of the part of the cells growing along the vertical walls of the grooves. In order to compare the biodegradable nature of the two polymers, we found that mesas of the micropatterns of PLA substrate were considerably degraded and was difficult to visualize under differential interface contrast (DIC) microscopy as compared to the mesas of the PS substrates which were visualized easily by DIC imaging. A somewhat higher number of cells immunolabeled with glial markers (S100β and p75NTR) on PLA substrate as compared to PS substrate points towards a possible effect of
degrading PLA on the differentiation of MSCs. Degradation of the mesas of PLA films was found to have no effect on alignment and elongation of both uMSCs and tMSCs as compared to the PS substrate. Thus, our results have shown that these patterned films can be used as a means to control the alignment and morphology of the cells without causing any negative effect on their differentiation potential.

Our research group has previously shown that nerve regeneration conduits produced using these biodegradable patterned films after seeding with SCs significantly improves the sciatic nerve regeneration in a rodent model of peripheral nerve injury [21]. Our future studies will include use of these films and transdifferentiated MSCs to test the synergistic potential of physical and biological cues on peripheral nerve repair in a rodent model.

7. Conclusions

Microfabrication techniques developed in our lab were successfully used for production of patterned PS and PLA films. Both polymers supported the growth of MSCs. Transdifferentiation of MSCs was carried out on these polymeric films and was characterized by immunocytochemical labeling of various glial protein (α-S100β and α-p75NTR) markers. MSCs undergoing the transdifferentiation protocol were found to have much higher percentage of glial protein immunostained cells as compared to undifferentiated MSCs. No significant differences were observed in the percentage of S100β and p75NTR-immunolabeled cells across the smooth and patterned PS and PLA substrates. Significantly higher AR of the cells on the patterned substrate implies that cells change their morphology because of the physical barrier and elongated in the direction of micropatterns. Not only do they elongate, but they also align themselves in the direction of micropatterns as compared to randomly oriented cells growing on smooth substrates. Cellular area of the cells growing on smooth substrates was found to be
higher than patterned substrates. However, no significant differences observed between smooth and patterned substrates with respect to transdifferentiation points towards no exceptional changes in the growth of the cells in terms of cellular area on patterned versus smooth substrates. Our results show that, micropatterning can be used as an effective tool for controlling the alignment and morphology of the MSCs without causing any deleterious effect on their growth and differentiation potential into SC-like phenotypes.

8. Acknowledgements

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Supplemental Figure 1. Transdifferentiated MSCs (tMSCs) on micropatterned PLA and PS films have a higher percentage of cells labeling with anti-p75NTR antibody than uMSCs. A nuclear staining of p75NTR was observed for uMSCs which was completely different as compared to strong cytoplasmic staining of tMSCs. A, A’ and A” represents the corresponding anti-p75NTR,
actin filaments (phalloidin) staining and merged image of tMSCs on patterned PLA substrate. B, B’ and B’’ represents the corresponding anti-p75NTR, actin filaments (phalloidin) staining and merged image of uMSCs on patterned PLA substrate. C, C’ and C’’ represents the corresponding anti-p75NTR, actin filaments (phalloidin) staining and merged image of tMSCs on patterned PS substrate. D, D’ and D’’ represents the corresponding anti-p75NTR, actin filaments (phalloidin) staining and merged image of uMSCs on patterned PS substrate. Abbreviations: uMSCs, undifferentiated mesenchymal stem cells; tMSCs, transdifferentiated mesenchymal stem cells; p75NTR, anti-p75NTR antibody; PLA, poly(lactic acid); PS, polystyrene; Merged, merged image of S100β, Alexa-488 Phalloidin, DAPI and DIC (Differential interference contrast). Scale bar = 100 µm
Supplemental Figure 2. Transdifferentiated MSCs (tMSCs) on non-patterned (Smooth) PLA and PS films have a higher percentage of cells labeled with anti-p75NTR antibody than uMSCs. A nuclear staining of p75NTR was observed for uMSCs which was completely different as compared to strong cytoplasmic staining of tMSCs. A, A’ and A’’ represents the corresponding anti-p75NTR, actin filaments (phalloidin) staining and merged image of tMSCs on smooth PLA substrate. B, B’ and B’’ represents the corresponding anti-p75NTR, actin filaments (phalloidin) staining and merged image of uMSCs on smooth PLA substrate. C, C’ and C’’ represents the
corresponding anti-p75NTR, actin filaments (phalloidin) staining and merged image of tMSCs on smooth PS substrate. D, D’ and D’’ represents the corresponding anti-p75NTR, actin filaments (phalloidin) staining and merged image of uMSCs on smooth PS substrate.

Abbreviations: uMSC, undifferentiated mesenchymal stem cells; tMSC, transdifferentiated mesenchymal stem cells; p75NTR, anti-p75NTR antibody; PLA, poly(lactic acid); PS, polystyrene; Merged, merged image of S100β, Alexa-488 Phalloidin, DAPI and DIC (Differential interference contrast). Scale bar = 100µm
Supplemental Figure 3. BrdU immunolabeling for uMSCs grown on PS and PLA smooth and patterned polymeric substrates. A, A’ and A” represents the corresponding anti-BrdU, nuclear (DAPI) staining and merged image of uMSCs on patterned PLA substrate. B, B’ and B” represents the corresponding anti-BrdU, nuclear (DAPI) staining and merged image of uMSCs on smooth PLA substrate. C, C’ and C” represents the corresponding anti-BrdU, nuclear (DAPI) staining and merged image of tMSCs on patterned PS substrate. D, D’ and D” represents the corresponding anti-BrdU, nuclear (DAPI) staining and merged image of uMSCs on smooth PS substrate.
substrate. PLA, poly (lactic acid); PS, polystyrene; Pattern, patterned substrate; Smooth, non-patterned substrate, BrdU, anti-BrdU antibody; Merged, merged image of BrdU, DAPI and DIC (Differential interference contrast). Scale bar = 100µm

10. References


38. Ni, H.C., et al., Fabrication of bioactive conduits containing the fibroblast growth factor I and neural stem cells for peripheral nerve regeneration across a 15 mm critical gap. Biofabrication, 2013. 5(3).
41. Carriel, V., et al., Combination of fibrin-agarose hydrogels and adipose-derived mesenchymal stem cells for peripheral nerve regeneration. Journal of Neural Engineering, 2013. 10(2).
CHAPTER 4: HIGH THROUGHPUT CHARACTERIZATION OF ADULT STEM CELLS ENGINEERED FOR DELIVERY OF THERAPEUTIC FACTORS FOR NEUROPROTECTIVE STRATEGIES

Adapted from manuscript:


1. Keywords

Mesenchymal stem cells, high throughput screening, genetic modification, cell tracking, neurotrophic factors, high content screening, HCS, neuroprotection

2. Short abstract

This study describes an experimental platform to rapidly characterize engineered stem cells and their behaviors before their application in long-term in vivo transplant studies for nervous system rescue and repair.

3. Long abstract

Mesenchymal stem cells (MSCs) derived from bone marrow are a powerful cellular resource and have been used in numerous studies as potential candidates to develop strategies for treating a variety of diseases. The purpose of this study was to develop and characterize MSCs as cellular vehicles engineered for delivery of therapeutic factors as part of a neuroprotective strategy for rescuing the damaged or diseased nervous system. In this study we used mouse MSCs that were
genetically modified using lentiviral vectors, which encoded brain-derived neurotrophic factor (BDNF) or glial cell-derived neurotrophic factor (GDNF), together with green fluorescent protein (GFP).

Before proceeding with in vivo transplant studies it was important to characterize the engineered cells to determine whether or not the genetic modification altered aspects of normal cell behavior. Different culture substrates were examined for their ability to support cell adhesion, proliferation, survival, and cell migration of the four subpopulations of engineered MSCs. High content screening (HCS) was conducted and image analysis performed.

Substrates examined included: poly-L-lysine, fibronectin, collagen type I, laminin, entactin-collagen IV-laminin (ECL). Ki67 immunolabeling was used to investigate cell proliferation and Propidium Iodide staining was used to investigate cell viability. Time-lapse imaging was conducted using a transmitted light/environmental chamber system on the high content screening system.

Our results demonstrated that the different subpopulations of the genetically modified MSCs displayed similar behaviors that were in general comparable to that of the original, non-modified MSCs. The influence of different culture substrates on cell growth and cell migration was not dramatically different between groups comparing the different MSC subtypes, as well as culture substrates.

This study provides an experimental strategy to rapidly characterize engineered stem cells and their behaviors before their application in long-term in vivo transplant studies for nervous system rescue and repair.
4. Introduction

A major issue with implementing useful therapies for treatment of nervous system disorders is in developing effective methods that prevent further degeneration and also facilitate recovery of function. An innovative strategy is to genetically engineer stem cells \textit{ex vivo}, for production of neuroprotective factors, prior to their transplantation. This combination of cell-based therapy, coupled with a type of gene therapy, provides a powerful method for the treatment of disease or injury-induced neuronal death in the nervous system.

Neurotrophic factors are essential for growth and survival of developing neurons as well as maintenance and plasticity of mature neurons. A number of studies have demonstrated significant roles of neurotrophic factors in promoting initial growth and differentiation of neurons in the central and peripheral nervous system (CNS and PNS) and they can also stimulate regeneration \textit{in vitro} and in animal models of neural injury \cite{1}. Brain-derived neurotrophic factor (BDNF) is highly expressed in the CNS and plays important roles in regulating neural development, synaptic plasticity and repair \cite{2}. Glial cell line-derived neurotrophic factor (GDNF) promotes survival of many types of neurons including dopaminergic and motorneurons \cite{3}. Thus, an important strategy for neural repair is to provide exogenous sources of neurotrophic factors to the injured or diseased regions of the nervous system.

Multipotent bone marrow-derived mesenchymal stem cells (MSCs) hold great potential for delivery of therapeutic proteins to treat the damaged or diseased nervous system. Transplantation of MSCs has attracted considerable attention in efforts to develop patient compatible cell-based therapies since they have a number of advantages including, 1) relative ease of isolation and maintenance, 2) multipotential capacity, 3) little ethical concerns, 4) ability to survive and migrate following transplantation and 5) potential for autologous transplantation.
Promising results have been reported with use of naïve and genetically engineered MSCs in animal models for a number of different neurodegenerative conditions, including spinal cord injury [6, 7], stroke [8, 9], myelin deficiency [10], and retinal degeneration [11-13]. Coupling cell transplantation with delivery of neurotrophic factors from genetically engineered stem cells is a novel and important neural repair strategy.

An essential step in developing cell-based therapeutic factor delivery systems is to determine the normal health of the engineered cells. As such, the principal purpose of this study was to evaluate general growth parameters of genetically engineered adult stem cells. An important approach to rapidly assess multiple cell parameters is to employ cellular image-based high-through screening (HTS), often referred to as high content screening (HCS) procedures[14]. This technology allows automated image acquisition and analysis and this approach is particularly well suited for stem cell research applications. In this project we developed a profiling platform that allows for the rapid characterization and optimization of cell substrate preferences and cellular functions with genetically engineered adult stem cells employing a HCS system.

5. Protocol

5.1. Substrate Preparation for 96-well Plates

1.1) Create a map of the 96-well plate outlining the different substrates and cell-types to be examined (Figure 1).

1.2) Obtain the stock solutions of different substrates [poly-L-lysine, fibronectin, collagen type I, laminin, and entactin-collagen IV-laminin (ECL)], a 96-Well Multiwell Plate and prepare a work station in a sterile cell culture hood.

1.3) Prepare individual substrates by diluting stock in sterile phosphate buffered saline (PBS) to a final concentration of 5 µg/ml. This concentration was previously determined based on a
substrate concentration-dependent assay for growth and proliferation of cells. Mix using a vortex before pouring into a sterile reservoir.

1.4) Add 100 µl of substrate solution into each well according to the 96-well map (Figure 1) (a 12- or 8-channel micropipette is convenient for micropipetting into a 96-well plate). Seal the lid to the 96-well plate using a strip of Parafilm and store overnight at 4 °C.

5.2. Cell Plating and Time-lapse Imaging

Mouse MSCs were isolated from the bone marrow of adult C57BL/6 mice and maintained as an adherent cell line. MSCs were infected using lentiviral vectors to engineer them to secrete brain-derived neurotrophic factor (BDNF; human cDNA) and glial cell-derived neurotrophic factor (GDNF; human cDNA) using lentiviral vector’s encoding BDNF (LV-BDNF; CMV-BDNF-IRES-GFP), GDNF (LV-GDNF; CMV-GDNF-IRES-GFP), and green fluorescent protein (GFP, LV-GFP; CMV-GFP).

Note: Culture media for mouse mesenchymal stem cells (MSCs) is Iscove's Modified Dulbecco's Medium containing 10% hybridoma-qualified fetal bovine serum, 10% equine serum, 2 mM L-glutamine, and 10,000 U/ml penicillin, 10 mg/mL streptomycin. The five different types of mouse MSCs (MSCs, GFP-MSCs, BDNF-GFP-MSCs, GDNF-GFP-MSCs and BDNF/GDNF-GFP-MSCs) were plated at about 30% confluence in T75 cell culture flasks.

5.2.1. Cell Plating

5.2.1.1) On the following day, remove the substrate solutions by aspiration and rinse each well with approximately 200 µl of sterile PBS, twice. Add cell culture media (200 µl/well) to each well after the final PBS rinse. Place the 96-well plate into a cell culture incubator set at 37 °C and 5% CO₂ for equilibration.
5.2.1.2) While the 96-well plate is equilibrating in the incubator, harvest the cells (MSCs in T75 flasks should be approximately 70% confluent at time of harvesting/plating) by collecting the growth media (referred to as conditioned media) from the T75 flask and storing in a 15 ml conical tube under sterile conditions (this conditioned media will be used in step 2.1.4 below).

5.2.1.3) Add 8 ml of sterile PBS to the flask and gently swirl and then pipette off the PBS and add 1 ml of 0.05% trypsin and 0.01% EDTA solution to detach the cells from the culture surface of the T75 flask. Monitor cell detachment by viewing the flask using an inverted microscope equipped with phase contrast optics.

5.2.1.4) When the cells have detached, immediately add 8 ml of the conditioned media (collected in step 2.1.2) to the flask. Collect the cell suspension and transfer to a 15 ml conical centrifuge tube and centrifuge for 4 min at 450 x g to pellet the cells.

5.2.1.5) Remove the supernatant and resuspend the cell pellet in 200 µl of fresh and warmed (37 °C) cell culture media.

5.2.1.6) Determine the number of cells in the cell suspension by performing a trypan blue viable cell count using a hemocytometer. Plate the cells at a density of approximately 300 cells/well into the appropriate wells of the 96-well plate.

5.2.1.7) Repeat these steps for each population of cells.

5.2.2. Time-lapse Imaging

5.2.2.1) Once all of the MSCs have been plated, place the 96-well plate into an incubator for 2 hr to allow the MSCs to attach to the substrate.

5.2.2.2) Start the HCS system and wait for 2 hr for the system to equilibrate. Set the environmental controller to 37 °C and connect a mixed gas cylinder containing 5% CO₂ in air to the HCS system environmental chamber supplying a constant air source.
5.2.2.3) Remove the 96-well plate from the incubator following the 2 hr equilibration period and place directly into the cell growth chamber of the HCS system. Allow 30 min for equilibration to account for any heat-related expansion of the plate and then start the image acquisition and analysis software to configure the plate settings.

5.2.2.4) Select the 20X objective for imaging. Select two wells per condition [i.e., GFP-MSCs on Fibronectin etc. (6 substrates X 5 MSC subtypes for a total of 30 conditions X 2 replicates = 60 wells in total)] for setting up time-lapse imaging. Choose two sites for imaging within each well.

5.2.2.5) Choose the correct light wavelengths for imaging. Note: Two different wavelengths (Phase contrast and GFP fluorescence) were selected for time-lapse imaging.

5.2.2.6) Focus on the well bottom using laser autofocus and take test images for multiple sites and multiple wells to find an optimized focal plane.

5.2.2.7) Once the focus has been established, begin capturing images every 5 minutes for 48 hours for all 60 wells (120 sites).

5.2.2.8) Feed the cells every 24 hr by removing the 96-well plate from the HCS system. Remove 75 µl of media from each well and add 100 µl of fresh media to each well (equilibrate this fresh media at 37 °C and 5% CO₂).

5.2.2.9) At the end of the time-lapse experiment, remove the 96-well plate from the HCS system. Under sterile conditions, collect the conditioned media samples from each well and transfer these samples to another 96-well plate. These samples can be used for further analysis by performing ELISA for neurotrophic factors.

5.2.2.10) Prepare the 96-well plate with cultured MSCs for additional assays such as Ki67 cell proliferation assay or Propidium iodide live/dead staining assay (see details below).
5.2.2.11) Perform time-lapse imaging analysis for cell migration/cell tracking as described in section 5 below.

5.3. Ki67 Cell Proliferation and Propidium Iodide Live/Dead Assay

5.3.1. Ki67 Cell Proliferation Assay (Immunocytochemistry)

5.3.1.1) Rinse the cell cultures with 0.1 M phosphate (PO₄) buffer for one minute two times. Fix the culture with 4% paraformaldehyde (PFA) for 20 min at room temperature. Remove the PFA and rinse the wells with PBS for seven minutes three times.

