The application of electrospun biofunctional scaffolds for stem cell differentiation and nerve regeneration

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**THE APPLICATION OF ELECTROSPUN BIOFUNCTIONAL
SCAFFOLDS FOR STEM CELL DIFFERENTIATION AND
NERVE REGENERATION**

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SCAFFOLDS FOR STEM CELL DIFFERENTIATION AND
NERVE REGENERATION**

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ABSTRACT

Tissue engineering is a promising strategy with great therapeutic potential intended to assist the nerve regeneration. In addition to basic research on organ transplantation, some researches focus on creating an artificial environment enabling cells to induce tissue regeneration. To best mimic extracellular-matrix (ECM), ideal scaffolds can not only supply mechanical support for cell growth, but also regulate the functions of various cellular activities. Electrospun fibrous scaffolds serve as ideal biomimetic scaffolds due to their unique physical properties that closely resemble that of the ECM. Electrospun fibrous scaffolds can be prepared with controlled structure, and are amenable to various functional modifications targeted towards enhancing stem cell survival and proliferation, directing specific stem cell fates, or promoting tissue organization. In addition, signaling molecules can be incorporated into electrospun fibers in a spatially defined manner and enables the manipulation of cellular behaviors for specific applications. Accordingly, the aim of this thesis is to develop biomimetic scaffolds capable of delivering topographical and biochemical signals to enhance tissue regeneration via electrospinning; and to understand the phenotypic changes of cells interacting with such biofunctional scaffolds.

In our works, we firstly demonstrated the combined effects of nanofiber topography and controlled drug release on enhancing MSC neural commitment. A scaffold-based drug delivery system was developed by encapsulating retinoic acid (RA) within aligned poly(ϵ -caprolactone) (PCL) nanofibers. A sustained released of retinoic acid was obtained for at least 14 days. Topographical cues presented by the electrospun fibers have profound impact on modulating cellular behaviors such as attachment, proliferation and differentiation. Compared with TCPS, nanofiber topography from aligned PCL nanofibers significantly up-regulated the expressions of neural markers at mRNA and protein levels. The synergistic effects of nanofiber topography and retinoic acid further enhanced the potential of MSCs to undergo neural differentiation. Despite lower totally amount of RA, scaffold-based controlled delivery of retinoic acid showed more significant enhancement in neural markers expressions and further enhanced MSCs neural differentiation, as compared to bolus delivery. The results highlight the advantage of the scaffold-based approach in enhancing the potential of MSC neuronal differentiation and demonstrated the importance of drug delivery approach in directing cell fate.

Meanwhile, we also developed a polysaccharide scaffold that can provide combined substrate topography and matrix compliance signals to direct cell fate. Pullulan/dextran (P/D) nanofibers were fabricated with variable

stiffness by in-situ crosslinking during electrospinning. Human first trimester mesenchymal stem cells (fMSCs) cultured on STMP14 P/D scaffolds (*Young's* modulus: 7.84 kPa) in serum-free neuronal differentiation medium exhibited greatest extent of differentiation. It highlights the combined effects of substrate topography and matrix compliance signals in enhancing the probability of fMSC motor neural commitment and may help us understand the influence of topography and matrix stiffness on fMSC differentiation.

Finally, we evaluated the feasibility of implanting the electrospun scaffolds *in vivo* and demonstrated the effects of substrate topography on nerve regeneration *in vivo*. Electrospun PCL conduits were used for peripheral nerve regeneration across a 15-mm critical defect gap in a rat sciatic nerve injury model. At three months post implantation, conduits with nanofiber topography (Nanofiber) resulted in significantly higher total number of myelinated axons and thicker myelin sheaths as compared to conduits with microfiber and flat topography (Microfiber and Film). Retrograde labeling also revealed a better nerve regeneration in the presence of Nanofiber conduits. In addition, motor-evoked responses showed better electrophysiological recovery in the Nanofiber conduit group as compared to the Microfiber group. Altogether, this study will help deepen the knowledge on basic cell biology involving fibrous scaffolds and broaden the application of fibrous scaffolds to general tissue

engineering and neural regenerative medicine.

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List of Abbreviations

aFGF: acidic fibroblast growth factors

AFM: atomic force microscopy

BDNF: brain derived neurotrophic factor

bFGF: basic fibroblast growth factors

BSA: bovine serum albumen

cAMP: cyclic AMP

CE: Conformat Europe

ChAT: choline acetyltransferase

CMAP: compound motor action potentials

CNS: central nervous system

CNTF: ciliary neurotrophic factor

CSPG: chondroitin sulfate proteoglycans

CTs: Collagen tubules

DAPI: 4',6-diamidino-2-phenylindole

DCM: dichloromethane

DMEM: Dulbecco's modified eagle medium

DMEM/F12: Dulbecco's modified eagle medium/Nutrient mixture F-12

DMSO: dimethyl sulphoxide

DMF: dimethylformamide

DRG: dorsal root ganglion

ECM: extracellular matrix

EdU: 5-ethynyl-2'-deoxyuridine

EEP: ethyl ethylene phosphate

EMSC: ectomesenchymal stem cells

ESEM: environmental scanning electron microscope

FBS: fetal bovine serum

FDA: Food and Drug Administration

FESEM: field emission scanning electron microscope

FGF2: fibroblast growth factor 2

fMSCs: human first trimester mesenchymal stem cells

FN: fibronectin

GDNF: glial cell-derived neurotrophic factor

GGF: glial growth factor

HA: hyaluronic acid

HMDS: hexamethyldisilazane

IGFBF-1: insulin-like growth factor-binding protein 1

IGF-I: insulin-like growth factor I

GalC: galactosylceramidase

GFAP: glial fibrillary acidic protein

LIF: leukemia inhibitory factor

LN: laminin

LN-1: laminin 1

MEM NEAA: MEM non-essential amino acids solution

MMA: methyl methacrylate

MAP2: microtubule-associated protein 2

MSC: mesenchymal stem cells

MSCGM: mesenchymal stem cell growth medium

NaOH: sodium hydroxide

NEFL: neurofilament light

NGCs: nerve guide conduits

NGF: nerve growth factor

NSC: neural stem cells

NT-3: neurotrophin-3

Olig2: oligodendrocyte lineage transcription factor 2

PAN/PVC: poly(acrylonitrile-co-vinylchloride)

PAN-MA: poly(acrylonitrile-co-methylacrylate)

PBS: phosphate buffered saline

PCL: poly (ϵ -caprolactone)

PCLEEP: poly(caprolactone-co-ethyl ethylene phosphate)

P/D: pullulan/dextran

PDGF: platelet-derived growth factor

PDLLA: poly(D,L-lactide)

PDMS: polydimethylsiloxane

PEVA: poly(ethylene vinyl acetate)

PGA: poly(glycolic acid)

PGCL: poly(glycolide-co-caprolactone)

PHB: poly(3-hydroxybutyrate)

PHEMA: poly(2-hydroxyethyl methacrylate)

PHEMA-MMA: poly(2-hydroxyethyl methacrylate-co-methyl methacrylate)

PLA: poly(lactic acid)

PLA-TMC: poly (L-lactide-co-trimethylene carbonate)

PLCL: poly(lactide-co-caprolactone)

PLGA: poly(lactide-co-glycolide)

PLLA: poly(L-lactic acid)

PNS: peripheral nervous system

PPE: poly(phosphoester)

PU: polyurethanes

PVDF: polyvinylidene fluoride

RA: retinoic acid

SC: Schwann cells

SDSC: skin-derived stem cells

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophores

SEM: scanning electron microscope

SHH: sonic hedgehog

SIS: small intestinal submucosa

STMP: sodium trimetaphosphate

TAE: Tris acetate-EDTA buffer

TBS: Tris buffered saline

TBST: Tris buffered saline and Tween 20

TCPS: tissue culture polystyrene

TFE: 2,2,2-trifluoroethanol

TH: tyrosine hydroxylase

Tuj-1: β -Tubulin III

VEGF: vascular endothelial growth factor

CHAPTER ONE:

INTRODUCTION

1.1. OVERVIEW

Peripheral nerve damage is a common problem associated with traumatic injuries and most patients require reconstructive surgery. However, functional recovery across large-gap lesions is often sub-optimal, particularly when empty synthetic nerve conduits are implanted. Therefore, despite the well-documented drawbacks related with autologous nerve grafts [1], these implants remain the gold standard for peripheral nerve injury treatments. In an attempt to enhance the performance of synthetic conduits, pores [2-4] and lumen fillers [5-8] have been introduced as potential modifications. Pores in the range of 1- 20 μm can enhance nerve regeneration by promoting nutrient transport and blood vessel infiltration [2-4, 9, 10] while reducing fibrous tissue invasion [11]. Lumen fillers, on the other hand, provide contact guidance and enhanced surface area for cell attachment and growth [1, 12].

In this context, fiber structures represent a potential class of materials for synthetic nerve guides due to their biomimicking architecture and have been implemented either as fillers within the lumens of nerve conduits [5-8]; or as the sole component of nerve guides to form fibrous porous conduits [13-18].

Much of the earlier related works have focused on evaluating the functionality of microfilaments and microfibers (diameter $\sim 10 - 250 \mu\text{m}$) in enhancing peripheral nerve regeneration [6, 19]. With the development of new techniques such as electrospinning, later works presented promising results involving fibers with diameters that are in the sub-micron to nanometer range [7, 8]. Part of the motivation to decrease fiber size stems from the fact that the natural extracellular matrix comprises of fibers in the nano-scale and that *in vitro* studies have suggested advantages of nano-size in promoting more physiologically relevant cellular behaviors [20].

Electrospun meshes serve as ideal biomimetic scaffolds due to their unique physical properties that closely resemble that of the extracellular matrix (ECM). Fibrous scaffolds as nerve guides offer the advantage of combining permeability, high surface area for cell attachment and growth, and possibly topographical signals for directing cellular functions. Depending on the method of fabrication, biochemical signals may also be included into fibrous scaffolds as an additional factor for enhancing nerve regeneration. Besides using naturally occurring fibers such as silk, electrospinning may be the most popular and versatile method adopted for the formation of fibrous nerve conduits for peripheral nervous system (PNS) regeneration. Although the advantages of fibers in enhancing nerve regeneration have been demonstrated, *in vivo*

evaluation of the effect of fiber size on nerve regeneration remains limited. Altogether, our studies may demonstrate the versatility of such fibrous scaffolds in enhancing nerve regeneration through a combination of topographical and biochemical signaling.

1.2. HYPOTHESIS

In this thesis, we hypothesize that the electrospun scaffolds endowed with biochemical signal and controlled macroporous architecture will provide a more favourable three-dimensional microenvironment to help better manipulate the fate of seeded cells and improve nerve regeneration.

Current studies have demonstrated some degree of success in mimicking the biochemical signals required during stem cell differentiation [21, 22], other works have suggested the critical and synergistic roles played by the ECM. In particular, substrate compliance and architecture signaling are important in directing cell fate [23, 24]. By encapsulating retinoic acid (RA) into electrospun fibers, we aim to develop a biomimicking drug-encapsulated platform which provides synergistic contact guidance and biochemical cues to direct stem cell fate and enhance tissue regeneration. The drug-encapsulated scaffold takes the advantages in providing localized availability of biomolecules to adherent cells for long-term effect. Furthermore, the drug-encapsulated scaffold provides

synergistic contact guidance and biochemical cues to control the fate of stem cells and facilitate regeneration process in the nervous system.

1.3. OBJECTIVE

The overall objective of this project is to develop biomimetic scaffolds capable of delivering topographical and biochemical signals to enhance tissue regeneration via electrospinning; and to understand the phenotypic changes of cells interacting with such biofunctional scaffolds. To accomplish this goal, the following objectives are set:

1.3.1. To evaluate the effects of combined nanofiber topography and sustained release of biochemical signal (RA) on stem cell fate.

a. Establish a robust system to fabricate RA-incorporated electrospun PCL/gelatin nanofibrous scaffolds and achieve sustained release of RA.

b. Evaluate the potential of RA-encapsulated PCL nanofiber constructs as a potential platform for directing stem cell fate.

1.3.2. To evaluate the effects of substrate compliance on fetal MSCs neural commitment

a. Fabricate biofunctional pullulan/dextran scaffolds that can provide combined substrate topography and variable matrix compliance signals.

b. Characterize substrate stiffness and evaluate the efficacy of the

scaffolds in directing fetal MSCs differentiation.

1.3.3. To evaluate the efficacy of fiber diameter in enhancing nerve regeneration *in vivo*:

- a. Establish a robust system to fabricate porous, fibrous nerve guide conduits comprising of different topography.
- b. Characterize the nanofibrous nerve guide conduits.
- c. Evaluate the efficacy of fiber diameter on peripheral nerve regeneration across a critical defect gap in a rat sciatic nerve injury model.

CHAPTER TWO:

RELEVANT BACKGROUND

*(Partially published with Shawn H. Lim, Hai-Quan Mao and Sing Yian Chew.
Experimental Neurology 2010;223(1):86-101)*

SUMMARY

Stem cell fate is known to be regulated by signals from the microenvironment, one of the environmental parameters being the extracellular matrix (ECM) to which stem cells adhere. The ability to harness the regenerative potential of stem cells via a synthetic matrix has promising implications for regenerative medicine. Electrospun fibrous scaffolds can be prepared with high degree of control over their structure creating highly porous meshes of ultrafine fibers that resemble the extracellular matrix topography, and are amenable to various functional modifications targeted towards enhancing stem cell survival and proliferation, directing specific stem cell fates, or promoting tissue organization. The feasibility of using such a scaffold platform to present integrated topographical and biochemical signals that are essential to stem cell manipulation has been demonstrated. Future application of this versatile scaffold platform to mesenchymal stem cells (MSC) for functional nerve repair and regeneration will further expand its potential for nerve

regeneration therapies.

2.1. INTRODUCTION

Compared to the adult central nervous system (CNS), the microenvironment surrounding an injury site in the peripheral nervous system (PNS) is often more permissive to axonal regeneration. Over relatively short distances, typically less than 5 mm, axons can spontaneously regenerate. However, although axonal regeneration occurs, functional recovery may not always be the ultimate clinical outcome due to misdirection of regenerating axons towards an inappropriate target. Nerve autografts have by far offered the best results in nerve regeneration. Donor nerves can include functionally less important nerves such as sural nerves, superficial cutaneous nerves or lateral and medial antebrachii cutaneous nerves [25]. However, the drawbacks of using autografts are also well-documented. Donor site loss-of-function, formation of potential painful neuromas, structural differences between donor and recipient grafts and the lack of graft material are just a few examples [1, 26]. Nerve entubulation is therefore often chosen as the method of treatment to act as a physical guide for nerve regeneration and also to minimize the competitive interactions between myofibroblasts and axon growth at the injured nerve stumps. As such, the emphasis on using artificial nerve guide conduits (NGCs) has increased over the years.

The use of artificial NGCs allow for the easy specification of conduit

sizes and their mass-production. Unfortunately, early studies involving empty or saline-filled nondegradable silicone nerve conduits have only resulted in minimal nerve repair over distances up to 1 cm [27]. At the same time, such non-biodegradable grafts often lead to chronic tissue response or nerve compression [26]. To circumvent these problems, tissue engineered scaffolds may serve as an alternative choice for implantation to facilitate nerve repair. The physical and chemical properties of artificial grafts can be tailored based in applications. Therefore, the design of tissue engineered scaffolds has to start from the understanding of underlying cellular behaviors, tissue specific environment, and cause of tissue degradation and injury. In terms of cellular behavior, for example, cells in various tissues respond differently according to diverse factors such as physical topography or rigidity [28], gradient of cellular adhesion sites, electric fields [29], and chemical factors [30]. Importantly, different cells may behave oppositely even under identical stimuli: fibroblasts migrate toward the more rigid region while neurons stretch neurite to the direction of softer site. Furthermore, the environmental effects originate from the organ-specific functions such as protection (skin), mechanical maintenance (bone), transmission of force (ligament and tendons), conduction of electrical signal (neuron), blood circulation (heart and vessels), and force generation (skeletal muscle).

Among the various factors described above, physical cues are of great importance since cells reside in a physical microenvironment with tissue specific topography and rigidity. Such physical structures are widely observed in the human body such as the neuronal network in the brain, aligned myofibrils in the heart and skeletal muscle, and collagen fibers in the skin. Since these structures are intended for tissue specific functions, pathology may change the physical nanostructures in these organs, which results in the malfunction or disconnection of signal or force transfer. For example, it is well known that neurons in the human brain have a radially spreading morphology connecting the cerebral cortex and white matter. In the case of physiological malfunctions such as Alzheimer's or Parkinson's diseases, neuronal interconnections are severely damaged, thus losing informational pathways [31]. The goals of regenerative medicine with the aid of nanostructures lie in facilitating guided cell migration and proliferation to restore physiological structures and functions and minimizing possible side effects.

Currently, various materials and fabrication methods have been used to fabricate the observed natural structures with distinct physical properties. Over the past decade, electrospinning has gained rising popularity as a means of fabricating scaffolds with micro to nanoscale features similar to the hierarchical structure of the ECM. The ability to mimic the ECM structural organization is

an important consideration in rational design of a cell-responsive scaffold platform upon which additional functionalities can be incorporated. Electrospinning offers great flexibility in terms of the choice of scaffold material, as well as finer control over the scaffold geometry. Fibers with diameters ranging from tens to hundreds of nanometers can be easily produced, where the diameter is adjusted empirically via modulation of spinning parameters such as flow rate and collecting distance, and polymer solution properties such as solvent, concentration, conductivity, and surface tension [32, 33]. Electrospun polymeric fibrous meshes which also offer a higher surface area for cell attachment, are easy and inexpensive to fabricate and scale-up, and are relatively reproducible. Synthetic polymer-based systems offer additional advantages with their adjustable mechanical properties, as well as ease of surface functionalization via protein coatings, or chemical conjugation of specific signaling molecules.

In this Chapter, we will review recent efforts in manipulation and control of stem cell fates via cellular interactions with substrate topography and stiffness compliance. We will also summarize recent developments on materials optimization, and functionalization of NGCs through the inclusion of biochemical signaling molecules and incorporation of physical features in the form of luminal fillers and the control of NGC porosity and pore size.

2.2. CONTROL OF STEM CELL FATE BY SUBSTRATE TOPOGRAPHY AND STIFFNESS

Stem cells interact with and respond to a myriad of signals emanating from their extracellular microenvironment to which stem cells adhere. The extracellular matrix (ECM) varies not only in composition, but also in physical parameters, including elasticity and topography. Such physical properties are known to affect the micro- to nano-topography of integrin ECM receptors and to influence a range of cellular processes through changes in cell shape and the actin cytoskeleton [34, 35].

Mesenchymal stem cells (MSC) are capable of differentiating into many mesodermal cell types [36, 37], and also transdifferentiate to neural lineage cells [38, 39]. During the transdifferentiation, retinoic acid (RA) is an essential signaling factor. RA plays important roles in the development, regeneration and maintenance of the nervous system. Remarkably, in association with sonic hedgehog (SHH) and basic fibroblast growth factor (bFGF), RA can promote the formation of motor neurons. At the same time, due to the presence of topographical signals from the scaffolds, the effects of neural differentiation should also be enhanced as compared to an unpatterned substrate [40, 41].

2.2.1. Substrate topography for stem cells fate

Stem cell differentiation could be strongly regulated by the topography features. Micro- and even nanoscale geometric cues from cells environment, such as molecular conformation, surface topography or roughness, fiber diameter, and other parameters can be potent regulators of stem cells growth and differentiation. For example, neurite outgrowth from neurogenically differentiated stem cells was significantly enhanced when grown within inert but highly porous 3D polystyrene scaffolds, as compared to traditional flat surfaces [42]. Similar influences have been observed on cell alignment, where the directional growth and differentiation of adult rat hippocampal progenitors cultured on micropatterned polystyrene substrates chemically modified with laminin exhibited over 75% alignment in the direction of the grooves (13 mm wide and 4 mm high), as well as significantly increased expression of neuronal markers [43]. These findings show that the 3D topography of the substrate, in synergy with matrix composition (laminin), can facilitate neuronal differentiation and neurite alignment. Interestingly, the ability of cells to recognize such architectural cues extends to nanoscale topographical features. The Leong group carefully examined the effect of topography features (gratings with various widths from about 0.4–10 μm) on neural differentiation of human MSCs derived from bone marrow [40]. They proclaimed a significant

upregulation of neuronal markers, such as microtubule-associated protein 2 (MAP2), on the patterned surface of smaller gratings compared to the surfaces unpatterned and of larger gratings. This topography effect can be enhanced by the biochemical cues, such as RA, and the smaller grating showed a stronger effect compared to using RA alone on unpatterned surfaces on neural differentiation. Furthermore, the Leong group found that the expression of zyxin, a focal adhesion component was downregulated on the smaller gratings, demonstrating that this topography feature decreased the mechanical forces acting on focal adhesions in hMSCs and thus regulated the stem cell function [44]. Lee *et al.* also presented evidence that about 0.4 μm scaled ridge/groove pattern arrays alone could effectively and rapidly induce the differentiation of human embryonic stem cells (hESCs) into a neuronal lineage without any differentiation-inducing agents [45]. Meanwhile, rat hippocampus derived adult neural stem cells grown on laminin-coated electrospun polyethersulfone fiber meshes with the diameter ranging from 283 nm to 1452 nm showed differentiation and proliferation responses that significantly depended on fiber diameter [46]. Cells stretched multidirectionally to follow underlying 283 nm fibers but when grown on larger fibers, extended along a single fiber axis. With decreasing fiber diameter, a higher degree of proliferation and cell-spreading and lower degree of cell aggregation were observed. An independent group, Xie

et al. demonstrated that, compared with unpatterned and random nanofiber control, aligned nanofiber substrates could discourage the differentiation of ES cells derived neuronal progenitor cells into astrocytes [47].

The mechanisms of nanotopographic cues influence stem cell proliferation and differentiation are not well studied. But they appear to involve changes in cytoskeletal organization and structure, potentially in response to the geometry and size of the underlying features of the ECM. The changes in the feature size of the substrate may influence the clustering of integrins and other cell adhesion molecules, and then altering the number and distribution of focal adhesions [48]. For example, the nanotopographic features of the ECM have been shown to alter the morphology and proliferation of human embryonic stem cells (hESCs) through cytoskeletal-mediated mechanisms. Polydimethylsiloxane (PDMS) gratings with 600 nm features and spacing were found to induce the alignment and elongation of embryonic stem cells [49]. This study also showed that nanotopographic cues altered the organization of various cytoskeletal components, including F-actin, vimentin, g-tubulin, and a-tubulin, and the observed changes in proliferation and morphology were abolished by the effect of actin-disrupting agents. Alternatively, the influence of nanotopographic features may be mediated through secondary effects, such as

alterations in the effective stiffness perceived by the cell or differences in protein adsorption due to the structural features of the substrate.

2.2.2. Substrate stiffness for stem cells fate

Cells have the ability to actively sense their microenvironment and to react to the properties of their surroundings [50]. In addition to the influence of topography, there is significant evidence that other physical properties of the ECM may also contribute to stem cell fate or lineage commitment. Numerous physicochemical features of the scaffolds, such as surface roughness, and mechanical stiffness or elasticity are reported to play important roles in stem cell attachment, proliferation as well as differentiation. Generally, anchorage-dependent cells are able to anchor onto the underlying substrate through focal adhesions formed by clusters of proteins, including integrins, that are trans-membrane cell adhesion proteins [51]. The cell cytoskeleton, mechanically linked to the focal adhesions, is a network of filamentous proteins that extends throughout the cell cytoplasm in eukaryotic cells, and consists of actin, microtubules, and intermediate filaments [52]. The mechanical connections between the matrix and the cytoskeleton allow cells to exert traction forces that are transmitted to the cell nucleus through intracellular pathways; the resulting force triggers signaling transduction into biochemical

signals that affect schwann cell (SC) response, for example, the synthesis of specific transcription factors in the nucleus [53].

The molecular mechanisms governing the interaction between stem cells and the anchoring nanomaterial scaffolds have been postulated to involve chiefly mechanotransduction, the process by which physical stimuli are converted into biochemical signals and subsequently integrated into the cellular responses [54]. Although the transduction mechanisms are highly complex and not completely understood, they have been greatly investigated by various groups. Most developments in this area are homed in the involvement of integrins, focal adhesion kinase (FAK) mediated Ras homolog gene family, member A (RhoA)/Rho associated coiled-coil protein kinase (ROCK) pathway and extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) pathway, which are considered to be responsible for the cellular response of stem cells upon mechanical loading by the underlying nanomaterials.

While it is not clear whether there are additional mechanotransduction pathways regulating stem cell differentiation, the key role of this mechanism is well supported by numerous studies demonstrating their activation or modulation in the presence of nanomaterials and the extent of interaction they make with the embedded cells. For instance, Namgung *et al.* reported that

aligned CNTs networks influenced the osteogenic differentiation of hMSCs through a FAK-mediated RhoA/ROCK pathway [55]. From the report of Biggs *et al.*, FAK and ERK/MAPK gene expression were significantly upregulated in skeletal stem cells that underwent osteogenic differentiation on the nanopit arrays topography [56]. It was also shown that integrins mediated FAK and ERK signaling, thus regulating the osteogenic differentiation of rat MSCs on TiO₂nanotubes [57]. In neural engineering, a study using neural progenitor cells (NPCs) expansion on aligned collagen nanofibers demonstrated that β -1 integrins mediated the interactions between cells and collagen nanofibers and activated endogenous MAPK to enhance NPCs proliferation [58]. Additionally, for NSCs on H-UNCD films, it was demonstrated the H-UNCD films promoted the differentiation of NSCs through β -1 integrins and FAK mediated MAPK /ERK pathways [59].

Following these principals, more recent studies have been able to directly test the hypothesis that stem cell lineage specification can be determined by the mechanical properties of the ECM. Engler *et al.* examined the stiffness effect on stem cell differentiation and found that the lineage commitment of human MSCs from bone marrow was significantly regulated by the stiffness of polyacrylamide gels [34]. On soft matrices that mechanically mimic the brain, a large number of stem cells committed to the neurogenic

phenotype; by contrast, MSCs on moderately stiff matrices that mimic muscle experienced a myogenic commitment, and rigid matrices that mimic collagenous bone proved osteogenic. Similarly, the effective stiffness of the underlying substrate has been shown to regulate the differentiation of neural stem cells. Using a synthetic, interfacial hydrogel culture system, adult neural stem cells were grown on substrates varying in moduli between 10 and 10,000 Pa [60]. Cell spreading, self-renewal, and differentiation were inhibited on soft substrates (10 Pa), whereas these cells proliferated on substrates with moduli of 100 Pa or greater and exhibited peak levels of a neuronal marker, beta-tubulin III, on substrates that had the approximate stiffness of brain tissue. Softer substrates (~100–500 Pa) promoted neuronal differentiation, whereas stiffer substrates (~1,000–10,000 Pa) led to glial differentiation.

In other studies, it was found that human MSCs could be kept quiescent by growing them on polyacrylamide substrates that mimicked the properties of marrow [61]. MSCs seeded sparsely on these gels stopped progression through the cell cycle and could be induced to re-enter the cell cycle when presented with a stiff substrate. These cells could also be induced into adipogenic or osteogenic pathways when cultured in the appropriate induction medium, suggesting that a soft substrate mimicking the bone marrow niche can maintain MSCs in a quiescent state while preserving their multilineage potential. An

important conclusion of this study was that mechanical signals from the elasticity of the ECM may serve as a critical factor in the bone marrow niche that allows the maintenance of MSCs as a reservoir for a long period.

In general, the interaction between nanomaterials and stem cells innervates mechanotransduction as a key regulatory control in the stem cell differentiation process. Therefore, it can be reasoned that different physicochemical properties of nanomaterial scaffolds may affect the quality of this interaction and in turn influence stem cell fate through the above mentioned mechanotransduction pathways. An understanding of the interactions of stem cells with nanomaterial scaffolds at the cellular and molecular levels could facilitate the rational design of new substrates toward successful stem cell-based therapies.

2.3. CURRENT APPLICATIONS AND FUTURE PERSPECTIVES OF ARTIFICIAL NERVE CONDUITS

2.3.1. Materials utilized for artificial nerve conduits

In designing the next generation of nerve conduits, as with other classes of biomedical devices, much consideration has to be placed in the selection of an appropriate biomaterial. Intrinsic materials properties determine the general biocompatibility of the device; synthetic materials chemistry techniques could

further be utilized for incorporating customized mechanical or degradation characteristics. In addition to tissue compatibility, a nerve conduit should possess sufficient toughness to resist compression or collapse, yet still be flexible and suturable [62]. Degradation kinetics of the conduit would ideally be tailored to match the nerve regeneration rate. Some evidence suggests that non-degradable conduits are less desirable, due to the possibility of chronic nerve compression, which may necessitate a second surgery to remove the conduit, or formation of a fibrotic capsule around the conduit that might compromise regeneration [63, 64]. Ease and reproducibility of the manufacturing process are also desirable. Finally, secondary conduit properties such as wall porosity, incorporation of luminal surface topography, or electrical conductivity may also serve to enhance axonal regeneration through the conduits.