5.3.1.2) After the final rinse, add 100 µl of blocker solution (phosphate buffer saline, 5% normal donkey serum, 0.4% bovine albumin serum, and 0.2% triton X-100) to each well and incubate at room temperature for one hour. Prepare the primary antibody, rabbit anti-Ki67, by diluting in blocker solution at a working ratio of 1:200.

5.3.1.3) Remove the blocker solution and apply 100 µl of the primary antibody solution to each well. Cover the 96-well plate and incubate the samples at 4 °C overnight.

5.3.1.4) On the following day, remove the antibody solution and rinse with PBS for seven min, three times.

5.3.1.5) Prepare the secondary antibody, donkey anti-rabbit Cy3 in blocking solution at a working ratio of 1:500. Add DAPI nuclear stain to the secondary antibody solution at a dilution of 1:100. After the removal of the last PBS rinse, apply 100 µL of the secondary antibody/DAPI solution to each well. Incubate at room temperature in the dark for 90 min.

5.3.1.6) Remove the secondary antibody/DAPI solution and rinse each well with PBS for seven min three times. Cover the 96-well plate and store at 4 °C until imaging.
5.3.2. Propidium Iodide Live/Dead Assay

Measure cell death by propidium iodide (PI) exclusion assay as described below.

5.3.2.1) Prepare the propidium iodide stain solution at a concentration of 1.5 µM in culture media.

5.3.2.2) Add 100 µl of 70% ethanol to one-well of the MSCs for two minutes with the intention of killing those cells. This well serves as a positive control for the PI stain. The majority of the MSCs will be PI-stained indicating cell death. Remove the ethanol solution.

5.3.2.3) Add 100 µl of propidium iodide stain solution to each well and incubate for 20 min at 37 °C in a 5% CO₂ incubator.

5.3.2.4) Rinse the cells with 0.1 M phosphate buffer for one min, two times. Fix the cells with 4% PFA in 0.1 M P0₄ buffer for 20 min at room temperature. Remove the PFA and rinse with PBS three times for seven minutes.

5.3.2.5) Incubate with DAPI solution (1:50) diluted in blocker solution for one hr at room temperature. Rinse all wells with PBS three times for seven minutes.

5.3.2.6) Remove the DAPI solution and rinse each well with PBS for seven minutes, three times. Cover the 96-well plate and store at 4 °C until imaging.

5.4. Automated Imaging and Multiwavelength Scoring Analysis

5.4.1) Load a 96-well plate (previously processed for Ki67 immunolabeling or propidium iodide-stained) into the HCS system and allow the plate to equilibrate for 20 min.

Open the HCS system image acquisition and analysis software.

5.4.2) Choose the acquisition settings for the 10X objective using camera binning at 1 and a gain setting of 2. Find the Z-plane in which the cells reside by utilizing the Auto Exposure function and calculate the offset for each wavelength of interest. For this analysis, capture images for
DAPI (W1), Cy3 (W2), and FITC (W3). Choose the maximum intensity level at which the negative control wells show no signal for image acquisition. Confirm this setting is appropriate for the positive wells.

5.4.3) Acquire plate. Capture images and store them in the image acquisition and analysis software’s database.

5.4.4) Once images have been acquired, open image acquisition and analysis offline software and review plate data from the images acquired above.

5.4.5) Select the Multi-wavelength scoring analysis. Configure the minimum and maximum intensities for each wavelength. Note: DAPI detection should mark the staining around each visible nucleus. Cy3 should detect the positive cells with Ki67 immunoreactivity (IR) and should not detect IR under the negative controls. For Cy3 Ki67 IR, the approximate minimum width was 7 µm, approximate maximum width was 30 µm, the intensity above the local background was 150 gray levels, and the minimum stained area was 50 µm².

5.4.6) Run analysis for all positions. Export the data to view in spreadsheet.

5.5. Cell Tracking

5.5.1) Open image acquisition and analysis program and click on “Review Plate Data [DB]…”, selecting the plate of interest.

5.5.2) View the data as “Time vs. Well”.

5.5.3) Choose one of the sites from the “Sites” section by left clicking on the desired selection. Select “Transmitted…” under the “Wavelengths:”. Right click on the target well in the 96-well plate template and click on “Load Images”.

5.5.4) Track the cells by clicking on the “Apps” and then “Track Objects”.
5.5.5) Use “Dynamic Data Exchange (DDE)” to choose the format to export the data, e.g., Microsoft Excel.

5.5.6) Choose the tracking data to export, e.g., elapsed time, object number, distance, time interval, velocity, absolute angle, distance to origin, delta x and delta y.

5.5.7) Tag each cell of interest with “Ctrl key + left click” on target cells.

5.5.8) Track cells. If necessary, stop/change improper tracking of cells with the “Esc” key and then adjust the settings.

5.5.9) After cell tracking is complete, save data with “Log Data”.

6. Representative results

MSC growth parameters were examined by culturing the different populations of MSCs on different substrates. The five different populations of MSC subtypes (MSCs, GFP-MSCs, BDNF-GFP-MSCs, GDNF-GFP-MSCs, and BDNF/GDNF-GFP-MSCs) were plated into 96-well tissue culture plates pre-coated with the different substrates as illustrated in Figure 1. After four days of culturing, the plates were fixed and immunolabeled and/or stained with the appropriate reagents and then examined using the HCS system and analyses conducted with the image acquisition and analysis software program.
Figure 1: 96-Well plate template for experimental design.

96-well plates were coated with various substrates and wells were seeded with engineered stem cells as shown in the template. As an example, only wells in rows B-F were used in this experiment. Rows A, G and H were left empty. Abbreviations – MSCs: mesenchymal stem cells; GFP-MSCs: green fluorescent protein-expressing MSCs; BDNF-GFP-MSCs: brain derived neurotrophic factor-GFP-expressing MSCs; GDNF-GFP-MSCs; Glial cell-derived neurotrophic factor-GFP-expressing MSCs; BDNF/GDNF-GFP-MSCs; BDNF and GDNF- GFP-expressing MSCs; ECL: Entactin-Collagen IV-Laminin).

Anti-Ki67 immunolabeling, followed by DAPI counterstaining, was used to evaluate whether the different substrates influenced proliferation of the different populations of engineered MSCs (Figure 2A). Expression of the Ki67 antigen occurs preferentially during late G1, S, G2 and M phases of the cell cycle, and is not detected in cells in the resting phase (G0), and therefore is
useful as a cellular marker for proliferation [15]. 4',6-Diamidino-2-Phenylindole (DAPI), is a commonly used nuclear and chromosome counterstain that emits blue fluorescence upon binding to AT regions of DNA [16]. The total number of cells in a field can be determined by counting the number of DAPI stained nuclei. As illustrated in Figure 2B, although there was variation in the percentages of proliferating MSCs, all substrates nevertheless supported considerable cell proliferation for each of the MSC subtypes.

Figure 2: Ki67 cell proliferation assay.

(A) Merged, double-fluorescent image of Ki67 immunolabeling (red) and DAPI (blue) nuclear staining. Many of the MSCs were immunolabeled with the Ki67 antibody (red). Scale bar = 50 μm. (B) Bar graph illustrating the percentages of Ki67 immunolabeled MSC subtypes grown on polystyrene (PS), poly-L-lysine (PLL), fibronectin, collagen type I, laminin, or entactin-collagen IV-laminin (ECL) substrates for 5 days in vitro (DIV). N = one experiment. Each bar represents averaged pooled data from 8 imaged sites from 2 wells for each condition.

Propidium iodide (PI) staining was used to evaluate whether different substrates influenced cell survival (Figure 3). Propidium iodide is a commonly used red-fluorescent nuclear and chromosome counterstain. Propidium iodide is membrane impermeant and generally excluded
from viable cells, and thus is useful to detect dead cells in a population. The proportion of dead cells within a given condition can be determined when combined with a general nuclear label such as DAPI to identify all cells within a field. The percentage of PI-positive cells was low on all substrates examined (Figure 3). As a positive control for the PI reagent, a few wells containing MSCs were incubated in 70% ethanol, a condition known to kill most cells, resulting in a high percentage of PI-labeled cells as illustrated in Figure 3B and 3C (Ethanol treated positive control).
Figure 3. Propidium iodide cell death assay.

(A) Merged, double fluorescent image for propidium iodide (red) and DAPI (blue) staining. Although the nuclei of all of the viable cells were stained with DAPI (blue), no propidium iodide staining was detected in the MSCs. (B) Virtually all MSCs were stained with propidium iodide following exposure to 70% ethanol. Scale bars in A and B = 100 µm. (C) Bar graph illustrating
the percentages of propidium iodide (PI) stained MSC subtypes grown on polystyrene (PS), poly-L-lysine (PLL), fibronectin, collagen type I, laminin, or entactin-collagen IV-laminin (ECL) substrates for 5 days in vitro (DIV). Ethanol Control: This condition served as a positive control for the PI staining reagent. Most cells subjected to PI stain following ethanol treatment are dead and thus positively stained for the PI reagent. N = one experiment. Each bar represents averaged pooled data from 8 imaged sites from 2 wells for each condition.

To investigate the possible influence of different substrates on the behavior of engineered MSCs, cell migration was analyzed using time-lapse digital microscopy and the transmitted light/environmental chamber system on the HCS system (see Supplemental Video 1). Multiple sites/well were time-lapse imaged and used to calculate cell migration rates for the different subpopulations of MSCs growing on the different substrates using the image acquisition and analysis software program. In general, as shown in Figure 4C, all subtypes of MSCs showed the fastest migration rate on the extracellular matrix-coated surfaces (Fibronectin, Collagen, Laminin and ECL) and the slowest on non-coated polystyrene surfaces.
Figure 4. Cell tracking and migration.

MSCs tracked with image acquisition and analysis software. Overlayed images of transmitted light and fluorescence images from (A) the start of time-lapse imaging and (B) at 29 hr later at the end of the time-lapse imaging session (see Supplemental Video 1). Cell migration tracks are indicated by the colored lines. Scale bar: 50 μm. (C) Bar graph illustrating the average migration rates (expressed as μm/hr) for MSC subtypes grown on polystyrene (PS), poly-L-lysine (PLL), fibronectin, collagen type I, laminin, or entactin-collagen IV-laminin (ECL) substrates for 2 days
in vitro (DIV). N = one experiment. Each bar represents the average of at least 10 imaged cells from 2 wells for each condition.

Taken together, these results provide preliminary evidence that these subpopulations of genetically engineered MSCs display similar growth properties. These results provide compelling evidence that the lentiviral mediated genetic modifications of these MSCs induced no dramatic detectable deleterious effects on the growth parameters investigated using this screening platform.

**Supplemental Video 1.**
Time-lapse digital video of MSC tracking using the image acquisition and analysis software program. The migration paths for two MSCs [indicated as 1 (green tracking line) and 2 (Blue tracking line)] are illustrated. Video captured during a 29 hr period. Images were captured every 5 min. Fluorescence images of GFP-expressing MSCs was used for time-lapse imaging in preparing the video. Calibration bar = 50 µm. This type of analysis is useful for investigating cell behaviors, including cell migration and cell division.

**Table 1: Materials and chemicals used during experiments**

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7. Discussion

Adult mesenchymal stem cells (MSCs) are an attractive cell type for development of an experimental strategy combining a cellular and gene delivery based therapy. MSCs are multipotent, capable of differentiating into cells of mesodermal lineage, and display considerable plasticity, differentiating/transdifferentiating into neuronal and glial lineages with the appropriate induction paradigms [17, 18]. Furthermore, MSCs have been transplanted and proven effective in preclinical studies for a number of disorders, including neurodegenerative conditions[19]. The therapeutic efficacy of MSCs is well known due to their beneficial anti-proliferative, anti-inflammatory and anti-apoptotic activities[20]. MSCs are also known to produce and secrete various neurotrophic and growth factors, which likely contributes to the neuroprotective qualities associated with naïve MSCs following transplantation at sites of injury or disease[21]. Importantly, MSCs can be genetically modified for sustained delivery of neurotrophic factors for combined cellular and gene therapy-based applications and have been used in a number of animal models of injury or disease to the CNS[11, 19, 22].

In developing a combined cellular and gene therapy-based strategy, it is important that the health of the cells is carefully assessed prior to their extensive use for in vitro, and especially in vivo
applications. As a proof of concept, we investigated multiple populations of engineered and control MSC lines in order to study the consequences of the genetic modifications on cell health and fitness using a high content screening (HCS) approach. In general, HCS refers to cellular image-based high throughput screening[14]. This screening approach permits a quantitative assessment of cellular phenotypes at multiple levels of spatial (cell to subcellular) and temporal (milliseconds to days) resolution across various experimental conditions. Using this approach we accessed possible differences in substrate preference on the following parameters: cell proliferation, expression of green fluorescent protein (GFP), cell death, and cell motility/migration. Experiments were designed in 96-well cell culture plate format. Within a single plate we routinely investigated possible substrate-related differences with respect to each parameter for the different MSC populations of lentiviral-transduced cells and compared the engineered MSCs with the original, non-transduced MSCs. This provided a means to directly compare the results for the different MSC subtypes with a battery of in vitro assays such as cell proliferation using Ki67 immunolabeling, live/dead cell viability assay using propidium iodide staining, and cell behavior by performing time-lapse digital imaging. As an extension of this HCS one can also perform ELISAs on conditioned media samples collected from individual wells to quantitatively determine secretion of neurotrophic factors. Conditioned media from different MSC subtypes may also be used in in vitro bioassays to determine biological activity of secreted factors [11, 23]. This type of HCS platform may also be used for in vitro measurements of neurite outgrowth from primary neuronal cultures and neural stem cell lines [24]. Overall, our results demonstrated that the subpopulations of the genetically modified MSCs displayed similar behaviors in comparison to the non-modified MSCs. The influence of diverse culture substrates on cell growth and cell migration was not dramatically different between the MSC subtypes, as
well as culture substrates. As such, the extracellular matrix substrates tested did not appear to play a critical role in modulating these aspects of cell behavior for these different engineered MSCs.

This study demonstrates the use of an HCS system for analyzing different aspects of cell behavior. However, it is not uncommon to encounter limitations associated with image analysis. On occasion, while analyzing the fluorescence images, it was somewhat difficult to determine the correct threshold value above which immunolabeled or stained cells would be counted as positively labeled/stained. Thus, to minimize subjective bias, the determination of threshold values was dependent upon a comparison with controls (negative controls for fluorescence imaging were carried out in parallel during all processing by the omission of the primary or secondary antibodies). Another limitation was encountered during the analysis of cell migration using time-lapse digital imaging. In some cases, the imaging software was not able to differentiate between random Brownian motion of a cell versus a cell actually migrating only a very short distance. Additional limitations were evident in situations where the analysis software was not able to distinguish the presence of multiple cells in very close proximity to one-another. To overcome this limitation required manual cell selection during the analysis rather than a fully automated analysis. Cell plating density can also result in skewing cell migration data between populations of cells that display greater preference to grow in clumps versus cells that grow in isolation from each other. These types of differences are in part likely a reflection of cell-substrate versus cell-cell preferences.

Using an HCS system to acquire images and perform data analysis provides an efficient and rapid means to assess multiple cell parameters. In addition, time-lapse digital videos for 30 different conditions (6 substrates and 5 different MSC subtypes) were routinely acquired for
periods ranging from hours to days (48 hr) while using the environmental chamber. This data was subsequently used to calculate and determine differences in cell migration rates across various cell lines on different ECM molecules.

In this report we have highlighted the implementation of a high content screening platform to assess cell health and function. This type of analysis is useful for developing rational strategies for designing cell-types, as well as polymer substrates to facilitate directed cell growth and neural regeneration. This is an essential step towards application of stem cell-based delivery of therapeutic factors prior to extensive in vivo preclinical studies using cell transplantation strategies.

8. Acknowledgements

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9. Disclosures

The authors declare they have no competing financial interests.

10. References


CHAPTER 5: DEVELOPMENT OF MULTIFUNCTIONAL FILMS WITH MICROPATTERNS, NERVE GROWTH FACTOR SURFACE GRADIENTS, AND NANOPARTICLE DELIVERY SYSTEMS FOR PERIPHERAL NERVE REGENERATION CONDUITS

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* (manuscript to be submitted to Journal of Controlled Release)

1. Abstract

Peripheral nerve injuries with large nerve gap defects present a challenging problem facing the field of neuroregeneration. A potential solution is the design of multifunctional nerve regeneration conduits offering combinations of guidance and trophic cues with controlled release of growth factors to enable guided and accelerated nerve growth and regeneration. In this study, multifunctional poly(lactic acid) (PLLA) porous films were designed and fabricated with longitudinal micropatterns, which could then be rolled into conduits with the patterns in the inner lumen. These films also sported a surface gradient of nerve growth factor (NGF), along with NGF-releasing, surface-eroding, biodegradable, and amphiphilic polyanhydride microparticles distributed into the film matrix, to address this challenging issue. PLLA films with micropatterns and a porous structure containing NGF encapsulated polyanhydride microparticles were
successfully obtained by dry phase inversion techniques. XPS analysis indicated the formation of a successful β-NGF surface gradient, enabling directional neurite outgrowth. The use of polyanhydride microparticles with different chemistries distributed throughout the scaffold matrix provided long-term controlled release profiles of NGF, while the surface attachment of NGF provided an initial burst release at the film surface. The experimental release kinetics profiles showed good fit with the theoretical mathematical models to yield estimates of diffusion coefficients. The diffusion coefficients for NGF transport through the conduit matrix varied between $6 \times 10^{-14}$ and $7.5 \times 10^{10}$ cm$^2$/min while diffusion coefficients for NGF transport at the surface varied between $1.21 \times 10^{-4}$ and $1.11 \times 10^{-3}$ cm$^2$/min. These multifunctional films successfully provided tunable release of bioactive NGF that promoted guided neurite extension from PC12 cells.