Materials used in the construction of artificial nerve conduits can be categorized as derived from either synthetic polymers or natural materials. Biologically derived nerve conduits from veins or muscle tissue have been used with varying degrees of success. Extracellular matrix (ECM) molecules purified from tissue such as collagen or laminin have the specific advantage of possessing cell-adhesive or signaling domains, which may present a more biologically relevant microenvironment. On the other hand, synthetic polymers

have the advantage over natural materials in that their specific properties such as degradation time can be tailored by altering their molecular weights or compositions. Both materials platforms are amenable to biofunctional modifications such as incorporation or surface-tethering of neurotrophic factors.

2.3.1.1. Synthetic materials

2.3.1.1.a. Aliphatic polyesters

Polyesters are a class of polymers that have been widely investigated for a variety of biomedical applications. Members of this family such as PGA, poly(lactic acid) (PLA), polycaprolactone (PCL) and their copolymers have been used widely in FDA-approved devices, including sutures, drug delivery systems, orthopedic screws and plates. The biocompatibility and safety of these materials *in vivo* have been well established. Polyesters are commonly synthesized by ring-opening polymerization, and biodegrade *in vivo* via hydrolysis of the ester linkages in the polymer backbone. A potential drawback to these materials is the release of mildly acidic degradation by-products; however, the ability to specifically tailor polymeric properties such as degradation rate and mechanical strength through copolymerization outweighs this disadvantage. For example, degradation behavior and rigidity of the a poly(lactide-co-glycolide) (PLGA) copolymer is highly dependent on its

molecular weight and crystallinity, which is in turn controlled by altering the proportion of glycolide to lactide monomers during synthesis [65]. Of this class of polyesters, only PGA and PLCL have been developed into FDA-approved, commercially available nerve guidance conduits (marketed as Neurotube and Neurolac, respectively). Even so, due to their desirable materials properties, there are continuing efforts to develop next-generation NGCs using polyesters as the base material.

The vast body of past research regarding material properties of PLGA and methods of controlling them render PLGA a very popular choice of material for the development of new nerve conduits. Bryan *et al.* processed 85:15 PLGA into a foamed conduit with 2 mm internal diameter and reported the successful regeneration of rat sciatic nerve over a 10 mm gap after 12 weeks *in vivo* [66]. Furthermore, the PLGA guide served as a suitable platform for the incorporation of additional signals to potentiate nerve regeneration, such as incorporation of glial growth factor or seeding with Schwann cells. In a follow-up study, the authors found that guides that had been primed by subcutaneous implantation for a week prior to suturing to the sciatic nerve further enhanced initial regeneration. Cellular infiltration into primed guides promoted the regeneration of significantly greater numbers of axons than unprimed guides at 4 weeks post-implantation [67]. In a separate study, Bini

and colleagues used a unique fabrication approach for creating microbraided 10:90 PLGA guides [68]. The intent was to overcome the inherent rigidity of the material, to achieve a nerve conduit that was flexible, porous and easily sutured to the nerve stump. Despite the textured exterior, the nerve conduit showed good biocompatibility without eliciting an inflammatory response after implantation into a 12-mm sciatic nerve gap in the adult rat. One month post implantation, 9/10 of the braided conduits contained regenerated axons, although the average axon diameter was only approximately 50% of the normal nerve. Most recently, Oh and colleagues blended PLGA with Pluronic F127 in a modified precipitation procedure to fabricate asymmetrically porous nerve conduits [2]. Pluronic was chosen as a porogen because it is water-soluble, thus eliminating the possibility of residual organic solvents contaminating the nerve guide. In a 12-mm rat sciatic nerve transaction study, the PLGA/Pluronic NGC had larger myelinated axons and faster CMAP nerve conduction velocity than pure PLGA or silicone control tubes.

NGCs fabricated from poly(L-lactic acid) (PLLA) were explored by Evans *et al.* as an alternative to PLGA [69]. The rationale was that PLGA tubes did not possess sufficient mechanical stability and had a tendency to collapse *in vivo*. In contrast, PLLA has high degree of crystallinity and is thus stiffer, yet remains biodegradable (43% mass loss after 8 weeks *in vitro*). PLLA NGCs

were fabricated using gas foaming/salt leaching techniques and implanted into a 12-mm sciatic nerve gap in the rat. At the end of 16 weeks, compared with their earlier study of PLGA, the authors found that PLLA had doubled the total number of axons in regenerated nerve cables. In a long term follow-up study, PLLA NGCs remained patent and supported axonal regeneration at a level that approached that of isografts, underlining their potential as an alternative material for NGCs [70].

There has been some interest in the use of poly(3-hydroxybutyrate) (PHB) as a potential NGC material candidate, largely due to the ease of synthesis via microorganisms and its potential biodegradability. Work done by Terenghi and associates demonstrated the feasibility of PHB NGCs in both the rat and rabbit common peroneal nerve regeneration models [71, 72]. However, there seems to be little follow-up on the use of PHB in the literature in favor of other more well-characterized polyesters likely due to their limited degradability *in vivo* [73] and concern over contamination from bacteria components.

2.3.1.1.b. Poly(phosphoesters)

One potentially important facet in the development of next-generation NGCs is the synthesis of novel biocompatible materials to supplement the

existing library of polymers currently under investigation. Potential drawbacks of existing materials such as loss of mechanical stability following implantation, lack of fine control of degradation rate, or release of acidic degradation products can be addressed via rational design of polymers. Additionally, there is now great interest in complementing polymeric NGCs with drug or cell delivery modalities to enhance axon regeneration or recovery of function. Materials capable of serving dual functions as both a polymeric conduit and a drug delivery vehicle considerably simplify the development of this new class of nerve guides.

One such example of a novel category of polyester modified to possess some attractive material properties is poly(phosphoester) (PPE). Such polymers contain a characteristic phosphoester linkage in the backbone, which is more easily cleaved via hydrolysis in physiologically relevant conditions than the ester linkage. Upon degradation, the phosphate, alcohol and diol by-products are milder than the acids released in polyester hydrolysis. Furthermore, the pentavalent phosphorus atom in the backbone is a highly flexible linkage site for the attachment of biofunctional pendant groups such as proteins or drugs [74, 75]. PPEs were tested for *in vitro* cell compatibility with a variety of cell types including primary neurons and neuronal cell lines, and the promising results prompted Wang and colleagues to test the suitability of one type of PPE,

poly((bis(hydroxyethyl) terephthalate-ethyl phosphoester) as a NGC material [76]. Conduits fabricated from two different molecular weights of the polymer were implanted into a 10-mm rat sciatic nerve gap and regeneration was evaluated after 3 months. At that time point, there was no visible inflammatory response elicited and only a thin fibrous capsule had formed around the tubes. 11 of 12 rats implanted with the higher molecular weight NGC showed positive reflex response suggestive of sensory recovery. The authors speculated that the lower molecular weight NGC underwent fragmentation too quickly following implantation, accounting for its poorer performance. Nevertheless, the biocompatibility of this polymer was established, and a secondary study later published by the same group used the same polymer NGC to complement delivery of microencapsulated nerve growth factor and showed that this system further improved peripheral nerve regeneration [77].

These studies suggest that the rapid degradation rate of PPE compromises its ability to support formation of the regenerating nerve cables immediately following implantation. In order to address this shortcoming, it would be useful to copolymerize polyphosphates with a more persistent, yet biocompatible and ultimately biodegradable polymer, thus prolonging degradation time while maintaining the desirable properties of PPEs. For example, Wen *et al.* copolymerized PLA with ethyl ethylene phosphate (EEP)

and achieved a copolymer with a moderate but steady degradation rate with 50% mass loss occurring over 30 days [78]. In a subsequent study, poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP) was prepared using the same synthesis scheme, and used to fabricate NGCs comprising a PCLEEP external film and an inner layer of drug-loaded electrospun PCLEEP fibers [8, 79]. Incorporation of more hydrophilic ethyl phosphoester groups into the backbone of the polymer was postulated to better facilitate loading and diffusion-dominated drug release from the polymer bulk phase, thus allowing the authors to combine both structural roles as well as drug delivery capabilities within the same NGC.

2.3.1.1.c. Polyurethanes

In addition to possessing the requisite stiffness to withstand collapse after implantation, another desirable mechanical characteristic of a NGC is flexibility. Such a NGC would be suitable for implantation, for example, in sites subject to constant flexion, such as the joint region. Elastic polymers such as polyurethanes (PU) are therefore a logical option for construction of such NGCs. On the other hand, PU is relatively soft, and needs to be either copolymerized or physically blended with another stiffer polymer to produce an NGC that can maintain its shape. Hoppen and colleagues first tested

PU-containing two-ply NGCs by blending commercially-available Estane 5701 PU with PLCL to form a biodegradable elastomeric NGC [80]. These NGCs were tested by Robinson *et al.* in 7-mm and 8-mm rat sciatic nerve transection models and showed comparable functional recovery with nerve autografts in the rats [81]. Additionally, the autografts elicited extensive endoneurial scarring, which was not observed in the NGCs.

Borkenhagen *et al.* used an elastomeric block copolymer, DegraPol, to fabricate NGCs by melt-extrusion under controlled temperatures [82]. It was hypothesized that the DegraPol PU would exhibit less swelling and biodegrade at a faster rate than the two-ply NGC. DegraPol was synthesized by coupling poly(R-3-hydroxybutyric acid-co-R-3-hydroxyvaleric acid)-diol and poly(glycolide-co-caprolactone)-diol (PGCL) with 2,2,4-trimethylhexamethylene diisocyanate. This copolymer was shown to be biocompatible and biodegradable. NGCs prepared from this copolymer were implanted to repair an 8-mm rat sciatic nerve gap. The degradation rate of the NGCs *in vivo* was found to correlate with the proportion of crystallizable PHB blocks in the polymer; that is, increasing the fraction of PHB (and thus crystallinity) decreased the degradation rate. All tubes gradually lost their elasticity after implantation, became brittle and started to fracture, as a result of faster degradation of the amorphous PGCL regions. Granulation tissue was

observed infiltrating through cracks in the tubes. Although the disintegration of the NGCs was not thought to have impeded regeneration of the nerve cable, further investigation into the long-term impact of the inflammatory reaction is warranted.

2.3.1.1.d. Piezoelectric polymers

Early evidence emerged to indicate that exposure to electrical charge might enhance nerve regeneration [83, 84]. This motivated investigations into the use of both electrically conducting, as well as piezoelectric materials as NGCs. Piezoelectric materials are by far more desirable as they generate transient charge in response to mechanical strain, rather than merely transmitting external electrical stimulation. Poled poly(vinylidene fluoride) (PVDF) NGCs were fabricated by stretching PVDF conduits (to mechanically align dipoles) and then subjecting them to an electric field [85]. The NGCs were then implanted into a 4-mm mouse sciatic nerve gap for 12 weeks. All the NGCs showed nerve regeneration, with significantly greater numbers of myelinated axons observed in the poled conduits. In a follow-up study, piezoelectric NGCs were fabricated from a copolymer of PVDF and trifluoroethylene (PVDF-TFE), so that NGCs did not require mechanical stretching to align the dipoles [86]. Positively-poled PVDF-TFE conduits

showed the best rat sciatic nerve regeneration over a 10-mm gap compared with negatively-poled or unpoled conduits, indicating that the piezoelectric effect was, in no small part, responsible for promoting axonal growth. Despite these encouraging results, however, the nondegradability of piezoelectric polymers, as well as their insolubility in most organic solvents (thus complicating the processing conditions) hinders their widespread adoption as a NGC material. Development of a synthetic method for production of biodegradable piezoelectric polymers would facilitate further progress in this area.

2.3.1.1.e. Hydrogel-based NGCs

Hydrogel-based nerve guidance channels were first designed with the repair of the spinal cord in mind. The hypothesis was that the intrinsically high water content of hydrogels would impart mechanical compliance that more closely matches to that of native nervous tissue, thus resulting in more efficient regeneration. Hydrogel tubes were fabricated by Dalton and colleagues from poly(2-hydroxyethyl methacrylate) (PHEMA), which is currently widely used as a material for soft contact lenses [87]. PHEMA was chosen as it is both relatively inert and elastic, and shown to be biocompatible with neural tissue [88, 89]. However, PHEMA homopolymer was deemed too soft to be suitable for implantation, and thus was copolymerized with methyl methacrylate (MMA)

to provide greater rigidity. These PHEMA-MMA tubes have a biphasic wall structure, with an outer gel-like layer and inner porous layer, created during the phase separation of polymerized particles from the solution bulk phase. The regeneration of rat sciatic nerve over a 10-mm gap was tested using these tubes as guidance conduits [90, 91]. After 8 weeks, PHEMA-MMA NGCs remained patent and had not elicited significant inflammation. After 16 weeks, 60% of NGCs supported nerve regeneration at a level similar to autografts, but most of the un-regenerated NGCs had collapsed. Electrophysiological recordings taken at 8 weeks showed no significant differences between NGCs and autografts; however, by 16 weeks the autograft yielded significantly better functional recovery as indicated by the differences in action potential velocity as well as amplitude. PHEMA-MMA NGCs permitted water and small molecule diffusion, and possessed sufficient stiffness required for suturing and to maintain their shape post-implantation over an 8-week testing period. However, in a subsequent long-term implantation study, 4 out of 14 tubes experienced tube wall collapse, and a small subset of tubes exhibited the presence of a fibrotic capsule and some inflammatory cells [90, 91]. Although they were largely biocompatible, the non-degradability of these NGCs, which may eventually lead to chronic foreign body reaction, presents a continuing concern that may limit their wider-scale application.

In order to address the concern regarding patency of PHEMA-MMA NGCs, Katayama *et al.* tested two modified NGC configurations in the 10-mm rat sciatic nerve transaction model: a hydrogel NGC in the shape of a corrugated tube, and a tube that was reinforced with an internal coil of PCL [92]. Coil tubes retained their original shape and remained patent over 16 weeks, whereas one-third of the corrugated tubes had collapsed at that time. The enhanced tube patency was also associated with significantly better functional recovery in animals implanted with the coil tube over those implanted with the plain PHEMA-MMA NGCs. This result unequivocally points to the importance of tube structural integrity in permitting efficient peripheral nerve regeneration.

2.3.1.2. Natural materials

2.3.1.2.a. Collagen

Collagen is a fibrous structural protein that is a major component of the extracellular matrix (ECM), comprising the bulk of connective tissue. The ubiquitous nature of collagen and its availability in a well-characterized, purified form has led to its widespread use as a biomaterial for tissue repair. NGCs fabricated from cross-linked bovine collagen I are currently commercially available in the form of the FDA-approved NeuraGen tube. The use of these tubes in a monkey median nerve transection model was first

described by Archibald *et al.* where they showed comparable performance to nerve autografts [93, 94], and ultimately superior to direct suturing of nerve stumps. Clinical studies using NeuraGen tubes for repair of nerve gaps up to 18-mm in the hand also showed promising results [95, 96]. Despite these clinical successes, the effectiveness of collagen NGCs in bridging larger nerve gaps has not yet been demonstrated. In a study done by Stang *et al.*, the authors demonstrated that rat sciatic nerve was not able to regenerate within a porcine collagen I/III NGC, when the gap length was 20 mm [97]. Regeneration was only observed when additional cues in the form of Schwann cells were supplemented to the collagen NGCs.

In an attempt to overcome the gap length limitation of collagen NGCs, Yoshii *et al.* tested bundled collagen filaments as a nerve guidance device without an external conduit wall [98-100]. Each bundle consisted of two thousand aligned 20 μ m-diameter collagen filaments made from purified bovine collagen I, which were crosslinked by polyethylene glycol diglycidyl ether to slow down the resorption rate. The ends of each bundle were sutured into a 20-mm rat sciatic nerve gap. The authors hypothesized that the improved flexibility and permeability of the collagen bundle as compared to a hollow tube would result in improved regeneration over long nerve gaps. Nerves repaired with fiber bundles contained a greater number of myelinated axons than those

repaired with the empty collagen tubes, but comparable to autografts. However, no significant functional recovery was observed in all experimental groups 12 weeks post-operatively.

Gelatin derived from denatured collagen has been widely investigated for a wide range of biomaterial applications, such as microcapsules and cell carriers for drug delivery and for tissue regeneration. The major drawback of gelatin-based scaffolds is that they are soft and prone to rapid clearance from the implantation site; thus crosslinking is required for mechanical stabilization. Gamez *et al.* fabricated conduits by photo-crosslinking styrenated gelatin [101, 102]; this platform was utilized as a facile method of incorporating within the conduit additional bioactive cues in the form of gelatin fibers, laminin, fibronectin or nerve growth factor [103]. In a 10-mm rat sciatic nerve gap model, there was no electrophysiological response recorded from the cerebral sensorimotor cortex following sciatic nerve stimulation, either at 6 or 12 months post-operatively in animals treated with the crosslinked gelatin conduit. However, somatosensory evoked potentials were recorded in animals treated with conduits containing either ECM molecules or gelatin guidance fibers. One potential explanation for the poor regenerative capacity of the UV-crosslinked gelatin conduits could be that their degradation profile is incompatible with the regenerating tissue. Lu *et al.* investigated peripheral nerve regeneration in a set

of genepin-crosslinked gelatin NGCs that had three different degrees of crosslinking [104]. The authors found that nerves regenerated within tubes with an intermediate crosslinking degree (and thus intermediate degradation rate) had the highest axon density and mature epineural and perineural organization. It was speculated that tubes with high crosslinking degraded too slowly, resulting in compression of the regenerating axon cable; on the other hand, tubes with low crosslinking fell apart too rapidly, releasing a large quantity of debris and evoking acute foreign body response.

2.3.1.2.b. Polysaccharides

Polysaccharides such as chitosan, alginate, and hyaluronan have been investigated in a variety of biomedical applications, especially as scaffolds for supporting the formation of new tissues [105-107].

Alginate is a naturally occurring block copolymer of β -D-mannuronate and α -L-guluronate that is commonly purified from seaweed. Although it is not known to perform any biological function in humans, it is generally well-tolerated and biodegradable *in vivo*. One particular feature of alginate is that it is readily crosslinked by calcium ions to form a weak hydrogel; however, the relatively high concentration of calcium required to achieve high degree of crosslinking is thought to be cytotoxic and detrimental to neural regeneration.

Nishimura and colleagues instead used ethylene diamine to covalently crosslink alginate that was further freeze-dried, resulting in a porous foam. This was subsequently implanted into a 7-mm sciatic nerve gap in the rat without suturing [108]. At 18 weeks post surgery, compound motor action potentials (CMAP) were recorded from the plantar foot muscles of rats receiving the alginate foam treatment following stimulation at the sciatic notch. In a subsequent study from the same group, alginate foams were tested alone or in combination with a protective PGA mesh in the treatment of a 50-mm sciatic nerve defect in the cat [109]. Within three months, CMAPs were successfully recording in both treatment groups, and after eight months, regenerated nerve cables were observed in all animals in both groups, although their myelinated axons were both smaller and had thinner myelin sheaths as compared to normal nerves. It is interesting to note that the alginate foams could promote nerve regeneration even without suturing to the nerve stumps, or enclosing within a tube structure.

Although it is abundant and readily purified from crustacean shells, chitosan is brittle in its dry form and has to be blended with a secondary material or chemically crosslinked prior to processing into a NGC. Xie *et al.* formulated conduits by blending chitosan with PLA, and showed that sciatic nerve regeneration across a 10-mm defect was superior to a silicone tube, but

still unmatched by autografts in terms of both area and number of regenerated axons [110]. Wang *et al.* implanted formaldehyde-crosslinked chitosan conduits containing PGA filaments into a 30-mm sciatic nerve gap in dogs, and demonstrated that after six months, animals treated with these dual-component conduits showed comparable functional histomorphometric recovery with the autograft control [111]. However, in this study the authors did not report implantation of a chitosan conduit without PGA filaments, so it was not possible to directly attribute the regeneration to either one of the two components.

2.3.1.2.c. Decellularized biomatrices

Despite the associated donor site morbidity, the nerve autograft remains the gold standard for peripheral nerve repair because it contains structural and biological components ideally matched to the requirements of the peripheral nerve. On the other hand, there is limited supply of autograft material, especially that suitable for treatment of long nerve gaps. Allografts present a viable solution to this challenge; however, the immune response of the host becomes a major consideration [112], necessitating the decellularization of the grafts prior to use [113]. Nevertheless, acellular nerve grafts retain the ECM and structural organization of the autograft, presenting a more biologically

relevant microenvironment suitable for regenerating axons. A number of groups have prepared cell-free nerve segments with preserved basal lamina tubes using detergent extraction [114-117]. Host Schwann cells rapidly repopulated these grafts, and axonal growth was supported, resulting in comparable functional recovery with autografts.

It was further postulated by Muir *et al.* that removal of neurite-inhibitory chondroitin sulfate proteoglycans (CSPG) from the basal lamina could potentially enhance regeneration through decellularized grafts. Axonal ingrowth into chondroitinase-treated grafts was markedly increased even at 4 days post-implantation, over untreated grafts [118]. When extended to 4-cm defects in the rat, chondroitinase-treated grafts showed some functional reinnervation and a small amount of axons were observed at the distal nerve stump after 12 weeks *in vivo* [119]. This is in dramatic contrast to untreated acellular grafts, where regeneration was markedly retarded and did not progress far beyond the midpoint of the grafts.

In addition to nerve tissue, there have also been some limited efforts at developing artificial grafts from other decellularized tissues such as small intestinal submucosa (SIS) [120, 121], blood vessels [122-124], or muscle tissue [125, 126]. As with allogenic nerves, these tissues are abundant in natural ECM and also, in the case of muscle tissue, contain longitudinally-oriented

basement membranes that serve as physical guidance cues for axons. Despite preliminary successes with regeneration across smaller gap sizes, there is general agreement that the lack of growth-promoting signals from non-neural tissues might pose an obstacle especially when applied towards longer nerve gaps. More efforts have thus been directed towards enhancing these acellular tissues with growth-stimulating neurotrophic factors or cultured Schwann cells.

2.3.1.3. Potential improvements in materials design

Despite the extensive work into development and testing of a variety of materials for generation of nerve conduits, there has not yet been a single material shown to have the potential to surpass the nerve autograft in terms of nerve regenerative potential. On the other hand, past studies have highlighted various design considerations that would contribute to improving the performance of artificial nerve guides. For example, it would be beneficial to be able to fine-tune the degradation profile of the NGC to match the rate of nerve regeneration. New polymeric biomaterials that break down into chemically neutral by-products are also advantageous. Processing techniques can be utilized to generate internal conduit microstructures that resemble the nerve basal lamina organization. However, the continued lack of success of nerve regeneration over long distances strongly suggests that merely providing

physical guidance through a conduit is innately insufficient, and additional trophic support in the form of biochemical or biophysical signals is required. Such modifications will be discussed in further detail in the subsequent sections.

2.3.2. Synthetic nerve guidance conduits with luminal fillers

The lack of success of empty synthetic nerve guides in promoting nerve regeneration across long lesion gaps has triggered immense development in new alternative designs. In particular, axonal regeneration through synthetic nerve guides across long lesion gaps requires additional growth-promoting factors, which may be broadly classified into biochemical and physical cues. In terms of biochemical signaling, growth/neurotrophic factors, cells, nucleic acids and ECM molecules such as collagen, laminin and fibronectin are often involved (Table 2.1). In terms of physical signals applied to PNS tissue regeneration, these often involve filaments and fibers (Table 2.2). For example, to enable nerve regeneration in defects of larger than 3 cm in rabbit peroneal nerves, luminal fillers such as Schwann cells were shown to return excellent growth even over 6 cm gaps [127, 128]. Favorable functional recovery is also observed in defects of up to 8 cm in canine peroneal nerves if PGA-collagen nerve conduits are filled with laminin-coated collagen fibers or

laminin-impregnated collagen sponge prior to implantation [129]. Such favorable nerve regeneration may reflect the typical scenario faced in human PNS injuries and clearly indicates the importance of luminal fillers in enhancing PNS regeneration.

Table 2.1. Summary of biochemical factors incorporated into nerve guide conduits.

Biochemical factor	Loading amount	Conduit material	Animal Model	Gap length	References
NGF	i. 100 ng in PPE microspheres; 50 ng in saline solution	i. Silicone or PPE	i. Rat sciatic nerve	10 mm	(Xu, <i>et al.</i> , 2003)
	ii. 20, or 50 ng/ml with heparin-containing fibrinogen polymerized matrices	ii. Silicone	ii. Rat sciatic nerve	13 mm	(Lee, <i>et al.</i> , 2003)
NT-3	i. 500 ng/ml NT-3 soaked fibrous, orientated fibronectin mats	i. Fibronectin mats	i. Rat sciatic nerve	10 mm	(Sterne, <i>et al.</i> , 1997)
	ii. 1 µg/ml in type I collagen gel (1.28 mg/ml)	ii. PHEMA-MMA hydrogel	ii. Rat sciatic nerve	10 mm	(Midha, <i>et al.</i> , 2003)
GDNF	i. 1 wt% in PEVA polymer rod	i. PEVA	i. Rat facial nerve	8 mm	(Barras, <i>et al.</i> , 2002)
	ii. 1 wt% in PEVA polymer rod	ii. PEVA	ii. Rat sciatic nerve	15 mm	(Fine, <i>et al.</i> , 2002)
	iii. 0.13 wt% in PCLEEP fibers	iii. PCLEEP	iii. Rat sciatic nerve	15 mm	(Chew, <i>et al.</i> , 2007)
	iv. 100 ng/ml with heparin-immobilized fibrin gel	iv. Silicone	iv. Rat sciatic nerve	13 mm	(Wood, <i>et al.</i> , 2009)
aFGF	i. 10 µg/ml in type I collagen hydrogel (1.28 mg/ml)	i. PHEMA-MMA hydrogel	i. Rat sciatic nerve	10 mm	(Midha, <i>et al.</i> , 2003)
bFGF	i. 0.1 wt% in PDLLA polymer solution	i. PDLLA	i. Rat sciatic nerve	15 mm	(Wang, <i>et al.</i> , 2003)
	ii. 50 ng in heparin/alginate hydrogel	ii. Heparin/alginate hydrogel	ii. Rat sciatic nerve	10 mm	(Ohta, <i>et al.</i> , 2004)
CNTF	i. 1 mg/kg in saline solution	i. Silicone	i. Rat sciatic nerve	10 mm	(Zhang, <i>et al.</i> , 2004)

	GGF	i.	1.25 µg/ml in 50:50 alginate-fibronectin hydrogel	i.	PHB	i.	Rabbit peroneal nerve	20-40 mm	(Mohanna, <i>et al.</i> , 2005)
	VEGF	i.	500-700 ng/ml in Matrigel	i.	Silicone	i.	Rat sciatic nerve	10 mm	(Hobson, 2000)
	LIF	i.	100 ng/ml in calcium alginate hydrogel	i.	PHB	i.	Rat sciatic nerve	10 mm	(Hart, <i>et al.</i> , 2003)
	IGF-I	i.	100 mg/ml in DMEM with osmotic pumps	i.	Autologous nerve grafts or acellular ECM	i.	Rat sciatic nerve	20 mm	(Fansa, <i>et al.</i> , 2002)
	PDGF	i.	0.375 µg in laminin containing Biomatrix, collagen, or methylcellulose gel	i.	Silicone	i.	Rat sciatic nerve	8 mm	(Wells, <i>et al.</i> , 1997)
Cellular components	Schwann cells (syngeneic)	i.	1.25×10^6 cells per conduit	i.	PLGA	i.	Rat sciatic nerve	20 mm	(Cheng and Chen, 2002)
	Schwann cells (syngeneic, heterologous)	ii.	4 to 12×10^7 cells/ml	ii.	PAN/PVC copolymer	ii.	Rat sciatic nerve	8 mm	(Guenard, <i>et al.</i> , 1992)
	Schwann cells (syngeneic)	iii.	2 to 16×10^7 cells/ml	iii.	PHB	iii.	Rat sciatic nerve	10 mm	(Mosahebi, <i>et al.</i> , 2001)
	Schwann cells (syngeneic)	iv.	2×10^6 cells/ml	iv.	Collagen (type I/III)	iv.	Rat sciatic nerve	20 mm	(Stang, <i>et al.</i> , 2005)
	Schwann cells (autologous)	v.	95,000 cells/conduit	v.	Silicone	v.	Rat sciatic nerve	10 mm	(Nilsson, <i>et al.</i> , 2005)
	Fibroblast-like MSCs	i.	1×10^6 cells/conduit in 2% gelatin	i.	Silicone	i.	Rat sciatic nerve	15 mm	(Chen, <i>et al.</i> , 2007)
	MSCs	ii.	2×10^6 cells/ml	ii.	Autologous muscle conduits	ii.	Rat sciatic nerve	20 mm	(Keilhoff, <i>et al.</i> , 2006, Keilhoff, <i>et al.</i> , 2006)
	Neural stem/progenitor cells	i.	5×10^4 cells/ml	i.	Chitosan-coated PDMS	i.	Rat sciatic nerve	10 mm	(Lin, <i>et al.</i> , 2008)
ECM molecules	Collagen	i.	Aligned collagen fibrils	i.	Silicone	i.	Rat sciatic nerve	5 mm	(Phillips, <i>et al.</i> , 2005)
		ii.	Sponge like	ii.	Collagen	ii.	Rat sciatic nerve	20 mm	(Stang, <i>et al.</i> , 2005)
	Laminin	i.	Laminin-soaked collagen fibers	i.	PGA	i.	Canine peroneal nerve	80 mm	(Matsumoto, <i>et al.</i> , 2000)
		ii.	Laminin-soaked collagen sponge	ii.	PGA	ii.	Canine peroneal nerve	80 mm	(Toba, <i>et al.</i> , 2001)
		iii.	Laminin conjugated to 1% agarose gel	iii.	Polysulfone	iii.	Rat sciatic nerve	10 mm	(Yu and Bellamkonda, 2003)
	Fibronectin	i.	Orientated Fibronectin Mats	i.	Fibronectin	i.	Rat sciatic nerve	10 mm	(Whitworth, <i>et al.</i> , 1995)

	ii. 0.05% Fibronectin conjugated to 2% alginate hydrogel	ii. PHB	ii. Rat sciatic nerve	10 mm	(Mosahebi, <i>et al.</i> , 2003)
Collagen, laminin, i. FN	2.4 mg/ml collagen, 1 mg/ml laminin and 1 mg/ml FN in a 2 : 1 : 1 volumetric addition in PBS	i. Silicone	i. Rat sciatic nerve	10 mm	(Chen, <i>et al.</i> , 2000)