2. Keywords
Nerve regeneration conduits, poly (lactic acid) (PLA), nerve growth factor (NGF), surface gradient, micropatterning, polyanhydride nanoparticles and microparticles, controlled release

3. Introduction
Severe damage to the peripheral nervous system (PNS) may cause malfunction of sensory and/or motor properties, leading to poor recovery of function and subsequent impaired quality of life for patients [1]. The PNS has the capacity for spontaneous nerve regeneration; however, it may not be successful in bridging peripheral nerve gaps larger than 10 mm. The use of bioengineered artificial nerve conduits based on synthetic biodegradable functional polymeric biomaterials has been proposed as a promising strategy for the regeneration of damaged nerves, circumventing the
limitations of autologous nerve grafting such as biological complexity, donor site availability, morbidity and requirement of multiple surgeries [1-8].

An ideal conduit should possess many features such as biodegradability, biocompatibility, mechanical properties, functional surface chemistry, and growth factor bearing capabilities [9-12] to overcome the current limitations in enabling peripheral nerve regeneration [5, 6, 13]. In order to further improve the conduit properties and enhance nerve regeneration, researchers have developed porous matrices to provide vascularization [14, 15], enable high permeability of nutrients/oxygen [14, 16, 17], fast drainage of nerve wound exudates, [18, 19] or neural cell alignment [20, 21]. In addition to these, our work has demonstrated the importance of surface nano/micropatterning the inner lumen to provide topographic guidance cues and alignment to create regenerative platforms for cells [22-33]. It has also been shown that the presence of neurotrophic factors within the conduit structure promotes neurite extension and growth of axons [34-36]. Among many neurotrophic factors, NGF has been found to promote the growth and survival of axons and protect neurons from injury-induced death in lesioned sciatic nerves [37]. However, considering short half-life, spatial distribution and the long duration required for regeneration of large gaps, strategies for sustained/controlled NGF release are needed in conduits [4].

Various loading and controlled release strategies exist for neurotrophic factors based on functional biodegradable synthetic polymeric biomaterials, including biodegradable micro/nano particles and nerve regeneration conduits [37-50]. Recently, the combined release and synergistic activity of multiple growth factors on axonal regeneration has also been applied as a strategy
Most of these studies used degradable polyesters as particles or conduits, such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) due to their low toxicity and rapid degradation into metabolites [51]. However, these polymers undergo bulk erosion [52, 53] causing burst and rapid drug release [54-56]. Although the erosion rate and, hence, the drug release can be controlled by backbone chemistry [54, 56], molecular weight [55, 56] and hydrophobic surface coatings [57], this brings about extra challenges in the preparation steps and leads to loss of growth factor activity and stability. Besides, none of these strategies considered the effect of directional guidance and distribution of multiple growth factors in a concentration gradient pattern enhancing the guidance of growth cones to their target tissues [58-63]. There are very few studies focusing on creating neurotropic factor concentration gradients on the conduit surface. Although these conduits possess porous structure and surface concentration gradients, they lacked surface nano/micropatterns facilitating the guidance and alignment [4, 64]. Furthermore, a significant drawback with these studies was that the concentration of growth factor loading was limited by the surface area and the conjugation/attachment efficiency. More importantly, the loading of the growth factors during conduit fabrication can lead to loss of control over conduit properties, including controlled release of growth factors, and potential denaturation and activity loss of the growth factors. As an alternative, the incorporation of micro/nanoparticles within a polymer scaffold matrix has been employed as a strategy allowing more controlled sustained release [65-68]. Despite efficient control over the release rates, these systems suffer from the lack of alignment and directed growth properties. Overall, the approaches mentioned above target only one or two specific challenges in nerve regeneration at a time. However, the development of an ideal conduit system for effective nerve regeneration requires a multifunctional combinatorial approach addressing several critical issues at once. To
the best of our knowledge, there is no other study combining all of the mentioned individual properties above into one single conduit.

Considering this motive, in this study, the combination of several of these guidance and trophic cues that aid nerve regeneration have been incorporated into one single conduit to provide effective controlled release of growth factors and stimulate cellular attachment guided nerve growth. For this purpose, β-NGF surface gradients were created on PLLA conduit surfaces with longitudinal micropatterns to provide initial burst β-NGF release and guided nerve growth while β-NGF encapsulated polyanhydride (PA) microparticles were incorporated into the biodegradable PLLA porous conduit matrix to provide sustained β-NGF release. This approach was also aimed at increasing the growth factor loading and furthermore, may also be used as a combined release strategy. PLLA was selected as conduit material due to low toxicity, controllable degradation and functional end groups allowing surface β-NGF modification [51, 54-56]. In contrast to other studies achieving porous structures mostly with the use of pore forming agents, in our work, the porous structure of the conduit was created through dry-phase inversion techniques, allowing not only β-NGF loaded microparticle distribution inside of the matrix but also efficient β-NGF surface concentration gradient modification leading to higher β-NGF loadings. Moreover, in this study, we focused on using biocompatible and biodegradable polyanhydrides for fabrication of β-NGF loaded nano/microparticles due to their advantages of providing β-NGF stability, surface erosion to better control sustained release over their polyester counterparts, and less acidic degradation by products. Polyanhydride copolymers were used to fabricate micro/nanoparticles for controlled release of β-NGF to stimulate the growing axons either alone or in combination with PLLA film matrix for the first time. In vitro β-NGF release
profiles and neurite extension and outgrowth against PC12 cells were evaluated to gauge the efficacy of these multifunctional conduits.

4. Materials and Methods

4.1. Materials

Poly-L-lactide (L-PL-ester terminated B6002-2) was obtained from Lactel Absorbable Polymers. Beta-nerve growth factor (β-NGF, 556-NG/CF) was provided from R&D Systems. Ovalbumin (OVA) was purchased from Invivogen. Methylene chloride and pentane were supplied from Fisher Scientific. β-NGF ELISA kit (ab100757) and OVA ELISA kit (EKU06441) was ordered from Abcam and Biomatik, respectively. RPMI-1640 cell culture media and supplies, fetal bovine serum (FBS) and heat inactivated horse serum (HS), and phosphate-buffered saline (PBS) were obtained from Invitrogen. Bovine serum albumin (BSA), paraformaldehyde (PFA) and triton-x100 were obtained from Fisher Scientific. Cultrex® Mouse Laminin I was purchased from Trevigen. Anti-βIII tubulin antibody conjugated with Cy3 (AB15708C3) was obtained from EMD Millipore while DAPI (4′,6-diamidino-2-phenylindole) dye was ordered from Invitrogen. All the other required buffers were prepared by using ultrapure water according to standard laboratory procedures.

4.2. Synthesis of copolymers and fabrication of bare and β-NGF loaded nano/microparticles

Random copolymers of CPH:SA and CPTEG:CPH (1, 6-bis (p-carboxyphenoxy) hexane (CPH), 1, 8-bis (p-carboxyphenoxy)-3, 6-dioxaoctane (CPTEG) and sebacic acid (SA)) were synthesized from the corresponding monomers with different copolymer ratios via a melt polycondensation reaction as described previously [69, 70]. Nuclear magnetic resonance spectroscopy (NMR) was used for resolving the structure and determining the molecular weight of the copolymer. Bare polymeric nanoparticles were fabricated by Solid/Oil/Oil (S/O/O) emulsion method, where
copolymers (200 mg) were first dissolved in methylene chloride (20 mL) and then rapidly nano-precipitated in anti-solvent pentane to obtain the nanoparticles. In order to obtain β-NGF encapsulated nanoparticles, polymer (200 mg) was dissolved in methylene chloride (20 mL) and different amounts of β-NGF were added. Then, the β-NGF-polymer mixture was precipitated in anti-solvent pentane. The amount of β-NGF used for encapsulation was 0.1% of the polymer weight used for fabrication of nanoparticles. In addition, polymeric microparticles were also fabricated using a benchtop spray-dryer (Buechi, Switzerland) to increase the β-NGF encapsulation efficiency. The polymer-protein solution was fed to the spray-dryer through a nozzle with argon as the feed gas at the feed temperature of 24°C, vacuum of -50bar. The particles formed were collected using a high efficiency cyclone. As a bigger batch is required for production of nanoparticles via spray drying, 0.05% loading was tested as opposed to 0.1% loading for S/O/O method. Scanning electron microscopy (SEM, Quant FEG 250) imaging was performed to determine the dimensions and structure of nanoparticles obtained.

4.3. β-NGF release from nano/microparticles

β-NGF loaded nano/microparticles (5 mg) and 200 µL PBS, supplemented with 0.5wt% BSA as stabilizer, were placed in an Eppendorf tube and incubated at 37°C with continuous shaking. Samples were collected on day 3, 6, 10, 14, 21, 28, 42, and 56. During the sample collection, the particles in the tube were precipitated via centrifugation (10 mins at 15000 rpm) and all 200 µL of the sample supernatant was collected and subsequently fresh 200 µL of 0.5w% BSA-PBS was added to the precipitated particles. The tube was vortexed and placed back to the incubator. The concentration of released β-NGF collected in the sample supernatant was analyzed using a β-NGF ELISA kit following the manufacturer’s procedure. Multiple ELISAs were performed to
optimize the dilution of the samples to bring the absorbance reading in the range of the ELISA kit.

4.4. Preparation and characterization of β-NGF/microparticle incorporated, porous and surface micro-patterned PLLA films

In this study, dry phase inversion technique was used to prepare β-NGF/microparticle-incorporated, porous and surface micro-patterned PLLA conduits. For this purpose, PLLA (1g) was dissolved in chloroform (10 mL) to create a 10 wt% polymer solution. At the same time, β-NGF-encapsulated polyanhydride particles (20 mg) were dispersed in 400 µL pentane (volatile non-solvent), which was used as a precipitator in the S/O/O technique, to provide a protective layer around loaded particles preventing their dissolution in chloroform and allowing their distribution in the PLLA film matrix. Then, 1 mL of 10 wt% PLLA solution was added on top of particles in pentane and shortly vortexed. This 1.4 mL casting solution was poured on a silicon wafer with longitudinal micro-patterns (4x4 cm² area) and spin for 10 min in a spin coater. Then the films were left to dry at room temperature for 6 h. During drying, the different evaporation rates of pentane (non-solvent) and chloroform (solvent) resulted in phase separation, and consequently, when the solvent and non-solvent were completely removed from the film, porous structure were obtained. The particles were also found to be uniformly distributed throughout the film. At the end of drying, the films were peeled off the wafer surface and rolled by using a carbon rod with diameter of 2 mm in such a way that the patterned surface remained in the inner lumen along the conduit. The structure of the films was characterized by SEM.
4.5. Formation of β-NGF concentration gradient on the film surface

For the formation of β-NGF concentration gradient on the film surface, the method described elsewhere was applied with modifications [4]. The PLLA films prepared as described above with dimensions of 1x3 cm² were placed upright in a tube as shown in Supplementary Information, Figure S.1. The β-NGF solution (10 µg/mL) was pumped into the tube at a velocity of 5 µL/min using a syringe pump in a cold room (at 4°C). With this approach, differential β-NGF exposure along the length of the films parallel with the walls of the tube was obtained. This differential β-NGF exposure created concentration gradients along the length of the film and also the surface micro-patterns (Supplementary Information, Figure S.1.). It was anticipated that the β-NGF molecules were attached to the film surface through the interaction between the ester ends of PLLA and amine groups of β-NGF [71]. Addition of β-NGF solution was stopped at the end of 6h. The membranes were stored at 4°C and rinsed with sterile PBS three times before use. The presence of a β-NGF concentration gradient on the film surface was determined by X-ray photoelectron spectroscopy (XPS) by checking for the presence of primary amine (–NH₂) groups on the surface. Analyses were performed on the film surface starting from section-1 to section-3 by taking at least 3 random measurements in each section (Supplementary Information, Figure S.1.). Throughout the analysis a monochromatic Al KX-ray source (1486.6 eV) with an Omni Focus III small area lens and multichannel detector was used. Measurements were taken at an electron take-off angle 45° from a normal sampling surface depth of ~50 Å. Survey scans were collected from 10 eV to 1100 eV with a pass energy of 187.85 eV. All spectra were referenced by setting the C 1s peak to 285.0 eV to compensate for residual charging effects.
4.6. β-NGF and OVA release kinetics from PLLA films

Throughout the release experiments, polyanhydride particles loaded with different amounts of model protein OVA (1, 3 and 5%) to distinguish between the protein released from the conduit surface and from the microparticles, were prepared by spray drying method. For the release kinetics experiments, PLLA films (1x3 cm²), containing OVA encapsulated particles in the film matrix and coupled with the β-NGF concentration gradient on the film surface, were cut into 3 sections (1x1 cm² each section) representing the higher, middle and lower β-NGF concentration gradient (Supplementary Information, Figure S.1.). Then, the sections were placed in 1 mL PBS buffer at pH 7.4 and 37 ºC. The simultaneous release of OVA from the PLLA film matrix and β-NGF from the film surface were observed. The release samples were collected at predetermined times and stored at -20 ºC till the day of analysis. The released amounts of OVA and β-NGF were detected using respective ELISA kits following the manufacturer’s procedure.

4.7. Determination of transport properties of PLLA films

Following the experimental data obtained through the release kinetics tests, the diffusion coefficient of OVA, released from the microparticles distributed in PLLA matrix, and dissolution constant of β-NGF, released from the PLLA film surface, were calculated using related mathematical models. The release rate of OVA was assumed to be controlled only by its diffusion through the microparticles distributed in PLLA film matrix, where Fick’s second law was used as the main transport equation to describe the change in the concentration of OVA in the film with respect to time and position. It was assumed that there was no chemical reaction between OVA and the film, the mass transfer is by diffusion only, the diffusivity of OVA is constant, and the thickness of the film does not change due to desorption of OVA. According to a solution of Fick’s second law with one initial and two boundary conditions applied[72], the
total amount of active compound desorbed from the film at any time \( t \), due to Fickian diffusion, \( M_t \) is given by the following expression.

\[
M_t = M_\infty \left[ 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2q_n} \exp(-Dq_n^2t/L^2) \right]
\]  

(1)

where \( M_\infty \) is the amount of protein desorbed at equilibrium, and the \( q_n \)'s are the non-zero positive roots of \( \alpha = \frac{V_{\text{solution}}}{A_{FLK}} \)

\[
\tan q_n = -\alpha q_n
\]  

(2)

The partition coefficient, \( K \), used in the definition of \( \alpha \) was defined as the ratio of OVA concentration in the film to that in the solution at equilibrium and it was calculated from the difference in the equilibrium and the initial concentrations of OVA measured in the solution, assuming that OVA does not degrade during the test.

Eq. (1) clearly indicates that in cases where the release is controlled only by Fickian diffusion, the diffusivity of OVA in the film is the main transport parameter and it was determined by minimizing the difference between the experimental data and the model predictions from Eq. (1).

\( \beta \)-NGF directly released from the film surface is based on the dissolution as described in the following equation.

\[
M_{\text{Dissolution}} = M_\infty \left[ 1 - \exp(-kt) \right]
\]  

(3)

\( M_{\text{Dissolution}} \) represent the released \( \beta \)-NGF amount at time, \( t \), and \( M_\infty \) is the amount of \( \beta \)-NGF released at equilibrium. ‘\( k \)’ is the dissolution constant.

4.8. Cell Culture of PC12 cells

PC12 cells (CRL-1721, ATCC), which are known to extend their neurites in the presence of \( \beta \)-NGF, were grown in complete cell culture media contained 85% RPMI-1640 Medium (30-2001, ATCC) supplemented with 10% (v/v) heat-inactivated horse serum and 5% (v/v) fetal bovine
serum at 37°C under a humidified atmosphere containing 5% CO₂. Cells were subcultured approximately every 2–3 days.

4.9. Bioactivity of β-NGF encapsulated microparticles on PC12 cells

Neurite extension assay of PC12 cells to assess the bioactivity of β-NGF released from 50:50 CPTEG:CPH microparticles were performed. Previously these microparticles were found to be nontoxic at a concentration of 125 µg/mL[73, 74]. Therefore, the bioactivity of released β-NGF were detected using the same non-cytotoxic concentration (125 µg/mL). Before the experiment, the 6 well plates were coated with mouse laminin (10 µg/cm²) and incubated at 37°C overnight to facilitate the attachment of PC12 cells. PC12 cells were then plated into the bottom of each well of the 6-well culture plates at a cell density of 2x10⁵ cells per well in 3 mL of complete cell culture media. The β-NGF encapsulated particles were then placed onto the membranes of transwell permeable support (BD Falcon) to perform a non-contact coculture assay. β-NGF at a concentration of 100 ng/mL was used as positive control while bare microparticles (without β-NGF encapsulated) and PC12 cells were used as negative control. After 2 days of incubation at 37°C under a humidified atmosphere containing 5% CO₂, cells were immunostained with anti-βIII tubulin antibody conjugated with Cy3 and DAPI to visualize neurite extension and nuclei of PC12 cells, respectively. Imaging of the cells was conducted using an ImageXpress Micro high content imaging system (Molecular Devices) and length of neurites for various conditions was quantified using a neurite outgrowth module of MetaXpress software (Molecular Devices).

4.10. Assessment of Bioactivity of β-NGF released from PLLA films using PC12 cells

The bioactivity of the released β-NGF from the PLLA films was assessed using PC12 cells. For this purpose, the PLLA films, incorporated with 0.5% β-NGF encapsulated 50:50 CPTEG:CPH microparticles and possessing surface β-NGF gradient were prepared, sterilized and sectioned
before the experiment. The 6 well plates were coated with mouse laminin (10 μg/cm²) and incubated at 37°C overnight to facilitate the attachment of PC12 cells. PC12 cells with a cell density of 2×10^5 cells per well were plated at the bottom of a six well plate in 3 mL complete cell culture media while the sectioned PLLA films were placed on top of transwell permeable support. Following 2 days of incubation at 37°C under a humidified atmosphere containing 5% CO₂, the cells were immunostained and imaged as described above.

4.11. Neurite alignment of PC12 cells on β-NGF gradients and micropatterned PLLA films
PLLAA films (1×3 cm²), incorporated with 0.5% β-NGF encapsulated 50:50 CPTEG:CPH microparticles and possessing surface β-NGF gradient were prepared, glued on a glass coverslip (micropatterned surface is upward) and sterilized. Then, the micropatterned films were placed into a 6 well plate, coated with mouse laminin (10 μg/cm²) and incubated at 37°C for 6 h to facilitate the attachment of PC12 cells. PC12 cells (1×10^4 cell/cm²) were seeded on section-1 (Supplementary Information, Figure S.1.), which contains the least β-NGF surface concentration, to observe both the effect of micropatterning and surface gradient on neurite alignment and extension. For this purpose, the cell suspension with a cell density of 1×10^4 was placed on section-1 with a small volume (~100 µL) and incubated for 2 h in order to let PC12 cells become attached to the film surface. Then, the well was filled with 2 mL of complete cell culture media. Following 2 days of incubation at 37°C under a humidified atmosphere containing 5% CO₂, the cells on the films were immunostained and imaged as described above.

4.12. Statistical Analysis
Throughout this study, significant differences between groups were evaluated using ANOVA analysis by Tukey’s method with 95% confidence interval. The results are presented as average ± standard deviation calculated from at least three independent experiments.
5. Results and Discussion

5.1. Characterization of polymers, particles and films

5.1.1. Polymer and Particle characterization

The NMR characterization shown in Supplementary Information Figure S.2 indicated that polymer molecular weight, structure, and composition were as intended and in agreement with previous studies [75-77]. Structure and size of the particles were assessed by SEM analysis. Nanoparticles and microparticles obtained were found to be of spherical shape and polydisperse in nature. Figure 1 shows SEM images of different types of nano and microparticles with and without encapsulated β-NGF. Size of particles fabricated using S/O/O double emulsion method was found to range from few nanometers to a micron (Figure 1 A and B) while the particles obtained by spray drying (Figure 1 C-I) possessed sizes ranging from a few nanometers to ~4 microns. Therefore, the method of particle fabrication significantly affected the size leading to the conclusion that spray drying resulted in larger sized particles. On the other hand, it was also observed that β-NGF or OVA encapsulation did not affect the particles in terms of shape, size and structure regardless the loaded amount (Figure 1 D-F and G-I). It was also noticed that the shape, size and structure of the particles were not altered by the type of protein loaded (β-NGF or OVA).
Figure 1. SEM images of different nano/microparticles. (A) 20:80 CPH: SA nanoparticles without β-NGF fabricated by S/O/O method (B) 20:80 CPH: SA nanoparticles with 0.1% β-NGF fabricated by S/O/O method. (C) 20:80 CPH: SA microparticles with 0.05% β-NGF fabricated by Spray drying. 20:80 CPTEG: CPH microparticles (D) blank (E) with 0.05% β-NGF loading and (F) with 0.1% β-NGF loading fabricated by spray drying method. 50:50 CPTEG: CPH microparticles (G) blank (H) with 1% OVA loading and (I) with 5% OVA loading fabricated by spray drying method.
5.1.2. Film characterization

Porous PLLA films with longitudinal micropatterns, containing 50:50 and 20:80 CPTEG:CPH microparticles loaded with different amounts of OVA, were prepared by dry phase inversion method. In this method, the different evaporation rates of non-solvent (pentane) and solvent (chloroform) resulted in phase separation during drying, and consequently, when the solvent and non-solvent are completely removed from the film, a porous structure is obtained. The advantage of this technique was to avoid the use of pore forming agent, which forms pores upon leaching in coagulation bath, and allow uniform distribution of particles in film matrix while creating pores. In addition, pentane, which is the precipitation agent in S/O/O method, was used to prevent the dissolution and facilitate dispersion of the OVA loaded particles. The particles loaded by various amounts of OVA were intended to be used to determine the release characteristics of the PLLA films while the particles loaded by different amounts of β-NGF were applied to observe the bioactivity of the PLLA films. The type and compositions of the prepared films used throughout this study is shown in Table 1.

Table 1. The type and composition of prepared PLLA films used in release tests.

<table>
<thead>
<tr>
<th>Film Type</th>
<th>PLLA</th>
<th>DCM</th>
<th>Pentane</th>
<th>Particle Type</th>
<th>OVA Loading</th>
<th>β-NGF Coating</th>
<th>Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA-1</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 50:50 CPTEG:CPH</td>
<td>1%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-2</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 50:50 CPTEG:CPH</td>
<td>3%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-3</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 50:50 CPTEG:CPH</td>
<td>5%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-4</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 20:80 CPTEG:CPH</td>
<td>1%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-5</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 20:80 CPTEG:CPH</td>
<td>3%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-6</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 20:80 CPTEG:CPH</td>
<td>5%</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+, represent the presence of β-NGF coating and micropattern)
The SEM images of the prepared film represented in Figure 2 A and B show the achieved micropatterns and surface porosity in both patterned and flat surfaces. Following the use of films for release experiments during 15 days, the pore size of the films in both sides increased indicating the degradation of PLLA (Figure 2 C and D). However, the pattern structure and shape maintained its solidity.

![SEM images of porous, OVA encapsulated 50-50:CPTEG-CPH particles incorporated PLLA films. (A) patterned (B) flat surfaces of unused films. (C) patterned (D) flat surfaces of used films. Films were maintained in PBS at 37°C for 15 days. Magnification: 2000x](image)

**Figure 2.** SEM images of porous, OVA encapsulated 50-50:CPTEG-CPH particles incorporated PLLA films. (A) patterned (B) flat surfaces of unused films. (C) patterned (D) flat surfaces of used films. Films were maintained in PBS at 37°C for 15 days. Magnification: 2000x

Upon the preparation of the films with protein-loaded polyanhydride particles, the film surfaces were modified by β-NGF by creating a surface gradient through ester amine interactions [71].
The peaks at 288.8 and 288.1 eV of the high-resolution C1s spectra are attributed to N–O–C=O and N–C=O, respectively, which resulted from the ester end groups of PLLA (Supplementary Information Figure S.3.A) [71]. Similarly, the XPS spectra of N1s indicate distinguishable increases at 402 eV of N–O as the portion of primary amines in the structure of β-NGF attached on the film surface (Supplementary Information Figure S.3.B)[71]. These XPS results indicated that ester terminated PLLA has the capability of forming covalent bonds with amines in the structure of β-NGF. The presence of N1s band on the β-NGF modified film (Supplementary Information Figure S.3.D) as compared to the plain film (Supplementary Information Figure S.3.C) indicates the presence of β-NGF on the surface.

The presence of a surface β-NGF gradient was also supported by XPS analysis as described in Section 2.5. Figure 3 indicates the changes in β-NGF concentration along the film sections on the surface. As the exposure time of β-NGF solution increases, the attached β-NGF amount increases along the sections (Sections-1: lowest, Section-3: highest) leading to the formation of concentration gradient as depicted in Figure 3.

The β-NGF loading on PLLA films through surface concentration gradient and microparticle distribution in PLLA conduit matrix enhances the loading capacity and control over the sustained release properties of the developed conduit system over its counterparts.
Figure 3. XPS analysis indicating the intensity of surface β-NGF gradient along different sections of the PLLA film (Section-1: Lowest β-NGF concentration, Section-3: Highest β-NGF concentration). *p<0.05 represents statistically significant difference.

5.2. β-NGF release from nano/microparticles

Various blank and β-NGF encapsulated polyanhydride copolymers with different ratios and chemistries, including poly SA, poly CPH:SA and poly CPTEG:CPH, were successfully fabricated and their β-NGF release properties were tested. The preliminary β-NGF release data obtained from different particles indicated that nano/microparticles of all copolymers except poly CPTEG:CPH showed none to very small amount of β-NGF release in PBS (data not shown). Only 50:50 and 20:80 CPTEG:CPH copolymer chemistry showed reliable β-NGF release in picogram levels in PBS. The released β-NGF amount was subsequently increased to nanogram levels by using 0.5w% BSA in PBS as stabilizer. Therefore, 50:50 and 20:80 CPTEG:CPH chemistries were used for the rest of the experiments. It was observed that the spray dried 50:50 and 20:80 CPTEG:CPH particles were able to release β-NGF for up to 8 weeks with the highest release amounts. Figure 4 shows cumulative release curves for 50:50 and 20:80 CPTEG:CPH particles. A sustained release of β-NGF was observed for almost 2 months where different
CPTEG:CPH polymer chemistries affected the release profiles. While the 50:50 CPTEG:CPH showed a slight burst release with higher amounts of β-NGF released at the start, the 20:80 CPTEG:CPH released lower amounts of β-NGF at the initial time points but the amount released at the later time points was found to be more than 50:50 CPTEG:CPH particles. This was mainly due to the increased hydrophobic CPH content (from 50 to 80) in the polymer chemistry leading to the slow surface erosion of the particles and thus, slow β-NGF release [78-82]. Blank control particles did not show any release. Overall, the particle chemistry has a major impact on surface erosion properties and, therefore, release characteristics. As opposed to the studies controlling the release through polyester based particles possessing bulk erosion or requiring excessive cross linking [37, 39], the polyanhydrides with surface erosion properties provided easy synthesis and better control over the protein release. In addition, the loading capacity of the synthesized polyanhydrides were significantly high.

Figure 4. Cumulative β-NGF release from 50:50 and 20:80 CPTEG:CPH particles in PBS buffer containing 0.5%BSA. N=3 and error bar represent standard error of the mean.
5.3. β-NGF and OVA release from films

The release characteristics of the films represented in Table 1 were also analyzed. OVA was used to observe the release characteristics of microparticles distributed in the PLLA film matrix, while β-NGF was released from the surface. Diffusion coefficients were calculated for each film and each protein (OVA and β-NGF) represented in Table 1 by using the equations mentioned in Section 2.7. The selected release plots, showing good fit of the experimental results with the theoretical calculations, are shown in Supplementary Information Figure S.4. This analysis was conducted for all of the cases and tabulated in Table 2. Most studies in the literature have not calculated the diffusion coefficients nor provided a detailed quantitative discussion about the release characteristics of the conduits. However, in this section, a detailed discussion regarding the release characteristics of the films for conduits is presented.

**Table 2.** The calculated diffusion coefficients of OVA and β-NGF released from each film.

<table>
<thead>
<tr>
<th>Film Type</th>
<th>Diffusion Coefficient of OVA (cm²/min)</th>
<th>Diffusion Coefficient of β-NGF (cm²/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Section-1</td>
<td>Section-2</td>
</tr>
<tr>
<td>PLLA-1 (50:50 CPTEG:CPH 1% OVA)</td>
<td>3.19x10⁻¹⁰</td>
<td>2.57 x10⁻¹⁰</td>
</tr>
<tr>
<td>PLLA-2 (50:50 CPTEG:CPH 3% OVA)</td>
<td>3.57x10⁻¹⁰</td>
<td>2.76 x10⁻¹⁰</td>
</tr>
</tbody>
</table>
The effect of the surface gradient, which was previously confirmed by XPS analysis (Figures S.3 and 3), was further supported by the release curves of OVA and β-NGF (Figure 5). An initial β-NGF burst release from the film surface was observed for all cases represented in Figure 5 and Table 2 due to the fact that the mass transfer resistance in the surface is low. It was noted that the released amount of β-NGF increased as the film sections changed from Section-1 (with the lowest β-NGF concentration) to Section-3 (with the highest β-NGF concentration) for both of the films PLLA-1 (Figure 5 A) and PLLA-4 (Figure 5 C) regardless of the type of the particles used, as expected. This was due to the increased β-NGF concentration going from Section-1 to 3. In a similar fashion, an increase in the diffusion coefficients calculated for β-NGF was observed for

<table>
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<tr>
<th>PLLA-3</th>
<th>(50:50 CPTEG:CPH 5% OVA)</th>
<th>7.51 x10^{-10}</th>
<th>6.51 x10^{-10}</th>
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<td>6.00 x10^{-14}</td>
<td>5.59 x10^{-13}</td>
<td>2.89 x10^{-13}</td>
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<tr>
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<td>(20:80 CPTEG:CPH 3% OVA)</td>
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<td>2.00 x10^{-12}</td>
<td>4.36 x10^{-13}</td>
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<td>1.70 x10^{-4}</td>
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<tr>
<td>PLLA-6</td>
<td>(20:80 CPTEG:CPH 5% OVA)</td>
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<td>1.21 x10^{-4}</td>
<td>2.63 x10^{-4}</td>
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</tbody>
</table>
both types of the particles used (PLLA-1 to 3, 50:50 CPTEG:CPH and PLLA-4 to 6, 20:80 CPTEG:CPH regardless the loading amounts) in Table 2, as the sections changed from 1 to 3 due to the increased surface β-NGF concentration. A consistent trend could not be established in Table 2 between the β-NGF diffusion coefficients and the type of the particles or their OVA loading contents. However, according to the release plots represented in Figures 5 A and C, although the presence of OVA loaded particles in the film matrix did not affect the creation of surface gradient on PLLA films and effective β-NGF release from the surface, the type of the microparticle did affect the β-NGF amount attached to and released from the film surface. For all of the sections, the released β-NGF amount from the surface were significantly lower in film PLLA-4, containing 20:80 CPTEG:CPH particles, than film PLLA-1, bearing 50:50 CPTEG:CPH particles. This could be due to the increased CPH content of the particles conferring more hydrophobicity and reducing the β-NGF attachment.

For all cases investigated, the release of OVA from the PLLA matrix was found to be slower than that of the release of β-NGF from the film surface due to the additional mass transfer resistance throughout the film matrix. Contrary to the case of β-NGF released from the surface, the amount of OVA released from the PLLA film matrix was highest at Section-1 (the lowest β-NGF concentration) and lowest at Section-3 (the highest β-NGF concentration) as indicated in Figures 5 B and D. This contradictory observation was due to the fact that during the β-NGF surface modification, as the β-NGF concentration level increases, the bottom part of the film (Section-3) was exposed to β-NGF solution for longer times (6 h) than the upper parts (Section-2 and 1), which resulted in loss of OVA, which was loaded into the particles and distributed across the PLLA matrix. This situation was true for all of the films regardless the type of the particle or
OVA loading percentage. Although the amount of OVA loss was small, the release curves in Figures 5 B and D indicated that it was enough to create another internal OVA concentration gradient through the film matrix. However, the formation of internal concentration gradients throughout the PLLA film matrix and loss of protein during the modification did not affect the overall β-NGF gradient of the films prepared by β-NGF loaded microparticles distributed in PLLA film matrix for bioactivity tests, due to the small losses (data not shown). The amount of OVA released from the film PLLA-4 was found to be lower than the film PLLA-1 for all sections. This could be due to the presence of 20:80 CPTEG:CPH particles with increased hydrophobic CPH polymer content in film PLLA-4 resulting in retarded surface erosion and therefore slow OVA release (Figures 5 B and D). The same situation is also valid for other cases shown in Figure 6. Similar trends can also be observed from the OVA diffusion coefficients presented in Table 2. The diffusion coefficients calculated for OVA release were significantly lower (varying between 1 to 4 order of magnitude) in films PLLA-4 to 6, with the 20:80 CPTEG:CPH particles, than films PLLA-1 to 3, with the 50:50 CPTEG:CPH particles, for the same sections and same OVA loadings (Table 2 and Figure 6). In addition, the decrease in diffusion coefficients from Section-1 to 3 can also be seen in Table 2.
Figure 5. The release profiles of PLLA-1 films containing 1% OVA loaded 50:50 CPTEG:CPH particles (A) β-NGF release from film surface (B) OVA release from film matrix. The release profiles of PLLA-4 films containing 1% OVA loaded 20:80 CPTEG:CPH particles (C) β-NGF release from film surface (D) OVA release from film matrix.