Legend: aFGF: fibroblast growth factors (acidic); bFGF: fibroblast growth factors (basic); CNTF: ciliary neurotrophic factor; EMSC: ectomesenchymal stem cells; GDNF: glial cell-line derived neurotrophic factor; GGF: glial growth factor; IGF-I: insulin-like growth factor I; LIF: leukemia inhibitory factor; LN-1: laminin 1; MSC: mesenchymal stem cells; NGF: nerve growth factor; NSC: neural stem cells; NT-3: neurotrophin-3; PAN/PVC: poly(acrylonitrile-co-vinylchloride); PCLEEP: poly(caprolactone-co-ethyl ethylene phosphate); PDGF: platelet-derived growth factor; PDLLA: poly(D,L-lactide); PDMS: Polydimethylsiloxane; PEVA: poly(ethylene vinyl acetate); PGA: Polyglycolic acid (or polyglycolide); PHB: polyhydroxy butyrate; PHEMA-MMA: Poly(2-hydroxyethyl methacrylate-co-methyl methacrylate); PLA-TMC: poly (L-lactide-co-trimethylene carbonate); PLGA: poly(lactic-co-glycolic acid); PPE: poly(phosphoester); SC: Schwann cells; SDSC: skin-derived stem cells; VEGF: vascular endothelial growth factor

Table 2.2. Summary of physical features incorporated into nerve guide conduits.

Physical features	Tube material	Animal model	Gap length	References
Filaments in conduit lumen	i. 8 polyamide filaments (ϕ : 250 μ m)	i. Silicone	i. Rat sciatic nerve	15 mm (Lundborg, <i>et al.</i> , 1997)
	ii. 8 collagen or PLA copolymer filaments (ϕ : 150-200 μ m)	ii. Silicone	ii. Rat sciatic nerve	15 mm (Itoh, <i>et al.</i> , 2001)
	iii. 16 PLLA filaments (ϕ : 60 – 80 μ m)	iii. Silicone, PLA	iii. Rat sciatic nerve	14 mm, 18 mm (Cai, <i>et al.</i> , 2005)
	iv. PGA filaments	iv. Chitosan	iv. Rat sciatic nerve	10 mm (Hu, <i>et al.</i> , 2008)
Fiber bundles/ conduit lumen	i. 10 bioglass 45S5 fibers (ϕ : 25 μ m)	i. Silicone	i. Rat sciatic nerve	5 mm (Bunting, <i>et al.</i> , 2005)
	ii. Gelatin fibers (ϕ : 10 – 100 μ m)	ii. Gelatin	ii. Rat sciatic nerve	10 mm (Gamez, <i>et al.</i> , 2004)
	iii. 2000 collagen fibers	iii. NIL	iii. Rat sciatic nerve	20 mm (Yoshii and Oka, 2001)

		(ϕ : 20 μm)				
		iv. 2000 or 4000 collagen fibers	iv. NIL	iv. Rat sciatic nerve	30 mm	(Yoshii, <i>et al.</i> , 2003)
		(ϕ : 20 μm)				
Fibrous scaffolds as conduits	i.	Aligned PCLEEP fibers	i. PCLEEP	i. Rat sciatic nerve	15 mm	(Chew, <i>et al.</i> , 2007)
		(ϕ : 4-5 μm)				
	ii.	Silk fibers	ii. Silk	ii. Rat sciatic nerve	10 mm	(Yang, <i>et al.</i> , 2007)
		(ϕ : 10 μm)				
	iii.	PAN-MA fibers	iii. PAN-MA	iii. Rat sciatic nerve	17 mm	(Kim, <i>et al.</i> , 2008)
		(ϕ : 400 and 600 nm)				
Permeability	i.	Porosity range from 66% to 84%	i. PLGA	i. Rat sciatic nerve	10 mm	(Chang and Hsu, 2005, Chang, <i>et al.</i> , 2007)
	ii.	Microporous (1-10 μm pore diameter) and macroporous (10-230 μm pore diameter)	ii. PCL	ii. Rat peroneal nerve	6 mm	(Vleggeert-Lankamp, <i>et al.</i> , 2007)

Legend: ϕ : fiber diameter; PAN-MA: poly(acrylonitrile-co-methylacrylate); PCL: Polycaprolactone; PCLEEP: poly(caprolactone-co-ethyl ethylene phosphate); PGA: Polyglycolic acid (or polyglycolide); PLA: Polylactic acid; PLGA: poly(lactic-co-glycolic acid); PLLA: poly l lactic acid

2.3.2.1. Biochemical signaling

2.3.2.1.a. Neurotrophic factors

Neurotrophic factors play important roles in controlling the survival, migration, proliferation and differentiation of various neural cell types, hence promoting nerve regeneration when used in nerve repair. In particular, nerve growth factor (NGF), neurotrophin-3 (NT-3), glial cell-line derived neurotrophic factor (GDNF) and fibroblast growth factors (acidic and basic, aFGF and bFGF) are among the most common choices.

Nerve growth factor (NGF): Nerve growth factor has been shown to enhance neuron survival and stimulate nerve regeneration [130, 131]. The mere inclusion of NGF-saline solution into plain silicone nerve guides can enhance the magnitude of motor nerve conduction velocities in rats after 6 weeks post-treatment of a 5-mm sciatic nerve injury [132]. In order to prolong the availability of neurotrophic proteins to targeted tissues, controlled release systems are often used. By encapsulating NGF in microspheres comprising of PPE copolymers of phosphoesters and ethylene terephthalate, sustained release of bioactive NGF was achieved for 20 days [77]. The potential of the protein delivery system in promoting PNS regeneration was then evaluated using a 10-mm rat sciatic nerve injury model. After 3 months, PPE or silicone nerve conduit comprising of saline solution-suspended NGF microspheres produced significantly more nerve fibers and a higher nerve fiber density than the control groups of empty nerve guides and conduits loaded with microspheres encapsulating a non-bioactive protein, bovine serum albumen (BSA). In a separate study, Lee *et al.* used heparin to immobilize NGF and to control its diffusion from a fibrin matrix [133]. The nerve guidance conduit, which was loaded with 20 ng/ml or 50 ng/ml of NGF, produced similar outcomes in terms of number of regenerating nerve fibers and fiber diameter as compared with an autograft in a 13-mm rat sciatic nerve injury model. On the other hand, the

empty NGCs and NGCs comprising of the delivery system alone without NGF were significantly less effective. These results clearly indicate the possibilities of including NGF into nerve guide designs and its effectiveness in assisting nerve regeneration and partial functional recovery in the PNS.

Neurotrophin-3 (NT-3): NT-3 plays an important role in supporting the survival, growth and differentiation of neurons and in encouraging the formation of neuronal synapses [134]. Several studies have developed and evaluated the efficacy of NT-3 incorporated NGCs for PNS regeneration. By impregnating fibronectin mats with NT-3 (500 ng/ml), biofunctionalized nerve guidance conduits could be grafted into a 10-mm rat sciatic nerve defect to enhance nerve regeneration [135]. In the presence of NT-3, axonal regeneration was significantly increased at day 15 as compared to the control group comprising of plain fibronectin mats. By 8 months after surgery, although both NT-3 and control groups resulted in the formation of axons of similar diameters and G-ratios, the presence of NT-3 supported a significantly greater number of myelinated axons. In a separate study, Midha *et al.* used PHEMA-MMA porous tubes filled with collagen matrix supplemented with either NT-3 (1 µg/ml), brain-derived neurotrophic factor (BDNF, 1 µg/ml), or acidic fibroblast growth factor (FGF-1, 1 or 10 µg/ml) to bridge a 10-mm gap in the rat sciatic nerve.

Nerve regeneration, as assessed at 8 weeks after repair by histomorphometric analysis, indicated the efficacy of NT-3 in enhancing nerve regeneration to a similar extent as BDNF. Although the NT-3 results were inferior to those of FGF-1, the presence of NT-3 promoted tissue regeneration to a greater extent than empty tubes and those containing plain collagen matrix [136].

Glial cell-derived neurotrophic factor (GDNF): GDNF is a neurotrophic factor secreted by Schwann cells after nerve injury, which is known to improve motor/sensory neuron survival, neurite outgrowth, Schwann cell migration, and in particular, the survival of dopaminergic neurons [137, 138]. The inclusion of GDNF into NGC designs to promote nerve recovery in the PNS has been attempted by several groups. Poly(ethylene-co-vinyl acetate) (PEVA) nerve guides comprising of GDNF-embedded PEVA/BSA rods have been used in the repair of an 8-mm rat facial nerve defect gap. At 6 weeks post-grafting, GDNF enhanced the formation of myelinated axons and motoneurons as compared to the inclusion of NT-3 or BSA [139]. By the same method, Fine *et al.* demonstrated enhanced motor and sensory neuronal regeneration across a 15-mm rat sciatic nerve defect gap in the presence of GDNF as compared to the inclusion of NGF and BSA at 7 weeks post-implantation [140]. The efficacy of GDNF in PNS regeneration was also

demonstrated by Chew *et al.* [8]. By using degradable NGC with GDNF encapsulated within electrospun polymer fibers enhanced nerve regeneration and functional recovery at 3 months post-implantation across a 15-mm critical defect gap in rats as compared to nerve guides without GDNF inclusion. Similarly, by using a mini-osmotic pump for protein delivery, Boyd *et al.* delivered GDNF and BDNF to the injury site of rat tibial nerves [130]. While GDNF showed no dose dependency within the tested range (0.1–10 µg/day), a synergistic effect with BDNF on enhancing nerve regeneration was clearly observed. In a recent study, Wood *et al.* embedded GDNF (100 ng/ml)-heparin immobilized fibrin matrix within a silicone nerve guide to bridge a 13-mm rat sciatic nerve defect [141]. The presence of GDNF-fibrin resulted in the largest myelinated/unmyelinated area measurements, and comparable extents of nerve regeneration as an isograft in terms of nerve fiber density and percent neural tissue regeneration. Altogether, from the comparative studies carried out thus far, it appears that GDNF is a potent candidate for inclusion into NGC designs for enhancing PNS regeneration.

Fibroblast growth factors (acidic and basic, aFGF and bFGF):

Fibroblast growth factors stimulate mitogenesis in a wide variety of cell types, and enhance regeneration following injuries in the peripheral nerve and spinal

cord. In an early study, purified aFGF incorporated into collagen-filled nerve conduits increased the formation of myelinated axons and primary sensory and motor neurons across a 5-mm sciatic nerve gap of a rat as compared to aFGF-free control groups [142]. Using PHEMA-MMA porous tubes filled with 10 µg/ml of aFGF dispersed within a collagen matrix to bridge 10-mm rat sciatic nerve gap, Midha *et al.* also demonstrated enhanced nerve regeneration at 8 weeks post-surgery. The presence of aFGF resulted in significantly better regeneration than the other experimental groups, which comprised of PHEMA-MMA plain tubes, tubes filled with collagen gel alone, tubes filled with 1 µg/ml of NT-3 in collagen gel and tubes filled with 1 µg/ml of BDNF in collagen gel [136]. Similarly, the efficacy of bFGF in enhancing peripheral nerve regeneration was also demonstrated by Wang *et al.* [143]. A semi-permeable and biodegradable two-ply nerve conduit comprising of poly(D,L-lactide) (PDLLA), with a porous inner PDLLA tube containing bFGF was used to repair a 15-mm gap in the rat sciatic nerve. At 4 months post implantation, significant enhancement in nerve regeneration was observed in the presence of bFGF. In further studies, Ohta *et al.* also developed an alternative system consisting of bFGF embedded in a heparin/alginate hydrogel [144]. At 16 weeks post surgery, these hydrogels with bFGF exhibited faster axonal regeneration, larger myelinated fiber diameter, and better-developed

vascularization across a 10-mm gap in the rat sciatic nerve, as compared to the case where ordinary alginate gel with bFGF was used.

Other factors: A number of other growth and neurotrophic factors, although less commonly used in the PNS, have also been demonstrated effective in enhancing peripheral nerve regeneration. Such include ciliary neurotrophic factor (CNTF) [145], glial growth factor (GGF) [146], vascular endothelial growth factor (VEGF) [147], BDNF [148], leukemia inhibitory factor (LIF) [149], insulin-like growth factor I (IGF-I) [150], and platelet-derived growth factor (PDGF) [151]. In general, these proteins are either injected directly into the lumen of nerve guidance conduits [145] or embedded within a hydrogel as nerve guide lumen fillers [147, 152]. Although most studies thus far do not analyze in detail the effects of protein dosage on nerve regeneration, those that do, most often demonstrated a dose-dependency. With VEGF presented between 500-700 ng/ml, Hobson *et al.* demonstrated a non-linear dose response of blood vessel regeneration towards VEGF [147]. In the case of BDNF, low doses (0.5-2 µg/day for 28 days) promoted axonal regeneration of chronically axotomized motoneurons, but had no detectable effect after immediate nerve repair. However, when high doses (12-20 µg/day for 28 days) were used, significant inhibition of motor axonal regeneration was

observed [153].