Besides the effect of different particles, the OVA loading also had a significant effect on the release kinetics and diffusion coefficients. As shown in Figure 6, the increase in OVA content from 1 to 5% resulted in higher amount of OVA release for all sections and particle types. Similarly, the increased OVA loading caused higher diffusion coefficient values due to the increased concentration difference promoting the faster release for all sections and particle types (Table 2). However, the OVA loading dependent increase in diffusion coefficients for 20:80 CPTEG:CPH particles were found to be more drastically changed (at least 2 orders of
magnitude change) than that of 50:50 CPTEG:CPH particles (remains in same order of magnitude) in Table 2 due to the slower release of 20:80 CPTEG:CPH particles with increased hydrophobic CPH content.

Figure 6. OVA release from Section-3 of films (A) PLLA-3, 5% OVA loaded 50:50 CPTEG:CPH and PLLA-6, 5% OVA loaded 20:80 CPTEG:CPH (B) PLLA-2, 3% OVA loaded 50:50 CPTEG:CPH and PLLA-5, 3% OVA loaded 20:80 CPTEG:CPH (C) PLLA-1, 3% OVA loaded 50:50 CPTEG:CPH and PLLA-4, 1% OVA loaded 20:80 CPTEG:CPH.

The effect of successful surface concentration gradients was clearly observed from the presented data. The β-NGF release from the surface and OVA release from the particles in PLLA matrix improved the control over the release rates. As an alternative to the studies mentioning co-delivery systems based on cross-linking and layering approaches [34-36], this strategy can also be used for simultaneous combined release of multiple growth factors providing different release rates for each individual growth factor to increase the flexibility.

5.4. Bioactivity of β-NGF released from nano/microparticles

The bioactivity of released β-NGF from spray dried 50:50 CPTEG:CPH particles was tested against PC12 cells. The differentiation of PC12 cells in the presence of β-NGF encapsulated microparticles was observed for 2 days in vitro. Since the released β-NGF amount from 20:80
CPTEG:CPH particles was low to trigger the differentiation of PC12 cells within 2 days of incubation (Figure 4), only 50:50 CPTEG:CPH spray dried particles, providing a burst release in 2 days, were tested for this assay. A non-contact co-culture assay was performed where microparticles at non-cytotoxic concentration were placed on cell culture inserts to observe their influence on PC12 cells growing at the bottom of the wells. It was observed that both β-NGF released from microparticles and soluble β-NGF, directly added to PC12 cells in culture media, caused significant neurite extension in PC12 cells, whereas the PC12 cells growing in media alone and in contact with blank particles (without β-NGF) had little to no neurites (Figure 7 A-C). Similarly, the quantitative measurement of neurite length per cell indicated that β-NGF released from microparticles caused ~6.5 µm long neurite extension per cell which was close to the effect of soluble β-NGF, directly added to PC12 cells in culture media (Figure 7 D). This assay showed that β-NGF released from 50:50 CPTEG:CPH microparticles was indeed stable and bioactive and showed a neurite extension pattern similar to that of free recombinant β-NGF in PC12 cell culture media. Furthermore, the process of β-NGF loading and storage time (one to two weeks) did not cause a significant decrease in bioactivity. Not only that, β-NGF encapsulated 20:80 CPH:SA particles, which did not show any β-NGF release during the ELISA test, were still able to cause neurite extension in PC12 cells (data not shown). This shows that most of the β-NGF released from the particles degrades in discontinuous systems, like release kinetics assays, and the amount quantified using ELISA kits is actually just the minimum amount that can be detected while the actual cumulative amount released is way higher than the detected amount. However, in continuous systems, like the non-contact co-culture assay with PC12 cells, the released β-NGF likely to be taken up by the cells immediately and shows a bioactive effect where released β-NGF was actually not detectable with ELISA tests.
Figure 7. Bioactivity of β-NGF released from 50:50 CPTEG:CPH microparticles (125 µg/ml) on the neurite extension of PC12 cells. PC12 cells density: 2x10^5 cells per well. Incubation time: 2 days. Neurites of the cells in every condition were immunostained with βIII-tubulin along with Cy3 secondary (shown in red), DAPI for nuclei (shown in blue). (A) Differentiated PC12 cells by the influence of β-NGF released from microparticles. (B) soluble β-NGF, directly added to PC12 cells in culture media (100 ng/mL). (C) PC12 cells only. (D) Quantification of neurite extension using ImageXpress Micro high content screening system and MetaXpress software. (N=3 independent experiments and Scale bar = 100 µm)
5.5. Bioactivity of β-NGF released from films

The bioactivity of released β-NGF from films containing 0.05% β-NGF loaded 50:50 CPTEG:CPH particles and β-NGF surface concentration gradients was tested using PC12 cells. 50:50 CPTEG:CPH microparticles were used for bioactivity tests considering their relatively faster β-NGF release than the 20:80 CPTEG:CPH microparticles, thereby providing sufficient amounts of β-NGF to trigger neurite outgrowth in PC12 cells. The quantitative neurite extension analysis shown in Figure 8.A indicated that as the surface concentration of β-NGF along the film surface sections (Sections-1 to 3) increased, the neurite length per cell increased as expected. The film bearing only β-NGF loaded particles showed similar effect with the Sections-2 and 3 of the film possessing only β-NGF surface modification while causing larger neurite extension than the Section-1 of the film possessing only β-NGF surface modification. The films with both β-NGF surface modification and β-NGF loaded 50:50 CPTEG:CPH particles produced the highest neurite extension in PC12 cells. It may be noted that the presence of β-NGF loaded 50:50 CPTEG:CPH particles in PLLA film matrix did not alter the overall β-NGF surface gradient. It can be anticipated that following the initial burst β-NGF release, the 50:50 CPTEG:CPH particles provided sustained β-NGF release, leading to higher and longer NGF activity. The images presented in Figure 8.B-G also showed neurite extensions upon exposure of released β-NGF. Therefore, the β-NGF loaded in particles and PLLA film matrix retained its stability and activity during the production steps.
Figure 8. (A) Bioactivity of β-NGF released from PLLA films possessing β-NGF surface gradient alone, containing 0.05% β-NGF encapsulated 50:50 CPTEG:CPH microparticles alone and combination of the two, both containing 0.05% β-NGF loaded 50:50 CPTEG:CPH particles and possessing β-NGF surface gradient, on the neurite extension of PC12 cells. PC12 cells density: 2x10^5 cells per well. Incubation time: 2 days. Same letters and * represents the statistically significant difference (p<0.05). Bioactivity of β-NGF released from PLLA films. PLLA films possessing β-NGF surface gradient alone (B) Section-1 (C) Section-2 (D) Section-3.
PLLA films possessing both 0.05% β-NGF encapsulated 50:50 CPTEG:CPH microparticles and β-NGF surface gradient (E) Section-1 (F) Section-2 (G) Section-3. PC12 cells density: 2x10^5 cells per well. Incubation time: 2 days.

The alignment of the neurite extension in one direction was also achieved by surface micropatterning. Figure S.5. in Supplementary Information clearly indicates the alignment of the extended neurites parallel to the direction of the micropattern. The additional effect of the surface gradient on the directional growth of the neurite extension was also explored; however, the fast β-NGF release likely resulted in the formation of homogeneous β-NGF concentration in the cell culture media preventing the observation of a surface gradient effect.

6. Conclusions
The overall results indicated that the multifunctional PLLA films possessing porous structure with longitudinal micropatterns as would be seen in the inner lumen of conduits, with β-NGF surface gradients and containing β-NGF-encapsulated surface eroding biodegradable and amphiphilic polyanhydride microparticles distributed into the film matrix were successfully produced. The change in particle type, protein loading amount and presence of β-NGF surface gradient succeeded in providing controlled release with several tunable parameters. It was shown that the NGF diffusion coefficient can be controlled over a wide range of values. The films and particles showed significant bioactivity with PC12 cells, causing effective neurite growth. Thus, these films bearing surface eroding polyanhydride particles are promising materials for peripheral nerve regeneration. The \textit{in vivo} efficiencies of the designed conduits will further be investigated.
7. Supporting Information

The schematic for NGF surface modification, NMR of synthesized polymers, XPS results for surface NGF coating, representative plots for the applied mathematical model, and effect of surface patterning in neurite alignment.

8. Acknowledgments

The authors would like to acknowledge the US Army Medical Research and Materiel Command under contract W81XWH-11–1–0700 and the Stem Cell Biology Fund for funding this work. We are also grateful to the Stanley Endowed Chair for providing additional funds in support of this work.

9. References


CHAPTER 6: PROTEOMIC ANALYSIS OF MESENCHYMAL STEM CELLS TRANSDIFFERENTIATED TOWARDS SCHWANN CELL-LIKE PHENOTYPIC CELLS

(manuscript in preparation in collaboration with Dr. Howard Gendelman’s group at UNMC)

1. Introduction

Peripheral nerve injuries can result in complete disruption of neuronal signals from peripheral limbs to the central nervous system (CNS) and vice versa and may cause paralysis in extreme cases. Anterograde (Wallerian) degeneration initiates a cascade of degenerative as well as regenerative events starting with infiltration of macrophages and Schwann cells (SCs) to help remove the debris created by degradation of axonal tissue, axonal fibers and myelin sheath of the distal end of the nerve segment. Schwann cells present in the distal end secrete neurotrophic factors that attract the axons regenerating from the proximal end. If sufficient support is provided to the growth cones they regenerate through the nerve gap and then grow into the remaining skeleton of the degenerated distal neural tissue. Various strategies are presented to provide support for regenerating neural tissue and commonly, a tube specifically called as a “nerve guidance conduit” is used for providing support for regenerating axons. Nerve guidance conduits filled with SCs have been shown to enhance regeneration significantly [1-3]. However, there is no good source of SCs, and usually, a healthy nerve is sacrificed to isolate SCs. Also, once isolated, isolation and enrichment of SCs is difficult process and can take weeks to months to obtain an enriched population of SCs devoid of other cell types such as fibroblasts and ultimately results in a limited number of SCs to transplant [4, 5]. To avoid such complications, various researchers have used other cell types such as mesenchymal stem cells [6-8], neural stem cells[9,
olfactory ensheathing cells [11, 12] and various other stem cell types [13, 14] to replace lost native SCs after nerve injury. Such cellular transplantation has also been shown to improve nerve regeneration significantly as well [2, 3]. One promising strategy that has been studied and shown to work effectively is the usage of mesenchymal stem cells (MSCs) as an alternative cellular source for transplantation at the site of nerve defect. Mesenchymal stem cells can be easily obtained from various sources [15] including bone marrow and adipose tissue using common surgical procedures. Mesenchymal stem cells provide benefits such as autologous transplantation, ability to continuously grow in cell culture environment, secretion of growth factors, and ability to transdifferentiate into SC-like phenotypic cells [16-18]. Transdifferentiated MSCs (tMSCs) used along with a nerve regeneration conduit resulted in enhanced nerve regeneration in a few studies [7, 8, 19]. After transdifferentiation, the MSCs show various morphological and molecular changes similar to native SCs and these changes are assessed using various experimental techniques such as immunocytochemistry, western blots, Enzyme-linked immunosorbent assays (ELISAs) and Reverse transcription-polymerase chain reaction (RT-PCR). However, in previous transdifferentiation studies, there have been no detailed proteomic analysis to understand the differences in the proteome of transdifferentiated MSCs (tMSCs) vs. undifferentiated MSCs (uMSCs). This study will help us in understanding the proteins which get regulated during transdifferentiation process and how proteome of differentiated cells differs from control cells. In this study, we are performing a proteomics analysis to identify and quantify proteins that may be differentially expressed by tMSCs and uMSCs, and to assess further the regulation of various pathways associated with identified proteins.
2. Materials and Methods

2.1. Culturing of bone marrow-derived mesenchymal stem cells

Mesenchymal stem cells were isolated and cultured from Brown Norway rats using previously established procedures [17]. Cells isolated were propagated in maintenance media containing alpha minimum essential media (αMEM, Gibco BRL| Thermofisher Scientific, Waltham, MA, USA), supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA, USA), 2% 200 mM solution GlutaMAX (Gibco BRL), and 1% antibiotic–antimycotic (Invitrogen| Thermofisher Scientific, Waltham, MA, USA) and maintained in incubators at 37°C/5% CO₂. Cells were harvested when they were approximately 80% confluent and Trypan Blue (T8154, Sigma–Aldrich, St. Louis, MO, USA) viable cell count performed using a hemocytometer prior cell platings. To harvest the cells, conditioned media was collected in a 15 ml centrifuge tube and then washed with PBS (Gibco BRL) followed by trypsinization using 0.05% trypsin-EDTA (catalog number 25300, Gibco BRL). After cell dissociation, conditioned media collected initially was used to neutralize the trypsin and collect the cells to transfer back to the centrifuge tube. The cell pellet was obtained by centrifuging the tube at 500 RPM. After centrifugation, the supernatant was aspirated, and cells were resuspended in the desired amount of fresh maintenance media.

2.2. Transdifferentiation of mesenchymal stem cells into Schwann-like cells

Protocol for in vitro transdifferentiation of MSCs into SC-like cells was obtained from Dezawa et al. [8] and was previously used to successfully transdifferentiate MSCs into SC-like cells on micropatterned substrates in our research group [17]. To start transdifferentiation, MSCs were allowed to grow under normal growth conditions and grown till they reached a confluence of about 30-40% before being subjected to 1 mM β-mercaptoethanol (BME; Sigma–Aldrich, St.
Louis, MO, USA) in αMEM for a day. After incubation for a day in BME, media from the flask was removed and then cells were washed with PBS before subjecting them to media containing αMEM, 10% FBS and 35 ng/mL all-trans-retinoic acid (ATRA (R2625); Sigma) for three days. Finally, after three days of incubation in retinoic acid supplemented medium, cells were again washed with PBS and further subjected to media containing αMEM supplemented with 20% FBS, 14 μL forskolin (FSK; EMD Millipore, Billerica, MA, USA), 5 ng/mL platelet-derived growth factor (PDGF; Sigma), 10 ng/mL basic fibroblast growth factor (bFGF, Promega Corporation, Madison, WI, USA) and 200 ng/mL heregulin β1 (HRG; Calbiochem, EMD Millipore) for 8 days in vitro (DIV).

2.3. Immunocytochemistry

In order to quantify protein expression after immunostaining using a high throughput imaging system, transdifferentiated MSCs (tMSCs) and undifferentiated MSCs (uMSCs) were plated in 96 well plates (655090, Greiner Bio-One, Monroe, NC, USA) at a density of 2000 cells per well at day 12 of transdifferentiation. Cells were first washed with 0.1M PO₄ buffer twice and then fixed for 20 minutes in 4% paraformaldehyde in 0.1M PO₄ buffer. After fixation, cells were washed with PBS three times every ten minutes and then blocked with a blocking solution prepared using PBS containing 5% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA, USA), 0.4% bovine serum albumin (BSA; Sigma) and 0.2% Triton X-100 (Fisher Scientific) for one hour. Various antibodies specific to glial, neuronal and proliferation markers (Table 1) were diluted at desired concentration in blocking solution and then applied to the cells overnight at 4°C. On the following day, cells incubated with primary antibodies were again washed with PBS three times every ten minutes before applying secondary antibodies Donkey-α-Mouse-Cy3 (1:500, Jackson ImmunoResearch) or Donkey-α-Rabbit-Cy3 (1:500,
Jackson ImmunoResearch) along with DAPI (1:50) for 60-90 minutes in the dark. Finally, as the last step, cells were washed with PBS again three times every ten minutes to remove any non-bound secondary antibody. 200 µl PBS was left in the wells to keep the immunostained cells hydrated for fluorescence imaging. Plates were imaged using an ImageXpress Micro high content screening system (Molecular Devices, Sunnyvale, CA, USA) and percent immunostaining for each antibody was quantified using multiwavelength cell scoring module of the MetaXpress software (Molecular Devices, Sunnyvale, CA, USA).

Table 1. Antibodies used for characterization of transdifferentiated MSCs

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Specificity</th>
<th>Vendor (catalog number)</th>
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<tr>
<td>Rab-α-S100</td>
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<td>Glial marker (SC marker)</td>
<td>Sigma-Aldrich (S2644)</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>Neuronal marker</td>
<td>R&amp;D systems (MAB1195)</td>
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<tr>
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<td>Proliferation marker</td>
<td>Abcam (ab16667)</td>
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</tbody>
</table>

2.4. PC12-TrkB cell culture

PC12-TrkB cells (obtained from…) were grown in maintenance medium containing RPMI-1640 Medium (30-2001, ATCC, Manassas, VA, USA) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. PC12-TrkB cells were genetically modified to express the BDNF receptor, TrkB[20]. The PC12-TrkB cells are capable of differentiating in the presence of both nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF). Cells were grown in 37°C/5%CO2 incubators. For passaging, cells were transferred to 15 ml centrifuge tubes, and the cell pellet was obtained by centrifuging tubes at 800 RPM. The supernatant was
aspirated before suspending the cell pellet in fresh maintenance media to count the number of cells using a hemocytometer. For differentiation experiments, multi-well plates were coated with laminin (10 µg/ml) before plating the cells.

2.5. PC12-TrkB and tMSCs/uMSCs non-contact co-cultures

To test out the differentiation of PC12-TrkB cells in the presence of soluble factors secreted by tMSCs or uMSCs, we employed a non-contact co-culture system using cell culture inserts in six well plates. For non-contact cocultures, PC12-TrkB cells were plated on laminin coated six-well plates at a density of 100,000 cells per well. tMSCs and uMSCs were plated on top of cell culture inserts (CLS3450, Sigma) at a density of 125,000 cells per well. From a previous experiment (data not shown), we determined that a 70:30 ratio of PC12-TrkB maintenance media and uMSCs maintenance media supports growth of all three cell types. A control condition was also maintained where PC12-TrkB cells were grown in maintenance media alone. After two days of differentiation, PC12-TrkB cells were immunostained with βIII-tubulin antibody conjugated to Cy3 along with DAPI to stain the nuclei. Imaging was performed using the ImageXpress Micro high content screening system and average neurite extensions across each condition was quantified using the neurite outgrowth module of the MetaXpress software.

2.6. Nerve growth factor-β enzyme-linked immunosorbent assay (ELISA)

A nerve growth factor-β (NGFβ) ELISA Kit (ab100757, Abcam, Cambridge, MA, USA) was used for quantification of released NGFβ from tMSCs and uMSCs. Similarly, a BDNF Elisa kit (BDNF Emax® ImmunoAssay System, Promega, Madison, WI, USA) was used to quantify the BDNF release. ELISAs were performed based on the instructions provided by the kit manufacturer. Cells were plated in 6 well plates at an initial plating density of 30,000 cells/well
and then grown for two days. After two days, conditioned media samples were collected, cells were fixed and stained with DRAQ5 (nuclei stain) (ab108410, Abcam) to assess the number of cells present at the time of sample collection.

3. Results

3.1. Expression of Schwann cell and neuronal marker proteins

Transdifferentiated MSCs (tMSCs) showed a higher expression of Schwann cell marker proteins such as S100, S100β and p75 as compared to undifferentiated MSCs (uMSCs) after performing immunocytochemical staining. tMSCs also showed higher expression of βIII-tubulin which is a neuronal marker. The presence of both glial and neuronal markers points out that after transdifferentiation MSCs appear to pass through a hybrid multipotential neuronal-glial fate. Despite higher expression of glial and neuronal markers, Ki67 immunostaining showed that tMSCs were proliferating at a much lesser rate compared to uMSCs. Figure 1 shows quantification of immunostaining for various marker proteins. S100 and TUJ1 were expressed significantly higher in tMSCs showing their neuronal-glial nature while Ki67 was expressed significantly higher in uMSCs showing that transdifferentiated cells proliferate slower than undifferentiated cells. S100β and p75NTR expression was found to be slightly higher in tMSCs but no significant differences were observed between tMSCs and uMSCs for these two markers.
3.2. PC12-TrkB neurite outgrowth quantification

PC12-TrkB cells (100,000 cells) cocultured with tMSCs/uMSCs (125,000 cells) under non-contact coculture conditions resulted in longer neurite outgrowth. PC12-TrkB cells growing in tMSCs coculture condition had significantly longer neurites as compared to uMSCs coculture (p≤0.1) and the control media condition (Figure 2). From these experiments, we showed the functional benefits of using tMSCs for growth and differentiation of PC12-TrkB cells as compared to uMSCs by showing that PC12-TrkB cells growing in the presence of tMSCs displayed longer neurite outgrowth as compared to PC12-TrkB cells growing in the presence of uMSCs.
3.3. Release of Nerve growth factor-β (NGFβ) and Brain derived neurotrophic factor (BDNF)

In parallel to cocultures, tMSCs and uMSCs were also plated in 6 well plates at an initial plating density of 30,000 cells per well to measure the release of nerve growth factor. Cells were stained with DRAQ5 and quantified to compare relative cell numbers for tMSCs and uMSCs at the time of collection of conditioned media samples. Media was collected after two days of cell growth and then the amount of NGFβ released from tMSCs vs. uMSCs was quantified using the NGFβ ELISA kit. Figure 3 and 4 illustrates the relative amount of NGFβ and BDNF released, respectively from tMSCs and uMSCs. Both BDNF and NGFβ release from tMSCs was found to be significantly higher as compared to uMSCs at p≤0.05.
Figure 3. The amount of nerve growth factor-β (NGFβ) released from transdifferentiated MSCs (tMSCs) and undifferentiated MSCs (uMSCs) after two days of cell growth. N=3 and error bars represent standard deviation. NGFβ release was found to be significantly higher from tMSCs as compared to uMSCs (p≤0.05).

Figure 4. The amount of brain derived neurotrophic factor (BDNF) released from transdifferentiated MSCs (tMSCs) and undifferentiated MSCs (uMSCs) after two days of cell
growth. N=3 and error bars represent standard deviation. BDNF release was found to be significantly higher from tMSCs as compared to uMSCs (p≤0.05).

4. Discussion and conclusion

Immunostaining of tMSCs using various SC marker proteins showed that tMSCs had a higher expression of SC marker proteins as compared to undifferentiated MSCs confirming that transdifferentiation of MSCs was successful. NGFβ ELISA showed that tMSCs released a significantly higher amount of neurotrophic factor NGFβ as compared to uMSCs pointing out another similarity of tMSCs with SCs. Finally, neurite outgrowth assay using PC12-TrkB confirmed that tMSCs provides a better functional benefit as compared to uMSCs and causes higher neurite extension in PC12-TrkB cells. A detailed proteomic analysis is currently in progress and will help to determine differences in regulation of proteins and pathways between tMSCs and uMSCs. Initial proteomics analysis has revealed 1025 proteins at the Global 1%FDR. Using PEAKView software, 808 proteins were included in the SWATH analysis and out of these 808, 388 proteins were found to be significantly changed by performing a Ztest for comparing tMSCs with control uMSCs values. Also, protein list was uploaded to Ingenuity pathway analysis (IPA) program to identify the protein and pathways of interest. Table 2 shows the pathways and protein of interest after performing IPA analysis and detailed analysis will be conducted on these proteins.
Table 2. Proteins and pathways of interest after performing IPA analysis

<table>
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<tr>
<td>Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency</td>
<td>PDLIM7</td>
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<tr>
<td>Mouse Embryonic Stem Cell Pluripotency</td>
<td>ACTR2</td>
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<td>Neurotrophin/TRK Signaling</td>
<td>ACTR3</td>
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5. References

CHAPTER 7: ONGOING AND FUTURE WORK

(In vivo transplantation of conduit and behavioral test were performed by Dr. Svitlana Zbarska)

The work described in the previous chapters is leading to the potential of transplantation of transdifferentiated MSCs seeded conduits in vivo in a sciatic nerve transection model with immunomodulation of macrophage phenotype using Interleukin-4 releasing micro/nanoparticles. This work is currently undergoing, and more studies will be conducted in the future.

Section 7.1 and 7.2 describes some of the preliminary data generated while conducting these studies.

7.1. In vivo experiments to test the therapeutic effect of transdifferentiated MSCs pre-seeded in micropatterned PLA conduits

7.1.1. Conduit implantation

Our initial pilot study to optimize the in vivo experiments was conducted on sixteen animals. The main goal of these experiments was to test and compare survival and possible therapeutic effects of tMSCs and uMSCs preseeded in the micropatterned biodegradable conduits for the sciatic nerve regeneration model. Young adult Brown Norway rats were used for these experiments. The right sciatic nerve of each animal was transected and a 10 mm segment of the nerve removed thus creating a gap that was bridged with micropatterned PLA conduits preseeded with either GFP-expressing tMSCs or uMSCs 24 hours before transplantation (20000 per conduit). The left sciatic nerve remained intact and used later as a control. Aseptic techniques were used during surgeries to ensure sterility. To eliminate bias, a double-blind study was performed. The conduits were prepared, preseeded with cells and given a number corresponding to the cell type. All procedures followed NIH guidelines for the care and use of laboratory animals and had the approval of the IACUC. The rats were tested weekly for functional recovery for 12 weeks after
conduit implantation. Shortly after surgery, one animal was excluded from the study and euthanized due to the right foot autotomy.

7.1.2. Functional tests: walking track test.

All animals were tested using the walking track test for footprint analysis to quantify possible functional recovery of the sciatic nerve. This non-invasive method has been shown to measure sensory and motor recovery. It can also be repeatedly used to detect the dynamics of functional recovery in an animal. For this analysis, we used video recordings using a mirror placed 45 degrees beneath the rat runway (Fig.1).

The video recording method used a Plexiglas runway running 100 cm long and 10 cm wide and was attached to a darkened cage at the end of the runway. The mirror underneath the runway reflects the image of the animal’s footprints. A running animal was video recorded using an HD Sony camcorder (Fig. 2, A) and then the video files were converted to images (30 frames per second). Afterward, the images were selected, and footprint analysis was performed using Avanti and VirtualDub software.

![Diagram of gait analysis system](image)

Figure 1. Diagram of gait analysis system. Setting up a mirror at 45 degrees helped with two different orientations (Figure 2A and 2B) of rat walking on the system.
Figure 2. A: Image of running rat during the walking test. B: the image of a footprint with represented measurements: PL – print length; ITS – intermediate toe spread; OTS – outer toe spreading.

The footprints were evaluated for three footprint parameters (Fig. 2, B): the distance between the first and fifth toe – outer toe spread (OTS), the distance between the second and fourth toes – intermediate toe spread (ITS), and the distance between the heel to the tip of the third toe – print length (PL). Six prints from each hind foot were measured and then averaged. The ratio of (injured-uninjured)/uninjured hind feet parameters was calculated. Based on these parameters, the sciatic functional index (SFI) was calculated according to the following formula:

\[
SFI = -38.3 \times \frac{EPL - NPL}{NPL} + 109.5 \times \frac{ETS - NTS}{NTS} + 13.3 \times \frac{EITS - NITS}{NITS} - 8.8
\]

Where:

EPL – experimental print length,
NPL – normal print length
ETS – experimental toe spread,
NTS – normal toe spread
EITS – experimental intermediate toe spread
NITS – normal intermediate toe spread.
The sciatic functional index is a common parameter that is calculated based on the empirically derived formula and is widely used for walking track analysis. The index score of 0 is defined as normal and an index score of -100 indicates total impairment. The measurements were taken weekly for two weeks before surgery and for 12 weeks after in order to observe functional recovery after sciatic nerve transection. For data analysis, data from all animals were pooled together due to the blind study requirements. On the group level, SFI dropped dramatically after surgery, and there was a little increase of SFI observed during the 12-week recovery period (Fig. 3).

![Figure 3. Changes of Sciatic Functional Index before and 12 weeks after sciatic nerve transection and PLA rolled conduit implantation (N=15).](image)

These data are similar to the SFI results of the previous group of animals that were implanted with empty conduits suggesting a limited therapeutic effect of preseeded cells. Following
histological analysis of extracted conduits revealed the absence of the GFP-expressing cells in the conduits 12 weeks after surgery. This might explain the low rate of recovery of motor function.

7.1.3. Functional tests: reflex test

All animals were tested using the hindlimb withdrawal reflex from a hot water bath. This water test is used to quantify sensory recovery of the sciatic nerve following injury. To conduct this test, the rat’s toes on the hind limbs were alternately dipped into water maintained at a temperature of 55°C and the time of the limb withdrawal reflex was measured using a video recording system. Withdrawal reflex time was defined as the period between the moment of crossing the water level and the first upper movement. To determine the average withdrawal reflex time, four trials were performed alternating between each limb with a minimum 30 sec time interval. Water temperature was constantly monitored. Our results indicated that the average time for normal withdrawal reflex of Brown Norway Rats is about 1-1.2 second (Fig. 4). While withdrawal reflex time (WRT) for the affected limb increased up to 2.91±0.96 s after surgery (Fig. 4). WRT for the affected limb decreased up to 1.89±0.37 s at the end of the 12-week period.
Based on these pilot experiments, we have established sterile surgical procedures for sciatic nerve transaction and conduit implantation at the injury site. Two functional tests, the walking track analysis, and withdrawal reflex test, were used to measure the motor and sensory functions of the affected nerve. The video recording method was used to capture the footprints during walking track test. This method was chosen because of its high accuracy, visibility, and minimum stress for animals. At the end of the 12-week recovery period, all animals were perfused with 4% paraformaldehyde. Conduits and the unaffected sciatic nerve from the contralateral side were extracted, and histomorphological analysis is underway.

A morphological analysis of the conduit with regenerated nerve and surrounding tissue is being performed to evaluate the conduits and possible extent of regenerated nerve tissue. Conduits with proximal and distal nerves attached were removed, examined qualitatively (Fig. 5, A), post-fixed, cut into four segments, and embedded in paraffin. Each segment of the conduit was given the name according to the position in the conduit (Fig 5, B): most proximal (MP), proximal (P), distal (D) and most distal (MD).

![Figure 5. A: Image of the isolated fixed conduit with tissue inside. B: Diagram of the cut conduit.](image)

Each segment was sectioned on a microtome at 5 µm transverse sections and placed on glass slides. Currently, we finished sectioning all MP and MD pieces from all 15 conduits and continue sectioning of P and D pieces.

After visual evaluation of the conduits possible regenerated nerve tissue was observed inside every conduit along its entire length (Fig.5, A).
As the first step in tissue characterization, the comparison of the most distal sections of MP piece and most proximal pieces of MD piece was performed. The corresponding 20 consecutive sections of MP and MD pieces of conduit and control nerve were imaged using dark field microscopy. Figure 6 shows representative images of the dark field imaging of MP section and MD section of the conduit. The presence of regenerated tissue was observed inside all MP as well as MD sections.

Figure 6. Dark field microscopy images of most proximal (MP) and most distal (MD) cross sections of the conduit. Outlined area was used for the area quantification. The errors point to the placement of the conduit layers.

The regenerated area inside of the conduit was outlined and quantified in mm². Group results are presented in Figure 7.
Figure 7. Area of regenerated neuronal tissue in mm² within most proximal and most distal conduit pieces and for contralateral control sciatic nerve. N=3

On average there was no significant difference between the area of regenerated tissue inside MP piece and inside MD piece (p=0.1562). Also, no significant difference was observed between MP and MD control sections (p=0.9478). On average the area of regenerated tissue inside of the conduit was two and half times smaller than cross-section area of control sciatic nerve.

7.1.5. Immunohistochemical analysis of regenerated tissue inside of conduits.

As the next step the standard immunohistochemical procedure was applied to regenerated tissue inside of the conduits using the antibodies listed in Table 1:
Table 1. Antibodies used for immunohistochemistry to examine nerve regeneration in the conduit.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Antibody target</th>
<th>Antigen-binding site</th>
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<tbody>
<tr>
<td>TUJ1</td>
<td>Neuronal marker</td>
<td>B-Tubulin microtubule</td>
</tr>
<tr>
<td>NF-68</td>
<td>Light neurofilaments</td>
<td>Cytoskeletal components of neurons</td>
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<tr>
<td>NF-200</td>
<td>Heavier neurofilaments</td>
<td>Cytoskeletal components of mature neurons</td>
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<tr>
<td>S100</td>
<td>Astroglial and Schwann cells</td>
<td>Calcium binding protein, mark Nucleus and cytoplasm</td>
</tr>
<tr>
<td>IBA1</td>
<td>Microglia marker- macrophages</td>
<td>Ionized calcium binder in cytoplasm</td>
</tr>
<tr>
<td>ED-1</td>
<td>Microglia marker- macrophages</td>
<td>Single chain glycoprotein of 110 kD</td>
</tr>
<tr>
<td>GAP43</td>
<td>Axonal growth cones in developing or regenerating neurons</td>
<td>Growth-associated protein</td>
</tr>
<tr>
<td>GFP</td>
<td>GFP-expressing cells</td>
<td>Green fluorescent protein</td>
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Cell nuclei were marked with 4’,6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to A-T rich regions in DNA.

Images were captured using a compound fluorescence microscope with 40x and 100x magnification. Also, double staining was performed to immunolabel the same regenerated area inside of the conduit with neuronal markers (TUJ1, NF200) and glial cell marker (s100). Figures 8 and 9 show representative images of TUJ1-s100 double staining of regenerate tissue inside MP and MD pieces of the conduit and control sciatic nerve correspondingly. TuJ1 antibody staining was highly effective in conduit sections as well as control sciatic nerve sections.
Figure 8. Regenerated tissue inside of the MP and MD pieces of the conduit double-labeled for TUJ1 Class III β-Tubulin (red) and SC-marker proteins s100 (green). DAPI (nuclei) staining is shown in blue. DIC images reflect the morphology of the neuronal tissue.
Figure 9. Regenerated tissue inside of the MP and MD pieces of the control sciatic nerve double-labeled for TUJ1 Class III β-Tubulin (red) and SC-marker proteins s100 (green). DAPI (nuclei) staining is shown in blue. DIC images reflect the morphology of the neuronal tissue.

TUJ1 staining was present in MP sections as well as MD sections revealing presence of the neuronal fibers along the entire length of the conduit (Fig. 8). Comparison between DAPI staining of conduit sections and control sections illustrate that the number of cells inside the conduit is considerably higher compared to the intact sciatic nerve. Colocalization of s100 staining around TUJ1 staining on control sections corresponds to Schwann cells wrapping around bundles of neuronal fibers (Fig. 9) Some s100 staining was present in conduit tissue but with the much smaller extent and was less organized (Fig. 8). DIC images reflected the morphology of the neuronal tissue inside of the conduit and in the sciatic nerve. Inside of the conduit, there was a higher number of fiber bundles smaller in diameter compared to the control.
Similar results were observed when tissue was double stained with neurofilament marker NF-200 and Schwann cell marker s100. Figures 10 and 11 show representative images of this staining on regenerated tissue inside MP and MD pieces of the conduit and control sciatic nerve correspondently.