With the increase in understanding of the mechanisms involved in peripheral nerve regeneration, researchers have also tried to devise more appropriate nerve conduits to mimic the natural process of protein secretion during nerve repair to enhance nerve regeneration. The combination of two or more growth factors may offer additional benefits. For instance, the combinations of NGF and IGF-I showed synergistic effects on neurite growth [154]. By combining 50 ng/ml of NGF with 10 ng/ml of GDNF and 10 ng/ml of CNTF, Deister *et al.* induced the highest level of neurite outgrowth from young rat dorsal root ganglion (DRG) as compared to other combinations (Deister and Schmidt, 2006). Collagen tubules (CTs) covalently linked with BDNF and CNTF (CT/BDNF/CNTF) displayed the best functional recovery and the largest axon diameters, greatest amplitude and fastest nerve conduction velocities, as compared to CT/BDNF, CT/CNTF and CT groups at 90 days post surgery [155]. Similarly, the combination of GDNF and BDNF via osmotic pump delivery to the cross-suture of rat tibial nerve for 8 weeks resulted in more significant motor axonal regeneration than either factor alone; and this effect was most pronounced following long-term continuous treatment [130].

Altogether, studies involving the inclusion of neurotrophic factors in NGC designs clearly indicate the effectiveness of this approach. It is also

apparent that more detailed understanding of neurotrophic factor dose response and their combinations on nerve regeneration is necessary for optimal scaffold designs.

2.3.2.1.b. Cellular Components

Cell transplantation and its incorporation into nerve conduit designs are alternative strategies to neurotrophic factor delivery, to create a favorable microenvironment for nerve regeneration. Transplanted cells may be tailored to secrete a cocktail of neurotrophic factors to further enhance regeneration. Other cellular components may also support and guide the regeneration of injured nerves [156].

Schwann Cells: Schwann cells play an indispensable role in supporting axonal regrowth and migration following PNS injuries. They offer a highly preferred substrate for axon migration, release bioactive factors, promote blood vessel formation, and possibly influence the embedding of connective tissues [157]. Upon injury, Schwann cells in the injured region produce various ECM molecules such as laminin and collagen, and express many cell adhesion molecules and receptors, including neural cell adhesion molecules. Moreover, after Wallerian degeneration, Schwann cells form longitudinally oriented

strands (bands of Büngner) that serve as a guiding rail for regrowing axons [158, 159]. As a result, they are by far the most favored cellular components for transplantation purposes to enhance PNS regeneration. However, in order to ensure the survival of Schwann cells at the site of injury, proper structural support is often required. Hydrogels, in particular, are commonly used as supporting substrates for delivering Schwann cells into the lumen of NGCs. Alginate/fibronectin [160], gelatin [161], collagen [162], and Matrigel [163] are among the common choices. Besides hydrogels, an alternative way is to improve the inner surface topography of NGCs to enhance the surface area available for cell attachment and growth. By using PLGA fiber and biomembrane, Cheng *et al.* seeded Schwann cells on the fiber and membrane, then wrapped them as conduits to bridge a 20-mm critical gap of rat sciatic nerve [164]. After 3 months, the conduit promoted nerve regeneration, and the number of axons and the area of nerve fibers were comparable to the autograft group. In a separate study, micropatterned biodegradable PDLLA conduits pre-seeded with Schwann cells also demonstrated reduced recovery time and sciatic functional index after bridging a 10-mm rat sciatic nerve defect gap [165]. The results were more favorable as compared to the control groups, which comprised of conduits with micropatterned inner lumens without Schwann cells, unpatterned conduits pre-seeded with Schwann cells, and

conduits without micropatterned inner lumens and Schwann cells.

As observed in multiple studies, the presence of Schwann cells typically results in enhanced nerve regeneration and functional recovery. However, the origin and seeding density of Schwann cells should also be considered as they play significantly impact the outcomes of nerve regeneration. Using a 60:40 acrylonitrile vinylchloride (PAN/PVC) copolymer tubing filled with syngeneic or heterologous Schwann cells suspended in Matrigel, Guenard *et al.* bridged an 8-mm gap in rat sciatic nerve [166]. The presence of syngeneic Schwann cells supported extensive axon regeneration and resulted in the formation of more myelinated axons than the empty conduits, or conduits filled with Matrigel alone. By varying the cell loading density between 40 and 120 millions of cells per ml (correlating to a total of 0.32 to 1.28 million cells per conduit), a positive correlation between the number of myelinated axons and the concentration of syngeneic Schwann cells was observed. In contrast, heterologous Schwann cells elicited strong immune reactions and resulted in only a limited growth of myelinated axons. To examine the importance of Schwann cell seeding density, Mosahebi *et al.* filled syngeneic Schwann cells into a resorbable PHB conduit to repair a 10-mm gap in the rat sciatic nerve. The length of the regenerated nerve, as measured at 3 weeks after implantation, demonstrated maximal regeneration at a cell density of 80×10^6 cells/ml and a slightly decreased extent

of recovery at 160×10^6 cells/ml [167].

While the enhancement in nerve regeneration in the presence of Schwann cells is obvious in most cases, it has also been demonstrated that this approach is effective only when the microenvironment surrounding the injury site is conducive for tissue re-growth. Stang *et al.* analyzed the effects of tube diameter on nerve regeneration using collagen nerve guides of 2.5 mm and 1.5 mm inner diameters to bridge a 20-mm sciatic nerve gap in the rat model [97]. The authors also evaluated the effects of including a collagen scaffold or incorporating Schwann cells into the lumen of the tube in promoting nerve regeneration. Eight weeks post implantation, through morphological and sensory function evaluation, NGCs with 1.5 mm diameter fared better. However, the presence of the collagen scaffold as a filler in the lumen impeded the regeneration. In addition, empty collagen NGCs did not bridge this large gap. The authors demonstrated that Schwann cells can aid nerve regeneration only when the microenvironment is conducive.

Although the aforementioned results indicate the ability of Schwann cell-loaded NGCs in enhancing nerve regeneration more significantly than acellular conduits, the clinical use of Schwann cells poses several pragmatic difficulties; the greatest amongst which is the source of autologous Schwann cells. To obtain a sufficient number of viable cells, 3-10 weeks of *ex vivo*

expansion and purification of the Schwann cells are often needed [168]. Such attempts increase cost, the risk of contamination, and most importantly, presents a time lag between injury and repair, which often leads to neuronal cell death in the dorsal root ganglion, thereby reducing the potential for recovery [169]. To shorten and facilitate the process of Schwann cell extraction and expansion, Nilsson *et al.* attempted a new approach [170]. By removing two to three millimeters of nerves from the proximal and distal ends of the severed nerve, a total of 95,000 cells (of which, 80% are Schwann cells) could be obtained. The freshly harvested cells were then injected into a silicone nerve conduit for bridging a 10-mm rat sciatic nerve gap. Although the number of transplanted SCs was much lower than the optimized cell concentration as reported by Mosahebi *et al.* [167], the conduits that contained the cell suspension mediated superior regeneration as compared to acellular nerve guides. Another method to enrich Schwann cell population is the use of allogeneic cells. Unfortunately, such approaches typically require the systematic administration of immunosuppressors [163].

Bone marrow stromal cells (or mesenchymal stem cells, MSCs): MSCs are multipotent stem cells derived from the bone marrow, which have been shown to transdifferentiate into neural cells [40]. Due to the ease in obtaining

MSCs via bone marrow aspirates and expanding these cell types in culture [171], MSCs can serve as the potential alternative to Schwann cells. Zhang *et al.* seeded GFP-labeled undifferentiated MSCs in a Matrigel-containing chitosan nerve guide to bridge a 5-mm gap in the rat sciatic nerve [172]. After 6 weeks, nerve conduction velocity and sciatic functional index were significantly improved in the presence of MSCs as compared to MSC-free NGCs. Additionally, nerve regeneration in the presence of MSCs was comparable to the case of Schwann cell transplantation. While the exact mechanisms behind the enhanced nerve regeneration in the presence of undifferentiated MSCs remain to be elucidated, it is possible that undifferentiated MSCs may contribute to nerve regeneration by secreting growth factors and depositing basal lamina components [173]. Cuevas *et al.* demonstrated that even though less than 5% of MSCs differentiated into Schwann cells and most of the implanted MSC population remained in an undifferentiated state [174], MSC-treated rats exhibited significant improvement in nerve regeneration and function recovery at 180 days after surgery, as compared to MSCs-free controls [175]. In another independent study, MSCs were shown to have the potential to differentiate into Schwann cell-like cells, thus improving nerve regeneration [176].

Compared with undifferentiated MSCs, nerve conduits loaded with

differentiated stem cells can significantly enhance nerve regeneration. Mimura *et al.* suspended differentiated MSCs in Matrigel and transferred the cells onto hollow fibers to bridge a 10-mm rat sciatic nerve gap. After 6 months, significant improvement in motor nerve conduction velocity and sciatic functional index were observed in the differentiated MSCs-transplanted group as compared to control nerve guides that were loaded with undifferentiated MSCs [177, 178]. Besides that, by transplanting fibroblast-like MSCs to treat a 15-mm rat sciatic nerve injury, the significant contribution of neurotrophic factors produced by MSCs on enhancing nerve regeneration and functional recovery was demonstrated [173]. Although the effect of differentiated MSCs on enhancing nerve regeneration has been shown to be inferior to Schwann-grafted conduits and autologous nerve grafts [177], it has also been demonstrated that such unfavorable outcomes may be reversed by combining MSCs with muscle- [176] or nerve-derived [179] allografts. In such cases, similar extent of nerve regeneration as Schwann cell-seeded grafts and autografts may be achieved.

Olfactory ensheathing cells (OECs): OECs can be regarded as peripheral nerve progenitor cells, since they develop from a peripheral origin, the olfactory placode [180]. OECs are able to self-renew and differentiate [156]

to promote axonal re-growth and remyelination in the CNS and PNS under experimental conditions [181]. By using silicon tubes filled with OECs, successful axonal regeneration was observed in 50% of rats with a 15-mm sciatic nerve injury gap and in 79% of the animals with a 12-mm gap [182]. Such therapeutic effects of OECs are also supported by other studies [183-185]. However, the potential for the therapeutic transplantation of OECs in peripheral nerve injury is still debatable, as there are many pragmatic difficulties to overcome when applied to the clinical setting, such as the limited source of autologous OECs.

Other cellular components such as embryonic stem cells [186], neural stem cells [187, 188], fibroblasts [162], skin-derived stem cells [189], hair follicle stem cells [190], and ectomesenchymal stem cells [191] have also been included into NGCs to promote nerve regeneration. Although they differ in nerve regeneration efficacy, their beneficial effects such as supporting axonal regrowth, secretion of neurotrophic factors and ECM molecules, can contribute to a better microenvironment for nerve regeneration.

2.3.2.1.c. Gene Therapy

Gene manipulation to neural cells is another attractive strategy due to

the prolonged production of therapeutic proteins in their bioactive form, particularly for more labile growth factors. By combining NGCs with the delivery of genes that encodes proteins that help in preventing motor neuron loss; enhancing initial axonal sprouting; preserving motor endplate morphology; and promoting accurate reinnervation, this approach can provide attractive platforms for enhancing nerve regeneration [192]. The two common methods are direct *in vivo* gene transfer and modification; or the isolation, purification, followed by genetic modification of specific cells *ex vivo* prior to implantation.

As an example of the direct *in vivo* gene alteration, Sakamoto *et al.* injected recombinant adenoviral vectors encoding several neurotrophic factors at the stylomastoid foramen after facial nerve avulsion. After 4 weeks, the expression of foreign neurotrophic factors could be detected, and the survival of axotomized motor neurons after avulsion injury was enhanced [193]. In contrast to the use of viral vectors, Barati *et al.* used monoclonal antibody MC192-poly-L-lysine/pGDNF complex to induce rat GDNF expression in hypoglossal motor neurons after nerve crush. Expression of GDNF was detected for up to 8 weeks, which helped rescue the targeted motor neurons [194]. By improving the delivery efficiency and transgene expression duration, this method will find wider application in neural regeneration.

Several cell types such as MSCs [195], neural stem cells [196] and

Schwann cells have been used for the *ex vivo* gene transfection approach. Nonetheless, Schwann cells are often the preferred cell type due to their importance in PNS regeneration. Over-expression of GDNF was achieved by retroviral transduction in Schwann cells, and these cells were introduced in silicone tubes and used to repair a 10-mm defect in rat sciatic nerve. Significantly increased functional and morphological recovery was observed [197]. In a separate study, Shi *et al.* also transduced NSCs with a lentiviral vector encoding enhanced green fluorescent protein (EGFP) and GDNF for the treatment of facial nerve gaps in rats for 12 weeks [196]. By filling PLGA NGCs with the transfected cells, nerve action potential amplitude, axonal area, and axonal number were greater with NGCs containing GDNF expressing NSCs. However, NGCs containing GDNF expressing Schwann cells yielded less degree of regeneration. While gene transfection approach potentially enhances nerve regeneration, the choice of targeted proteins is important. Transfected Schwann cells over-expressing high-molecular isoforms of bFGF (21 and 23 kDa) significantly enhanced nerve regeneration across a 15-mm critical defect gap in rat sciatic nerves 4 weeks post implantation. However, when using the 18-kDa isoform of bFGF, an inhibitory effect on the myelination of axons was observed [198]. In summary, NGCs loaded with genetically modified cellular components represent a promising strategy for the

treatment of peripheral nerve injuries.

2.3.2.1.d. ECM molecules

After a nerve is transected, fluid from the damaged nerve ends often accumulates, mainly consisting of a fibrin clot matrix [1]. The inclusion of such ECM molecules into NGC design has therefore been adopted by several groups in attempt to mimic the post-injury microenvironment. The hypothesis is that such biomimetic environment should enhance nerve regeneration. Using magnetically aligned fibrin matrix as lumen fillers within a nerve conduit for neuronal contact guidance, Dubey *et al.* demonstrated improved nerve regeneration [199]. By utilizing hyaluronic acid (HA) as a luminal filler Seckel *et al.* showed that the presence of HA produced better conduction velocity, higher axon counts and a trend towards earlier myelination as compared with saline-filled conduits [200]. Various other ECM molecules, such as collagen, laminin and fibronectin have also been evaluated as possible candidates for NGC luminal fillers.

Collagen: Using a perforated silicone conduit to tether and align type I rat tail collagen that fills the conduit lumen, Phillips *et al.* aligned cells from DRG and Schwann cells along the longitudinal direction of the nerve conduits

[162]. Following that, enhanced presence of neurons and Schwann cells were observed at the distal end of the 5-mm injury gap in the rat sciatic nerve. Although this study demonstrated the alignment of Schwann cells in collagen filler *in vitro*, the small gap size of only 5 mm makes it difficult to extrapolate to critical defect model due to the lack of direct evaluation of nerve regeneration and functional recovery at 8 weeks post implantation. In addition, the effect of aligned collagen filler on nerve regeneration *in vivo* remains to be illustrated due to the lack of proper controls. Nonetheless, this study represents one of the attempts of using collagen fillers to enhance nerve regeneration.