Figure 10. Regenerated tissue inside of the MP and MD pieces of the conduit double-labeled for neurofilament NF200 (red) and SC-marker proteins s100 (green). DAPI (nuclei) staining is shown in blue. DIC images reflect the morphology of the neuronal tissue.
Figure 11. Regenerated tissue inside of the MP and MD pieces of the control sciatic nerve double-labeled for TUJ1 Class III β-Tubulin (red) and SC-marker proteins s100 (green). DAPI (nuclei) staining is shown in blue. DIC images reflect the morphology of the neuronal tissue.

Thus far, MP and MD immunolabeled sections of three animals imaged with 100x magnification and were quantified using MetaXpress software. All averages presented further were calculated based on quantification of 10 images per condition per animal; a total number of animals included in group analysis equals three. First, the number of cell nuclei was counted based on the DAPI staining (Fig.12).
Figure 12. Averaged number of cell nuclei per 0.1 mm$^2$ of regenerated tissue in the MP, MD pieces of the conduit and MP, MD pieces of control sciatic nerve.

Group DAPI quantification support the individual examples on Figures 8-11, and a higher number of DAPI staining was observed in the conduit tissue than in the control sciatic nerve.

Also using the same software number of TUJ1 and NF200 immunolabeled neuronal bundles were quantified (Fig. 13)
Figure 13. Averaged number of neuronal fibers per 0.1 mm² of regenerated tissue in the MP, MD pieces of the conduit and MP, MD pieces of control sciatic nerve immunolabeled with A: neuronal marker TUJ1; B: neurofilament marker NF200.

Figure 14. Neuronal area immunolabeled with neuronal markers presented as a percentage of total area of regenerated tissue in the MP, MD pieces of the conduit and MP, MD pieces of control sciatic nerve A: neuronal marker TUJ1; B: neurofilament marker NF200.
Group results of TUJ1 and NF200 immunolabeling revealed that some neuronal bundles in the conduit were higher than in the control sciatic nerve (Fig.13). Also, the total area of immunostaining for TUJ1 and NF200 was quantified as a percentage of total area of regenerated tissue inside of the conduit or total area of control sciatic nerve (Fig.14). On average the stained area inside the conduit was larger than in sciatic nerve.

Besides neuronal markers TUJ1 and NF200 and SCs-marker s100, tissue was immunostained with IBA1 and ED1 antibodies for the presence of macrophages. Almost no staining was observed inside of the regenerated tissue as well as in control sciatic nerve.

Conduits preseeded with GFP-expressing cells sections were immunolabeled with anti-GFP antibodies to amplify GFP fluorescents. No GFP-expressing cells were observed inside of the conduit 12 weeks after surgery. This could be due to several reasons: cells might migrate out of conduit, they could lose their GFP expression, or they might not survive during the recovery period. To improve the survival of MSCs in conduits, we planned to improve the design of conduit by introducing the pores in the conduit wall to improve the transfer of nutrients and waste across conduit wall.

7.1.6. Characterization of tMSCs growth on porous micropatterned PLA films fabricated with 10% Salt.

To characterize cell adhesion, growth and proliferation on porous films GFP-expressing tMSCs were plated and cultured on films produced with 10% Salt. Before cell placement, films were exposed to rat laminin-5 for 12 hours to provide an extracellular matrix coating for cells. The initial cell seeding density was 6,000 cells per cm². Cells on films were cultured in TDM3 Schwann-like cells transdifferentiated media. To detect the changes in cell adhesion and
alignment two films with cells were fixed and stained after 1, 3 and 6 days culturing in vitro. Rhodamine phalloidin and DAPI fluorescent stains were used to visualize the cell’s cytoplasm and nuclei respectively (Fig.15). tMSCs adhered, aligned, and survived on 10% Salt porous micropatterned PLA films and no major decrease in the number of tMSCs was observed one week after plating. This experiment was repeated three times. The results were consistent between three experiments.

Figure 15. tMSCs adhered, aligned, and survived on a 10% Salt micropatterned porous PLA film after 3 and 6 days culturing in vitro (DIV). Cells were fixed and stained with Rhodamine Phalloidin (RhPh) (red) and DAPI (blue) after 3 days in vitro (A-D) and after 6 days in vitro (E-H).

7.1.7. PLA porous micropatterned conduits manufacturing.

To manufacture conduits for in vivo and in vitro tests, PLA porous micropatterned films were rolled about 2.5 times around carbon fiber rod and sealed with chloroform. On average the internal diameter of a conduit was equal 1.8 mm, the external diameter was 2.2 mm and the
length of each conduit was about 12.5 mm (Fig. 16). Conduits were produced using 10% Pluronic porous micropatterned PLA films as well as 10% salt porous micropatterned PLA films.

Figure 16. Image of 10% Salt porous micropatterned PLA conduit (conduit dimensions: L=12 mm, ID= 1.8 mm, ED=2.2 mm).

7.1.8. Characterization of tMSCs growth inside porous micropatterned PLA conduits fabricated with 10% salt.

To characterize cell adhesion, growth and proliferation on porous films, GFP-expressing tMSCs were plated and cultured inside of the conduits produced with 10% Salt. Before cell placement, conduits were exposed to rat laminin-5 for 12 hours to provide an extracellular matrix for cells. The initial cell seeding density was 60,000 cells per conduit. To achieve an even cell distribution inside of the conduit lumen during the seeding procedure, conduits were rotated ten times per minute for two hours right after cell suspension was injected into the conduit. Cells inside of the conduit were cultured in TDM3 Schwann-like cells transdifferentiated media. To detect the changes in cell adhesion and alignment two conduits with cells were fixed and stained after 1, 3, 7 and 20 days culturing in vitro. Rhodamine phalloidin and DAPI fluorescent stains were used to visualize the cell’s cytoplasm and nuclei respectively (Fig. 17). tMSCs adhered, aligned, and
survived inside 10% Salt porous micropatterned PLA conduits over 20 days culturing in vitro. This experiment was repeated three times. The results were consistent between three experiments.

Figure 17. tMSCs adhered, aligned, and survived inside a 10% Salt micropatterned porous PLA conduit after 3, 7 and 20 days culturing in vitro. Cells were fixed and stained with Rhodamine Phalloidin (RhPh) (red) and DAPI (blue) after 3 days (A-D), after 7 days (E-H) and after 20 days in vitro (I-L).

Because the results from the multiple experiments where tMSCs were cultured on 10% Salt porous micropatterned PLA films or inside of the 10% Salt porous micropatterned PLA conduits were consistent and positive it was proposed to test the cell survival inside of these conduits in vivo.
7.1.9. Implantation of 10% Salt porous micropatterned PLA conduit preseeded with tMSCs in Brown Norway rats.

To test the tMSCs survival inside of the 10% Salt porous micropatterned PLA conduits implanted in the animals a pilot in vivo experiment was recently initiated. Four young adult Brown Norway rats (150-250g) were used for these experiments. Two days before surgery prior to cell placement 10% salt porous micropatterned PLA conduits were exposed to rat laminin-5 for 12 hours to provide an extracellular matrix for cells. Then one day before surgery tMSCs were seeded inside of the conduits with a seeding density of 50,000 cells per conduit. Aseptic techniques were used during surgeries to ensure sterility. First, animals were anesthetized with isoflurane. Then, the sciatic nerve was exposed along the femur bone and transected at the mid-thigh (Fig. 18, A). A conduit was placed in the gap. Proximal and distal ends of the transected nerve were inserted and sutured to the lumen of the conduit using 18" micro nylon suture (Fig. 18, B). Then the wound was closed by muscles and skin suturing. The antibiotic ointment was applied on top of the sutured skin to prevent bacterial infections. To prevent autotomy, rat e-collars were placed on the animals’ necks.

Figure 18. A) the sciatic nerve exposed along the femur bone; B) the 10% Salt porous micropatterned PLA conduits bridging 10 mm gap in sciatic nerve.
7.1.10. Isolation of 10% Salt porous micropatterned PLA conduits from rats.

To test the tMSCs survival inside of the 10% Salt porous micropatterned PLA conduits in vivo implanted animals were euthanized 3, 8, 15 and 29 days after surgery. To recover the implanted conduits, rats were deeply anesthetized with isoflurane, sutures were removed, the previously implanted conduit was cleared of connective tissue and then cut proximally and distally with extra sciatic nerve tissue at the both ends (Fig 19, A). The conduit was immediately placed in 4% paraformaldehyde solution for 30 minutes to fix the tissue (Fig. 19, B). Then proximal nerve tissue with 2 mm of the proximal part of the conduit and distal nerve tissue with 2 mm of the distal part of the conduit were cut and paraffin embedded for the immunohistochemical analysis. Morphological analysis of the conduit inside revealed the presence of the tissue within all conduit lumen. The middle part of the conduit was cut longitudinally into eight pieces, and the standard immunocytochemical procedure was applied to them to reveal the presence of the implanted tMSCs.

Figure 19. 10% Salt porous micropatterned PLA conduit bridging 10 mm gap in sciatic nerve three days after in vivo implantation. A) Before conduit was removed from the animal, B), Isolated fixed conduit before the cut on proximal, middle and distal parts.
7.1.11. Immunocytochemical analysis of isolated 10% Salt porous micropatterned PLA conduits after 3, 8, 15 and 29 days in vivo.

As the next step, the standard immunocytochemical procedure was applied to the middle part of the 10% Salt porous micropatterned PLA conduits which were implanted for 3, 8, 15 and 29 days in vivo. After conduits had been removed from animals, they were cut into three pieces. Then the middle part was cut longitudinally into four pieces, and each piece was immunolabeled for the presence of GFP-expressing cells and macrophages. After 3 and 8 days in vivo conduits did not have much regenerated tissue formed inside but after 15 and 29 days formed tissue was observed, removed, sectioned and immunolabeled. To amplify GFP expression of tMSCs, the anti-GFP antibody was applied. Also, Rhodamine phalloidin and DAPI were used to visualize cell cytoplasm and cell nuclei. Immunocytochemical analysis revealed that GFP-expressing tMSCs survived inside of the 10% Salt porous micropatterned PLA conduits after 3, 8, 15 and 29 days in vivo (Fig. 20). After four weeks in vivo, the number of GFP-tMSCs decreased, and these cells were obscured by regenerated tissue overgrown on the internal surface of the conduit. (Fig. 21). Regenerated tissue inside of the conduit lumen showed the presence of many macrophages/microglia which were immunolabeled with the anti-IBA1 antibody (Fig. 22).
Figure 20. (A-E) tMSCs shown on 10% Salt micropatterned PLA conduit which was implanted in Brown Norway rat to bridge 10 mm gap in sciatic nerve for eight days. (F-J) tMSCs shown on 10% Salt Micropatterned PLA conduit cultured in TDM3 for nine days in vitro.

Figure 21. (A-E) tMSCs shown on 10% Salt micropatterned PLA conduit which was implanted in Brown Norway rat to bridge 10 mm gap in sciatic nerve for 29 days. (F-J) tMSCs shown on 10% Salt Micropatterned PLA conduit cultured in TDM3 for 30 days in vitro.
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Figure 22. (A-E) Macrophages /microglia immunolabeling in the regenerated tissue inside of the 10% Salt micropatterned PLA conduit which was implanted in a rat to bridge 10 mm gap in sciatic nerve for eight days. (F-J) tMSCs shown on 10% Salt micropatterned PLA conduit that was implanted in Brown Norway rat to bridge 10 mm gap in sciatic nerve for eight days.

7.1.12. Immunocytochemical analysis of regenerated tissue inside of isolated 10% Salt porous micropatterned PLA conduits after 3, 8, 15 and 29 days in vivo.

After 15 and 29 days in vivo, regenerated tissue was observed inside of the conduit. That tissue was removed, sectioned and immunolabeled for the presence of GFP-tMSCs, Neuronal tissue, glial cells, and macrophages. To amplify GFP expression of tMSCs, the anti-GFP antibody was applied. TUJ1 and NF200 antibodies were used to mark neuronal tissue. S100 and IBA1 antibodies were used to immunolabel glial cells and macrophages correspondently. Results of the immunocytochemical analysis revealed that after 15 days in vivo the tissue inside of the conduit mostly consists of glial cells and macrophages. No presences of neuronal marker or GFP tMSCs were observed (Fig. 23). After 29 days in vivo the immunocytochemical analysis of regenerated tissue inside of the conduit showed that some appearance of the neuronal tissue, glial cells and macrophages (Fig. 24) No GFP-tMSCs were observed four weeks after implantation.
Figure 23. Immunolabeling of regenerated tissue inside of the conduit at 15 days in vivo at 10x magnification. Immunolabeling of TUJ1 and GFP, DAPI staining, merged image of TUJ1/GFP/DAPI (Top row). (Immunolabeling of TUJ1 and IBA1, DAPI staining, merged image of TUJ1/IBA1/DAPI (Middle row) Immunolabeling of TUJ1 and S100, DAPI staining, merged image of TUJ1/S100/DAPI image (Bottom row)

Figure 24. Immunolabeling of regenerated tissue inside of the conduit at 29 days in vivo at 10x magnification. Immunolabeling of NF200 and s100, DAPI staining, merged image of
TUJ1/GFP/DAPI (Top row). Immunolabeling of TUJ1 and IBA1, DAPI staining, merged image of TUJ1/IBA1/DAPI (Bottom row)

This pilot \textit{in vivo} experiment showed that implanted GFP-tMSCs could survive inside of the PLA 10\%Salt porous conduits, at least, one month after implantation although the number of implanted cells decreased during the recovery period. Since GFP-tMSCs were observed only on the internal surface of the conduit and not inside of the regeneration tissue it is suggesting that not much migration of these cells occur \textit{in vivo}. The regenerated tissue started to form about two weeks after conduit implantation. These results have to be repeated on a higher number of animals for the longer recovery period. Future experiments will involve behavioral testing of motor and sensory recovery after implantation of 10\% salt porous micropatterned PLA conduit preseeded with tMSCs in the new set of Brown Norway rats for long-term peripheral nerve recovery. Also, a large number of IBA1 positive macrophages in the conduit shows that immune cells play a very important role during the process of peripheral nerve regeneration. However, it has been shown in the literature that modulating the macrophage phenotype to a pro-healing one (M2 macrophages) helps with the regenerative outcome. So, in parallel to our \textit{in vivo} work, we started working on fabrication of interleukin-4 (IL4) releasing micro/nanoparticles as it has been known that IL4 is capable of reprogramming macrophages into M2 phenotypic macrophages [1] and thus improves peripheral nerve regeneration.
7.2. Interleukin-4 (IL4) releasing nano/microparticles for modulating macrophage phenotype to enhance peripheral nerve regeneration

As an additional strategy to facilitate nerve regeneration, we are encapsulating therapeutic proteins and cytokines in nano/microparticles. Polyanhydride nanoparticles have been successfully used for encapsulating various proteins and also for providing a sustained release of protein during the time of biodegradation. There are certain advantages of using polyanhydrides for making this protein releasing nanoparticles as they degrade by a mechanism of surface erosion unlike polyesters (PLA) that degrade by acid hydrolysis. Also, the degradation products cause less acidity in the immediate microenvironment as compared to the polyesters. Here we are planning to use copolymers of 1, 6-bis (p-carboxyphenoxy) hexane (CPH), 1, 8-bis (p-carboxyphenoxy)-3, 6-dioxaoctane (CPTEG) and sebacic acid (SA) to fabricate micro/nanoparticles for controlled release of NGF and Interleukin-4 (IL4). NGF is known for stimulating the growth of regenerating axons while IL4 has been used to reprogram naïve macrophages (M0) into pro-healing phenotypes (M2) as opposed to pro-inflammatory phenotypes (M1). Our initial pilot in vivo experiments showed a massive infiltration of macrophages inside the conduit and in the literature it has been demonstrated that M2 phenotypic macrophages can have a significant effect on nerve regeneration. To reprogram the macrophages into M2 phenotype, we need only a short-term supply of IL4 to the macrophages that infiltrate the conduit at the start. Once reprogrammed, M2 phenotypic macrophages themselves release a higher amount of IL4 creating a loop for further conversion of M0 macrophages to M2 phenotype. For achieving a short-term release of IL4 we have decided to coat the particles with IL4, but for NGF, we chose to encapsulate the NGF inside the particles so that we have a long-term sustained release of NGF to stimulate the regenerating axons throughout the regeneration
period (See chapter 5). Our strategy is that these NGF encapsulated and IL4 coated nan/microparticles will be co-administered inside the conduit along with MSCs and will release NGF and IL4 as they degrade and stimulate the regenerating axons and reprogram the infiltrating macrophages into M2 phenotype. So far we have finished optimizing the properties of NGF loaded particles (chapter 4), and recently we started working on characterizing reprogramming of macrophages using IL4.

7.2.1. IL4 release kinetics from IL4 coated 20:80 CPTEG: CPH and 50:50 CPTEG: CPH nano/microparticles in 0.5% BSA-PBS buffer.