Chen *et al.* used silicone conduits filled with a mixture of collagen, laminin and fibronectin to bridge a 10-mm sciatic nerve defect gap in a rat model [201]. At 6 weeks post implantation, they recognized that the presence of the ECM protein fillers resulted in the formation of more mature myelinated axons and larger nerve cross-sectional area, compared with empty silicone tubes. Unfortunately, no significant difference at the morphological level was observed between the sample groups with and without ECM fillers. At 6 weeks post implantation, re-innervation of muscle was still poor in all experimental groups and no functional recovery was observed. The effectiveness of including collagen lumen fillers was further put into question by the observation that the presence of a bulk collagen scaffold in a conduit lumen appeared an

impediment to nerve regeneration, and such an effect could not be rescued even in the presence of Schwann cells [97].

Laminin: Laminin, a heterotrimeric ECM protein that regulates cell viability and function, plays a critical role in the myelination of peripheral nerves [202]. *In vitro*, laminin shows a strong, dose-dependent effect on neurite length and outgrowth [203]. It also increases Schwann cell attachment, which may be favorable for directing peripheral nerve regeneration [204]. These effects of laminin were also demonstrated by *in vivo* experiments. PGA-collagen tubes filled with laminin-coated collagen fibers could bridge axonal regeneration over an 80-mm gap in canine peroneal nerves. Although smaller regenerated nerve fibers with thin myelin sheaths and incomplete electrophysiological recovery were observed at 12 months after implantation, good recovery of behavioral function and close-to-normal walking patterns were achieved [129]. In a separate study, Yu *et al.*, utilized a semi-permeable polysulfone tube filled with laminin (LN) and NGF to bridge a 10-mm sciatic nerve gap in adult rats. Two months after implantation, LN and NGF-loaded NGCs enhanced nerve regeneration. A synergistic effect was also observed between LN and NGF with larger numbers of myelinated axons and higher success rate of regeneration occurring as compared to the use of either factor

alone. Furthermore, the LN/NGF NGCs performed comparably to autografts in terms of functional recovery [205].

Fibronectin (FN): FN is a large ECM glycoprotein, which promotes cell adhesion and migration [206]. It has been shown to increase the volume of sensory and motor neuron regeneration and enhance the influx of Schwann cells to the site of nerve injury [207]. Using orientated mats of FN as nerve conduits to bridge a 10-mm rat sciatic nerve defect, Whitworth *et al.* demonstrated the ability of FN in supporting a significantly faster rate of axonal growth than freeze-thawed muscle grafts within the first 10 days post surgery. Furthermore, from day 15 to day 60, FN conduits resulted in comparable amounts of regenerating axons and Schwann cells in the conduits as compared to nerve grafts [208]. In a separate study, Ahmed *et al.* impregnated FN mats with NGF and implanted it into a 5-mm gap in an arm radial nerve of *Macaca fascicularis* monkeys. Four months post implantation, the FN/NGF grafts showed larger diameters of regenerated nerves and greater number of myelinated fibers and cutaneous reinnervation as compared to the plain FN mats. The observed nerve regeneration reached similar level as the nerve autografts [209]. Mosahebi, *et al.* tested PHB NGCs filled with Schwann cells and alginate hydrogel matrix with FN, and showed that nerve regeneration across a 10-mm rat sciatic nerve gap

was enhanced at 2, 3 and 6 weeks post surgery compared to NGCs with only alginate matrix. An additive effect of SCs and FN was also observed concurrently, possibly due to the fact that FN contributed to supporting Schwann cell viability and augmented the effects of Schwann cells on enhancing axonal growth [160]. The presence of FN also appeared to attenuate the inhibitory effects of alginate hydrogels on neurite growth and Schwann cell activities [210].

While ECM molecules have been commonly used as fillers in nerve guidance conduits, their effect on nerve regeneration outcomes remain inconclusive. The mode of presentation of ECM molecules as conduit fillers may be an important factor influencing the nerve regeneration outcomes. Controlled amounts of fibrous structures seem to enhance nerve regeneration. In contrast, ECM proteins introduced in bulk or hydrogel form tends to provide more contradicting results. The exact roles played by such ECM lumen fillers, therefore, require further elucidation and more “standardized” experimental designs may help obtain more conclusive results.

2.3.2.2. Physical Signals - Filaments, Fibers and Contact Guidance

The motivation behind introducing contact guidance into the design of NGCs stems from the idea of enhancing Schwann cell alignment. Following

nerve injuries, the formation of Bands of Büngner normally precedes axonal regeneration. Unfortunately, the failure of non-neuronal cells, such as Schwann cells, to migrate across lesion gaps is often the reason for the poor regeneration outcome in non-filler-containing nerve conduits, particularly over large gap defects. Promoting early Schwann cell alignment may therefore hasten nerve regeneration.

With this hypothesis in mind, Lunborg *et al.* first used silicone NGCs filled with eight polyamide filaments with a diameter of 250 µm for the treatment of 15 mm rat sciatic nerve injury [211]. In the presence of filaments, successful bridging of the critical defect gap in rats was observed at 4 weeks post implantation. In contrast, empty silicone tubes did not yield significant nerve regeneration or functional recovery. The inclusion of filaments into the lumen of the silicone tubes also increased the overall cross-sectional area of the regenerated nerve fiber, enhanced the formation of myelinated axons and enabled the recovery of sensory nerve function. In a separate study, Itoh *et al.* also reported using a similar bridging graft (15 mm) made from silicone conduits containing eight collagen or polyester filaments. Unfortunately, these two reports did not give direct evidence concerning the ability of filaments to provide contact guidance for axon regrowth [212]. Nonetheless, these studies sparked the growing interests in using filament lumen fillers, either synthetic

[213-215] or biological in nature [99, 103, 216], for enhancing nerve regeneration.

Cai *et al.* tested PLA and silicone conduits, respectively, filled with Matrigel supplemented with sixteen PLA wet-spun fibers for the treatment of rat sciatic nerve injuries [214]. At 10 weeks after implantation, the presence of filaments significantly enhanced tissue regeneration across injury gaps of both 14 mm and 18 mm, with the effects of fiber inclusion being more drastically demonstrated in the group with an 18-mm injury gap. The study clearly indicated that filaments were more effective for bridging long nerve defect gaps and that Schwann cell migration was also more prominent in the presence of filaments over the gap of 18 mm. By accounting for the ratio of myelinated to unmyelinated axons, the combination of PLA conduit and filament scaffold had the highest ratio at all time points regardless of injury gap size, likely due to the presence of 10-25 μm -sized pores that were on the PLA conduits. Without filament bundles, Schwann cell cables formed discontinuously with few cells found in the center region of the nerve conduit, along with neuroma formation. However, as nerve regeneration was evaluated via gross examination instead of detailed quantitative evaluation, the quality of nerve regeneration could not be concluded. Also, the optimal packing density of filaments needed to fill the conduit lumens remains to be evaluated. While scaffold permeability may have

enhanced nerve regeneration across the PLA conduits as compared to silicone conduits, material surface chemistry may also influence cellular interactions with the implants. Lastly, it remains to be elucidated if contact guidance and/or increased cell attachment area contributed to the enhanced nerve regeneration in the presence of fibers.

Based on the same approach, Gamez *et al.* developed a photo-cured gelatin conduit contains gelatin fibers with diameters ranging from 10 to 100 μm and encapsulated NGF, laminin and FN, and evaluated its ability to bridge a 10-mm gap in a transected rat sciatic nerve by measuring locomotor activity and somatosensory-evoked potential for functional recovery and by histo-morphological changes at 6 and 12 months post implantation [103]. NGCs with gelatin fiber fillers yielded the fastest regeneration and functional recovery, followed by a similarly constructed gelatin conduit with one photo-cured gelatin rod (with the same diameter as the inner diameter of the conduit) and same set of biochemical molecules encapsulated in the rod, and a gelatin conduit without any fillers. Rats received gelatin fiber-filled NGCs exhibited mature and well-developed axons similar to those in normal nerve tissues at 12 months after implantation. Both groups received conduits filled with gelatin fibers and rod, respectively, showed latency and amplitude values close to normal values at 6 and 12 months post implantation, whilst the group received

conduits without lumen fillers was inferior even after 12 months. Although this study demonstrated that the combination of lumen fillers and biochemical cue delivery enhanced nerve regeneration, it remains unclear whether these conduits are effective for critical defect repair.

Yoshii *et al.* demonstrated the microfilament effect using bundles of collagen filaments with diameters of 20 μm to repair long lesion gaps of either 20 mm [99] or 30 mm in the rat sciatic nerve injury model [216]. Formation of myelinated axons in the distal nerve across the 20 mm injury gap occurred at 8 weeks post-implantation. At 12 weeks, nerve regeneration across the injury gaps was observed in all experimental groups. Comparing bundles of 4000 filaments to 2000 filaments, an increase in fiber number appeared to enhance the maturation of the regenerated nerve. Unfortunately, walking track analyses at 12 weeks did not show any functional recovery across the 30 mm defect gap. In the case of the 20-mm injury gap, no significant differences were observed between the collagen filament groups and the control groups (collagen conduits and autografts). Although the axons that regenerated across the collagen filaments had smaller cross-sectional area as compared to those found in an autografts, the results remained promising. The presence of plain collagen filaments, without a conduit and neurotrophic additives, was demonstrated to be sufficient in enabling nerve regeneration across defect gaps much larger than

the critical defect size in a rat model.

Various other attempts have also been made to utilize different materials as contact guiding filaments. Such include the use of commercially-available PGA filaments in chitosan nerve conduits [215] and bioactive glass fibers in silastic nerve conduits [213]. However, due to the lack of quantitative evaluation of nerve regeneration and functional recovery; and the use of small nerve defect gap sizes (5 mm to 10 mm in the rat sciatic nerve injury model) the advantages of using these different materials for enhancing nerve regeneration remains hard to extrapolate.

The above mentioned studies have demonstrated the advantages and potential of physical lumen fillers in promoting nerve regeneration. However, several factors remain to be elucidated. Firstly, there appears to be an optimal packing density of filaments included within the lumen of the NGCs. Using a silicone tube filled with only 8 polyamide filaments, Lunborg *et al.* [211] demonstrated that the resulting cross-sectional area occupied by the regenerated nerve was still relatively small. In contrast, Chew *et al.* demonstrated the regeneration of nerve across the entire cross-section of their nerve guide through the inclusion of electrospun fibers [8]. Secondly, the size of filaments may also play a role. While most studies carried out in the past utilizes filaments in the range of tens to hundreds of microns, recent fabrication

techniques, such as electrospinning, have allowed the reduction of filament diameters by at least one order of magnitude. It will be interesting and insightful to further evaluate such size effects on nerve regeneration. Lastly, while most studies claim that inclusion of filament fillers enhances nerve regeneration due to contact guiding cues, the potential effect of increased surface area for cell attachment and growth has not yet been decoupled. While the study by Chew *et al.* demonstrated increased surface area as the winning factor, further studies are required to obtain more conclusive information and mechanistic insights.

2.3.3. Control of NGC architectural designs

2.3.3.1. Effect of nerve guide permeability and pore size

Besides the inclusion of contact guiding cues into synthetic scaffolds to enhance nerve regeneration, various groups have also focused on controlling the permeability of nerve guides through phase separation techniques, the introduction of porogens and the use of fibrous scaffolds. The main motivation behind controlling scaffold porosity is to limit cell infiltration that may hinder axon extension, while promoting nutrient transport and blood vessel infiltration to support Schwann cell proliferation and nerve regeneration.

Using phase separation techniques, Chang *et al.* fabricated asymmetric

porous PLGA tubes with interconnected-microporous inner tube surface and macroporous outer tube surface [9, 217]. Depending on the proportion of solvents used during the fabrication process, the porosity of the conduits could range from 66 to 84 %. *In vitro*, such uni-directionally permeable scaffolds enhanced Schwann cell proliferation but inhibited fibroblasts division. When the porous grafts were seeded with Schwann cells to treat a 10-mm rat sciatic nerve defect, formation of more axons, larger axon area and more blood vessels were observed at 6 weeks post surgery as compared to scaffolds with symmetric porosity and plain silicone conduits. Unfortunately, as evaluated morphologically, nerve regeneration was inferior to an autograft and functional recovery in the animals was not evaluated. The lack of information on the exact pore sizes of the scaffolds also limits the translation of porosity effect observed in these studies to future nerve guide designs. Nonetheless, it was clear that porosity of a scaffold could have effects on cell viability and proliferation and may be accounted into future scaffold designs for enhanced nerve regeneration in the PNS.

In another study by Vleggeert-Lankamp *et al.*, the effect of nerve guide pore size was evaluated in bridging a 6-mm rat peroneal nerve gap. Interestingly, the microporous (1-10 μm pore diameter) PCL conduits yielded similar regeneration to an autograft in terms of regenerated nerve cross-sectional area,

axon number and axon density at 12 weeks post implantation, and better than the macroporous (10-230 μm pore diameter) and non-porous conduits that performed similarly [218].

As illustrated in the next section, electrospinning is a commonly used technique for fabricating fibrous scaffolds. By coupling electrospun scaffolds with or without a supporting film substrate, nerve conduit permeability can be altered [10]. Altogether, these results confirmed that good permeation of tubes to enable exchange of nutrients and metabolic waste is another important factor in nerve guide designs for peripheral nerve regeneration.

2.3.3.2. Fibrous scaffolds as nerve guides

Fibrous scaffolds as nerve guides offer the advantage of combining permeability, high surface area for cell attachment and growth, and possibly topographical signals for directing cellular functions. Depending on the method of fabrication, biochemical signals may also be included into fibrous scaffolds as an additional factor for enhancing nerve regeneration. Besides using naturally occurring fibers such as silk, electrospinning may be the most popular and versatile method adopted for the formation of fibrous nerve conduits for PNS regeneration.

The feasibility of using silkworm silk to form highly porous silk-based

nerve guides with fibrous architecture comprising of fibers with diameters ~ 10 μm in the inner surface was evaluated in a 10-mm rat sciatic nerve injury model [14]. At 6 months post-surgery, nerve regeneration and functional recovery within silk nerve guides were comparable to treatment with autografts. Retrograde labeling also indicated similar numbers of regenerated sensory and motor neurons within the two groups. Comparing the two nerve guides, it is likely that the enhanced surface area for cell attachment and growth, along with scaffold permeability provided by the fibrous silk scaffolds, supported favorable nerve regeneration.