Previously we have shown that NGFβ encapsulated 50:50 and 20:80 CPTEG: CPH particles in 0.5% BSA-PBS buffer, released NGFβ up to 8 weeks. To coat IL4 on the surface of particles, we used similar but blank, spray dried CPTEG:CPH particles. We dissolved 20 µg recombinant rat IL4 in 400 µl PBS and then incubated 5mg particles with this solution for a duration of 30 mins. After 30 mins, we centrifuged particles at the speed of 15,000 RPM for 10mins and followed by careful removal of supernatant without disturbing the pellet of particles. Another 200 µl of PBS was added to IL4 coated particles to wash out the excess IL4 before starting the release. Finally, release study was initiated by adding 200 µl of 0.5% BSA-PBS buffer, and samples were collected at 1, 4, 11, 24, 48, 72, 96, 120 and 144 hours after initial time point. Multiple ELISA’s were performed to optimize the dilution of the samples to bring the absorbance reading in the range of the ELISA assay. Figure 25 shows release kinetics data from IL4 adsorbed particles. Blank particles did not show any release.
Figure 25. Cumulative release curves for IL4 coated particles fabricated by adsorbing IL4 on the surface of 50:50 and 20:80 CPTEG: CPH particles. Blank 50:50 and 20:80 particles were used as a negative control. Both 50:50 and 20:80 NGFβ CPTEG: CPH microparticles showed a sustained release of IL4 for up to six days in 0.5% BSA-PBS buffer. Different release kinetics was observed for different copolymer chemistry. N=2 and error bars represent standard deviation.

7.2.2. Characterization of RWS 264.7 (Mouse Macrophages) cells reprogramming using soluble IL4

Different concentration of lipopolysaccharides (LPS) and IL4 was tested out to check conversion of RWS 264.7 macrophages into M1 and M2 phenotype respectively. Cells were plated in a 96 well plate at an initial concentration of 2000 cells/well and were left for growing in the plate for 24 hrs before adding LPS and IL4 at the desired levels. LPS was used at four different concentrations of 125, 250, 500 and 1000 ng/ml while IL4 was used at eight different concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 ng/ml. Positive M2 phenotypic markers
used were CD206 and CD163 while CCR7 was used for immunostaining M1 phenotypic macrophages. Additionally, double labeling was performed along with CD68 to immunostain all kinds of macrophages including M1, M2 and naïve (M0). Figure 26 shows immunostaining data for CCR7, figure 27 for CD163, figure 28 for CD206 and Figure 29 shows an average number of nuclei counted for each treatment condition.

Figure 26. Percent CCR7 (M1 phenotypic marker) immunostaining of RWS 264.7 macrophages treated with various concentrations of LPS and IL4. NT stands for non-treated control. Percent CD68 immunostaining was also quantified for all the conditions. LPS treated cells showed more CCR7 immunostaining as compared to IL4 treated cells.
Figure 27. Percent CD163 (M2 phenotypic marker) immunostaining of RWS 264.7 macrophages treated with various concentrations of LPS and IL4. NT stands for non-treated control. Percent CD68 immunostaining was also quantified for all the conditions. IL4 treated cells showed little to no staining as compared to LPS treated cell.
Figure 28. Percent CD206 (M2 phenotypic marker) immunostaining of RWS 264.7 macrophages treated with various concentrations of LPS and IL4. NT stands for non-treated control. Percent CD68 immunostaining was also quantified for all the conditions. IL4 treated cells showed little to no staining as compared to LPS treated cell.
Figure 29. Comparison of an average number of nuclei across various conditions. LPS was found to be cytotoxic in nature, and some cells after LPS treatment was found to be significantly lower as compared to IL4 or non-treated cells.

From our initial characterization experiments, we found that the IL4 that we used for treating the macrophages was not useful in reprogramming the cells. We repeated the experiment, and we still did not see any increase in expression of M2 phenotypic marker proteins (CD206 and CD163) in IL4 treated cells as compared to non-treated cells. Going further, we used other antibodies (CD206 from a different company) and CD11C (M1 marker) which we borrowed from Dr. Bratlie’s group and found that RWS 264.7 macrophages still did not show any expression of these M1 and M2 marker proteins. Previously, Dr. Bratlie’s group has shown that these macrophages reprogram themselves in the presence of IL4. Finally, we decided to try Mouse IL4 that Dr. Bratlie’s group used for reprogramming and planned to compare with the recombinant Rat IL4 (cross-reactive with Rat, Mouse, and Human) we were using for our experiments. When we used Mouse IL4 for reprogramming, we immediately saw evidence of
morphological changes in macrophages, and a fraction of macrophages acquired a bipolar morphology as compared to a round shaped naïve macrophages. We also stained the cells with CD206, CD163, and CCR7 and saw an increase in M2 phenotypic marker with Mo IL4 treated cells as compared to Rat IL4 and control cells (Figure 30). Also, in the presence of Mo IL4, we saw an increase in cellular proliferation and a higher number of macrophages were found in the wells treated with Mo IL4 as compared to Rat IL4 and non-treated macrophages (Figure 31).

Figure 30. Percent CD206, CD163 and CCR7 immunostaining of RWS 264.7 macrophages treated with 25ngs/ml of IL4. Macrophages treated with Mo IL4 showed the highest expression of M2 marker antibodies.
Figure 31. An average number of cells per site and mean cellular area for macrophages treated with Rat and Mouse IL4. Macrophages treated with Mouse IL4 proliferated a lot more as compared to Rat IL4 and non- treated cells pointing out that Mo IL4 is effective as compared to Rat IL4.

Because we plan to do our in vivo studies in rats, and recombinant rat IL4 was not found to be useful in the reprogramming of RWS 264.7 mouse macrophages, we purchased a rat specific macrophage cell line (NR8383) and test Rat IL4 to reprogram the new cell line.

7.2.3. Characterization of NR8383 (Rat alveolar macrophages) cells reprogramming using soluble IL4

Semi-adherent rat alveolar macrophage cell line (NR8383) was bought from ATCC and cultured for a week to freeze down stock vials of cells. For reprogramming of this cell line, Rat IL4 was used at a concentration 50ng/ml. In literature, it is shown that pro-healing phenotypic macrophages (M2) decreases the production of nitric oxide and increase the proliferation and
migration of Schwann cells, which in turn helps with nerve regeneration. So, we also wanted to test out such behavior of M2 macrophages, and we chose the following four assays.

- Production of nitric oxide (Griess assay)
- Proliferation of RT4-D6P2T (Schwannoma) cells (Ki67 immunostaining)
- Proliferation of RT4-D6P2T (Schwannoma) cells (CCK8 assay)
- Migration of RT4-D6P2T cells (Boyden chambers)

7.2.3.1. Production of Nitric Oxide
NR8383 cells were plated in wells of a 24 well plate at an initial seeding density of 40,000 cells per well in 300ul media. Cells were allowed to grow for a day and then IL4 was added to the cells at a concentration of 50ng/ml. IL4 treatment was done for a day to let the cells reprogram and after a day IL4 containing media was removed and fresh warm maintenance media was added to cells. After another 24 hrs, conditioned medium from the reprogrammed macrophages was collected and used for testing out nitric oxide production. 50 µl and 100 µl conditioned media was added to equal volume of modified Griess reagent in a 96 well plate, and the absorbance of the solution was read out after 15 mins using a spectrophotometer at a wavelength of 540 nm. M2 phenotypic macrophages released a lower amount of nitric oxide as compared to M0 macrophages, and Mo IL4 treated macrophages (Figure 32), showing that macrophages got converted to M2 phenotype after treatment with rat IL4.
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Figure 32. Griess assay to quantify the release of nitric oxide from reprogrammed macrophages vs. naïve macrophages. Macrophages treated with Rat IL4 released a lower amount of nitric oxide as compared to naïve macrophages and mouse IL4 treated macrophages.

7.2.3.2. Proliferation of RT4-D6P2T (Schwannoma) cells (Ki67 immunostaining or CCK8 assay)

RT4 cells were plated at an initial seeding density of 2000 cells per well in a 96 well plate in 50:50 RT4 medium and macrophages conditioned medium. Cells were grown for two days before fixation and then immunostained with Ki67 (proliferation marker) to compare the RT4 proliferation rate in various conditioned and control medium. RT4 cells treated with M2 macrophages (rat IL4 treated) conditioned medium was found to have higher Ki67 antibody staining as compared to naïve, or M0 IL4 treated macrophages conditioned medium. However, the staining was still found to be lower than control RT4 cells (100% RT4 cells medium) and RT4 cells growing in 50:50 Fresh RT4: fresh Macrophages medium (Figure 33). These results showed that FBS in the fresh medium affects the proliferation (Figure 34) and needs to be removed to make proper comparisons of conditioned mediums. We also conducted CCK8 assay
where we added 10% CCK8 dye to the RT4 cells after two days of growth and then after an hour, read the absorbance of different wells using a spectrophotometer at a wavelength of 450nms. CCK8 assay also showed a similar pattern of results compared to Ki67 immunostaining (Figure 35).

Figure 33. Percent Ki67 immunostaining of RT4 cells treated with different macrophages conditioned medium. Rat IL4 treated macrophages’ conditioned medium caused a higher proliferation as compared to Naïve and Mo IL4 treated macrophages’ conditioned medium. Control cells growing in the fresh medium had the greatest increase in cell number pointing out that, the FBS removal is required to test out the real effect of macrophage conditioned medium.
Figure 34. Average number of RT4 cells per site. Fresh macrophage medium caused a high proliferation of RT4 cells as compared to fresh RT4 medium and macrophages conditioned medium.
Figure 35. CCK8 assay. Rat IL4 treated macrophages’ conditioned medium caused a higher proliferation of RT4 cells than naïve, and Mo IL4 treated macrophages’ conditioned medium, but proliferation was still found to be lower than control cells growing in the fresh medium.

7.2.3.3. Migration of RT4-D6P2T (Schwannoma) cells (Boyden chambers)
Boyden chambers were used to study the effect of migration of RT4 cells in the presence of M2 phenotypic macrophages as compared to M0 phenotypic macrophages. After reprogramming, 50,000 RT4 cells were plated on top of Boyden chambers in a 24 well plate where different kinds of macrophages were growing in different wells. Soluble factors secreted by macrophages caused the RT4 cells to migrate from the top of the membrane to the bottom. After two days of migration, Boyden chambers were stained with sytox green (1:1000) and then ImageXpress Micro high content screening system was used to take images of the cells growing at the bottom of chambers using a 10X objective. Our initial data from these experiments shows that M2 macrophages (Rat IL4 treated) caused a higher migration of RT4 cells as compared to M0 macrophages and control RT4 cells (Figure 36).
Figure 36. Boyden chambers migration assay. M2 macrophages caused a higher migration as compared to M0 and control RT4 cells.

7.2.4. Future direction for IL4 releasing nano/microparticles study

7.2.4.1 Third replicate for IL4 release kinetics
So far, we have finished two replicates for IL4 release kinetics data. We will soon be conducting the third replicate for the same to get our replicate count to three.

7.2.4.2 Macrophage reprogramming using IL4 in serum free medium
Our initial experiments have showed that NR8383 cells are getting reprogrammed to M2 phenotype, but their effect on proliferation of RT4 cells is not clear because of the presence of FBS in the medium. In our future experiments, we will use the serum free medium to make sure proliferation is only affected by soluble factors released from reprogrammed macrophages.

7.2.4.3 Effect of M2 phenotypic macrophages on transdifferentiated MSCs (tMSCs)
Because of Schwann cell-like characteristics of tMSCs, we will also test out proliferation and migration of tMSCs in the presence of M2 and M0 phenotypic macrophages in vitro.
7.3. References

CHAPTER 8: SUMMARY AND CONCLUSIONS

We have successfully fabricated micropatterned poly (lactic acid) (PLA) films and polystyrene (PS) films and shown that these patterned films supported the growth of undifferentiated mesenchymal stem cells (uMSCs). We found that uMSCs proliferate at about the same rate across all four types of substrates (PLA patterned (PLA Pa), PLA smooth (PLA Sm), PS patterned (PS pa), PS smooth (PS sm)) and thus, micropatterns did not affect the proliferation of uMSCs. Further, by transdifferentiating MSCs to a Schwann cell-like phenotypic cells on these micropatterned films, we found that micropatterns do not cause any deleterious effect on the differentiation potential of MSCs. Percentages of S100β and p75NTR (markers of Schwann cells) immunostaining revealed that transdifferentiation of MSCs on these films resulted in a higher degree of differentiation (~80%) as compared to glass substrates (~40%). Percentage transdifferentiation was found to be similar on the patterned vs. smooth parts of both PS and PLA films. However, transdifferentiated MSCs (tMSCs) on the patterned part of the films were found to be highly elongated, spindly and the majority (~80%) were found to be oriented in directions of the patterns, while the tMSCs growing on the smooth substrate were considerably short and fibroblastic in nature. Aspect ratio, the ratio of cellular width to its height, was found to be 4-5 times greater for the tMSCs growing on patterned films vs. the tMSCs growing on smooth films. With this study, we found that micropatterned film is a suitable substrate for oriented growth of MSCs, and these films do not affect MSCs’ transdifferentiation capability and thus, can be used for fabricating an implantable conduit having micropatterned inner lumen. We were able to roll these films into conduits consistently, which were used for the in vivo studies.

Furthermore, before in vivo transplantation studies, we genetically modified MSCs for expression of the green fluorescent protein (GFP) and tested transdifferentiation of GFP-
expressing MSCs. GFP-expressing tMSCs exhibited a higher percentage of S100, S100β, p75\text{NTR} (Schwann cell marker protein) and βIII-tubulin (neuronal marker) immunostaining as compared to GFP-uMSCs showing that tMSCs behave like hybrid neuronal-glia像 cells. Also, GFP-tMSCs released a significantly higher amount of nerve growth factor-β (NGFβ) and brain-derived neurotrophic factor (BDNF) as compared to GFP-uMSCs. These GFP-tMSCs were found to benefit functionally as well and caused a significantly longer neurite outgrowth in PC12-TrkB cells compared to the control GFP-uMSCs.

Long-term continuous supply of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) has been found to neuroprotective in the case of many neurodegenerative disorders and has been shown to help even with nerve regeneration. We genetically modified mouse mesenchymal stem cells to secrete BDNF, GDNF, and BDNF/GDNF. We characterized the neurotrophic factor (BDNF, GDNF, BDNF/GDNF) secreting mouse MSCs on various extracellular matrix proteins to ensure that genetic modification does not affect the proliferation and migration capabilities of these cells negatively compared to control wild type and GFP-MSCs. We successfully achieved this by taking advantage of ImageXpress Micro high content screening (HCS) system that can be used for automated imaging and analysis of the samples in a multi-well plate format and showed that the cells were behaving normally via proliferation (Ki67 immunostaining), migration (time-lapse imaging) and cell survival assays (propidium iodide staining). We found that BDNF, GDNF, and BDNF/GDNF MSC subtypes secreted a significantly higher amount of respective therapeutic proteins as compared to control wild type and GFP-expressing MSCs. Conditioned media collected from these BDNF and GDNF MSCs caused a significant higher neurite extension in
PC12-TrkB and PC12 cells respectively showing the potential functional benefit associated with using these MSCs in vivo. Therefore, transplantation of genetically modified cells can be used as an alternative strategy for replacing native SCs during nerve injuries.

Using amphiphilic polyanhydride copolymers, we successfully fabricated NGFβ releasing micro/nanoparticles. Particles released protein for a period of two months, and different polymer chemistries resulted in different release profile. When these particles were used at a concentration of 125µgs/ml or lower, they did not show any cytotoxicity on mesenchymal stem cells. Finally, using noncontact coculture assays, we tested the therapeutic benefit of the released NGFβ from particles on the differentiation of PC12 cells and found significant higher neurite extension in PC12 cells as compared to control PC12 cells.

We have also successfully transplanted GFP-expressing transdifferentiated MSCs along with biodegradable porous micropatterned conduits in vivo and showed that transplanted cells survived for up to a month. After a one-month period, we also saw a huge infiltration of macrophages inside the conduit. Macrophages reprogrammed to M2 phenotype are known to enhance nerve regeneration. So, in vivo transplantation work and NGFβ releasing polymeric particle work, lead up to the work where we are fabricating cytokine (interleukin-4) releasing micro/nanoparticles.

In the future, in vivo transplantation studies will be conducted using a multifunctional nerve regeneration conduit incorporating various regenerative cues that we have characterized and developed during this dissertation. This multifunctional conduit will have following properties

- Biodegradability to avoid possibility of a second surgery
• Porosity to improve the flow of nutrients and waste from conduit microenvironment
• Longitudinal micropatterns to guide regenerating axons
• Patient-specific transdifferentiated mesenchymal stem cells as an alternative to Schwann cells
• Nerve growth factor-releasing microparticles to stimulate further the regenerating axons
• Interleukin-4 releasing microparticles to reprogram the macrophages infiltrating the conduit to wound healing M2 phenotype

From the work done during this dissertation, we have hypothesized that we can fabricate a multifunctional nerve regeneration conduit capable of providing effective mechanical, biological and trophic support to the regenerating nerves by combining strategies such as polymer scaffolding, nanoparticle delivery system, and stem cell engineering.