An alternative method of fabricating fibrous scaffolds as NGCs is electrospinning. Being a highly versatile technique, one may easily combine biochemical and topographical features into a single scaffold for enhancing nerve regeneration. The technique is also applicable to a wide variety of polymers ranging from synthetic polymers such as PLA, PLGA, and PCL to biopolymers such as collagen, polysaccharides, and silk. The use of electrospinning to fabricate biofunctional scaffolds encompassing growth factor delivery and topographical cues for nerve regeneration was first demonstrated by Chew *et al.* [8, 79, 219]. By encapsulating human nerve growth factor (NGF) into PCLEEP, the authors demonstrated that NGF could be released in a sustained manner from electrospun fibers for at least 3 months while retaining

its bioactivity [79]. Conduits with longitudinally-aligned electrospun PCLEEP fibers encapsulating human GDNF (diameter $\sim 5 \mu\text{m}$) were used to bridge a critical defect gap of 15 mm in the rat sciatic nerve injury model [8]. After 3 months, nerve regeneration was significantly enhanced in the presence of electrospun fibers and GDNF. The mere presence of plain electrospun fibers (diameter $\sim 4 \mu\text{m}$) was sufficient in promoting functional recovery in 33% of the rats as compared to the empty conduit group, which resulted in no functional recovery. Interestingly, by varying the orientation of the aligned fibers to constitute a circumferentially-aligned plain PCLEEP fiber group, the authors demonstrated that at least for their experimental setup, the increase in surface area for cell attachment, instead of contact guidance, appeared to be the main factor that promoted nerve regeneration. In another independent study, however, Kim *et al.* implanted poly (acrylonitrile-co-methylacrylate) (PAN-MA) fibrous conduits to across a critical defect gap of 17 mm in the rat model. After 16 weeks, axon regeneration and function recovery in the presence of aligned polymer fiber-based conduits were comparable to the autograft, and were much better than the random polymer fiber-based conduits [7].

The effect of enhanced surface area for cell attachment on nerve regeneration is further supported by the results from Wang *et al.* [10]. By comparing NGCs made from only chitosan films and bilayered nerve guides

comprising of an outer layer of chitosan film and an inner layer of chitosan micro/nanofiber mesh, improved tissue-implant integration was observed in the latter after 4, 8 and 12 weeks post surgery bridging a 10-mm defect gap in a rat sciatic nerve. The effect of scaffold permeability was also reinforced, with rats receiving plain electrospun fibrous tubes showing better nerve regeneration and functional recovery as compared to animals that received the bilayered electrospun tubes. By changing the degree of acetylation on chitosan between 78% and 93%, the authors also demonstrated that mechanical properties of the guides was crucial to retaining the shape of the nerve conduit and promoting nerve regeneration, similar to the observations reported in the literature [26].

Besides an increase in surface area for cell attachment, topographical cues from electrospun fibrous scaffolds could also enhance Schwann cell maturation as demonstrated in a separate study by Chew *et al.* [219]. By culturing human Schwann cells on PCL film, randomly-oriented PCL electrospun fibers (average diameter 2.26 μm), and aligned PCL electrospun fibers (average diameter 1.03 μm) for a period of 7 days, enhanced Schwann cell maturation on electrospun fibers was observed as indicated by gene expression changes. Significant down-regulation of gene expression for neurotrophin and neurotrophic receptors, and immature Schwann cell marker, NCAM-1 was observed on cells interacting with fibrous scaffolds. Concurrently,

significant upregulation of pro-myelination marker, MAG, was observed in the presence of the fibers. Aligned Schwann cells further expressed a significantly higher level of P0 as compared to randomly oriented Schwann cells on fibrous scaffolds and PCL film.

Altogether, these studies demonstrated the versatility of such fibrous scaffolds in enhancing nerve regeneration through a combination of topographical and biochemical signaling.

2.4. CONCLUSIONS

The wide availability of synthetic NGCs and versatile designs for incorporation of various cues important to regeneration are major advantages of these conduits over autografts. However, as compared to the autograft, the efficiency of synthetic NGCs to enhance nerve regeneration and functional recovery in the PNS remains to be further improved. It is generally accepted that synthetic NGCs require additional biochemical and physical factors to provide a more conducive microenvironment for PNS regeneration particularly over long lesion gaps. The proper choice of conduit materials and structural design, conduit lumen filler, incorporation of cell adhesion molecules, and sustained release of neurotrophic factors and cells are just a few important design factors to consider. Stem cell-based approach to provide an array of

relevant neurotrophic factors represents an exciting improvement over the current conduit designs.

Electrospinning has been proven to be a powerful tool to develop topographical features for mimicking the natural architecture of the ECM environment. Microscale topographical cues can be used to precisely control adhesion, morphology, migration, and differentiation in a wide variety of cell types. Importantly, the ability to engineer artificial ECMs that, through physical as well as molecular interactions, enable directed control of stem cell behavior may further extend our capabilities in engineering functional tissue substitutes. By controlling the nanotopography, mechanical properties, and mechanical loading environment of tissue engineering scaffolds, we may further improve the regulation of stem cell fate in bioartificial systems. Despite significant advances shown in these *in vitro* studies, the ultimate success of such approaches will require *in vivo* demonstrations of functional engraftment and tissue regeneration. The continuous advancements in the field of cell–substrate interactions will not only benefit fundamental biological studies, but it will have significant implications in the field of tissue engineering through engineering synthetic and implantable substrates with controlled features.

CHAPTER THREE:

NANOFIBER TOPOGRAPHY AND SUSTAINED BIOCHEMICAL SIGNALING ENHANCE HUMAN MESENCHYMAL STEM CELL NEURAL COMMITMENT

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SUMMARY

Stem cells hold great promise in enhancing nerve regeneration. In particular, human mesenchymal stem cells (MSC) represent a clinically viable cell source due in part to their abundance and accessibility. Unfortunately, current methods to direct stem cell fate remains largely limited to biochemical-based approaches on two-dimensional substrates with restricted efficacies. Here, we evaluated a scaffold-based approach for directing stem cell differentiation. We demonstrate the combined effects of nanofiber topography and controlled drug release on enhancing MSC neural commitment. By encapsulating up to 0.3 wt% of retinoic acid (RA) within aligned poly (ϵ -caprolactone) (PCL) nanofibers (average diameter $\sim$ 270 nm, AF750), a sustained released of RA was obtained for at least 14 days ($\sim$ 60 % released). Compared with tissue culture polystyrene, nanofiber topography from plain

PCL nanofibers significantly up-regulated the expressions of neural markers, Tuj-1, MAP2, GalC and RIP at mRNA and protein levels. Combined with sustained drug availability, more significant changes in cell morphology and enhancement in neural markers expressions were observed. In particular, scaffold-based controlled delivery of RA enhanced MAP2 and RIP expressions as compared to bolus delivery despite lower amounts of drug (> 8 times lower). The generally higher expression of mature neuronal marker, MAP2, as compared to glial markers at mRNA and protein levels suggested the enhanced potential of MSC neuronal differentiation. In addition, positive staining for synaptophysin was detected only in cells cultured on aligned scaffolds in the presence of RA. Taken together, the results highlight the advantage of the scaffold-based approach in enhancing the potential of MSC neuronal differentiation and demonstrated the importance of drug delivery approach in directing cell fate. Such biomimicking drug-encapsulated scaffolds may permit subsequent direct cell transplantation and provide guidance cues to control the fate of endogenously recruited stem cells.

3.1. INTRODUCTION

Stem cells hold tremendous potential in the treatment of nerve injuries and neural diseases [220-222]. Amongst the various cell types explored, mesenchymal stem cells (MSCs) probably hold the greatest promise for translational applications due in part to its greater abundance and easier accessibility as compared to neural stem cells and embryonic stem cells. However, neural differentiation in MSCs remains ambiguous [223-225]. While multiple studies have demonstrated the potential of MSCs to differentiate into progenitor cells expressing neural specific markers [21, 22] and the ability of these cells to enhance nerve regeneration *in vivo* [221, 226], there remains a search for a deeper understanding on the factors that govern MSC differentiation into non-mesenchymal lineages.

Furthermore, regardless of the choice of stem cell type, being able to specifically direct stem cell fate remains a limitation to the wide spread clinical application of stem cell therapy. One of the major obstacles lies in the recapitulation of the stem cell niche. While studies thus far have demonstrated some degree of success in mimicking the biochemical signals required during stem cell differentiation [21, 22], other works have suggested the critical and synergistic roles played by the extracellular matrix (ECM). In particular, substrate compliance and architecture signaling are important in directing cell

fate [23, 24]. Therefore, being able to combine several microenvironmental signals within a single scaffold construct would be attractive towards achieving the ideal artificial stem cell niche design.

Nanofiber matrices mimic the architecture and size scale of the natural ECM. As compared to 2D substrates, nanofiber constructs provide more three-dimensional biomimicking topographical signals to seeded cells and result in more physiologically relevant cellular phenotypes [227]. Applied to stem cell engineering, nanofibers alone support stem cell culture and neural differentiation, and effectively direct cell alignment and neurite extension along aligned fibers [228]. Endowed with sustained drug release capabilities, these nanofiber constructs enhance tissue regeneration [12, 228, 229] and may find useful applications in providing synergistic contact guidance and biochemical cues to direct stem cell fate [230]. Such biofunctional scaffolds may further permit subsequent direct cell transplantation and provide guidance signals to control the differentiation of endogenously recruited stem cells.

In this study, we attempt to understand the effects of nanofiber topography and sustained release of neuronal induction factor, retinoic acid (RA), on MSC neural commitment. We aim to evaluate the potential of RA-encapsulated poly(ϵ -caprolactone) (PCL) nanofiber constructs as an alternative platform for directing stem cell fate. Since MSC non-mesenchymal

differentiation remains controversial, basic studies to understand factors contributing to MSC phenotypic changes towards the neural lineage would be of scientific interest and relevance.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Acetonitrile (gradient grade for HPLC), all-trans retinoic acid (> 98%, gradient grade for HPLC), ammonium acetate (gradient grade for HPLC), bovine gelatin type B, bovine serum albumin (BSA), dichloromethane (DCM), dimethyl sulphoxide (biotechnology performance certified, DMSO), ethanol, fluoromount™ aqueous mounting medium, 10% formalin, glycine, hexamethyldisilazane (HMDS), poly (ε-caprolactone) (PCL, Mn 80,000), 2,2,2-trifluoroethanol (TFE) with 99.0% purity, tris acetate-EDTA buffer (TAE), Triton-X, Tween® 20, mouse anti-MAP2 monoclonal antibody, and goat anti-rabbit IgG-FITC were purchased from Sigma Aldrich. Antibiotic and antimycotic (100x), Click-iT® EdU Alexa Fluor® 488 Imaging kit, DAPI stock solution (5 mg/ml), Oregon green® 488 phalloidin, phosphate buffered saline (PBS, pH 7.4), TRIzol® reagent, Alexa Fluor® 488 goat anti-chicken antibody, and Alexa Fluor® 633 goat anti-mouse antibody were purchased from Invitrogen. RNeasy® Mini kit, Sensiscript® Reverse Transcription kit, and

HotStarTaq[®] Master Mix kit were purchased from Qiagen. Fast SYBR[®] Green Master Mix was purchased from Taqman. Mouse anti-Oligodendrocytes, clone NS-1 (RIP) monoclonal antibody and chicken anti-GFAP monoclonal antibody were purchased from Millipore. Rabbit anti- β -Tubulin III (Tuj1) polyclonal antibody was purchased from Covance. Mouse anti-synaptophysin monoclonal antibody was purchased from Dako. Mesenchymal Stem Cell Growth BulletKit[™] Medium (MSCGM) was purchased from Lonza. Fetal bovine serum (FBS) was purchased from Hyclone. Agarose was purchased from Bio-Rad and all primers were ordered from 1st base, Singapore. All materials were used as received without further purification unless otherwise noted.

3.2.2. Fabrication of scaffolds

PCL (8% w/w) and gelatin (1.5% w/w) were dissolved in TFE. Thereafter, PBS was added into the resulting solution at 1:5 (PBS:TFE) volume ratio to form solution A. Solution A was used for all samples subsequently.

To fabricate plain PCL/gelatin electrospun scaffolds, 2 ml of solution A was charged at 16 kV and fed at a flow rate of 0.8 ml/h. All fibers were then collected on a rotating drum (diameter = 12 cm) that was charged at -5 kV. To obtain randomly oriented plain PCL/gelatin fibers (denoted as RF, Table 3.1), the rotational speed of the drum was set at 500 rpm. To obtain aligned fibers

(denoted as AF, Table 3.1), a rotational speed of 2,500 rpm was used. The syringe tip-to-collector distance was set at 12 cm.

To fabricate RA-encapsulated electrospun fibers, a stock solution of 3 mg/ml was prepared by dissolving RA in ethanol. To obtain aligned fibers comprising of theoretical loading levels of 0.1 wt% and 0.3 wt% of RA (with respect to PCL, denoted as AF250 and AF750 respectively, Table 3.1), 85 μ l and 250 μ l of RA stock solution were added respectively into 2.2 ml of solution A for electrospinning. The resulting drug-loaded polymer solution was then charged at 15 kV and fed at a flow rate of 0.8 ml/h. All fibers were finally collected on the negatively charged rotating drum (-5 kV, 2,500 rpm) that was set at 12 cm away from the polymer supply. The electrospinning process was protected from light to minimize degradation of RA.

Table 3.1. Optimized electrospinning parameters and average fiber diameters of scaffolds.

Sample	Applied voltage (kV)	Retinoic acid loading level (%)	Rotating speed (rpm)	Fiber Φ (nm)
RF	16 / -5	--	500	279 $\pm$ 30
AF	16 / -5	--	2,500	239 $\pm$ 37
AF250	15 / -5	0.1	2,500	270 $\pm$ 45
AF750	15 / -5	0.3	2,500	243 $\pm$ 93

All samples were fabricated using polymer solution comprising of 8% PCL/ 1.5% gelatin, dispensed at 0.8ml/h. The tip-to-collector distance was 12 cm.

3.2.3. Characterization of nanofibers

The morphology of the electrospun fibers was investigated using a scanning electron microscope (SEM, JEOL, JSM-6390LA) at an accelerating voltage of 15 kV after 90 seconds of gold coating. The fiber diameters were measured using Image J (NIH). Five images at 10,000 $\times$ magnification were used and at least 100 fibers were measured for each sample.

The distribution of RA within PCL/gelatin fibers was analyzed by electrospinning nanofibers directly onto a glass coverslip. Thereafter, the autofluorescence of RA was detected by using an Olympus IX71 microscope with a FITC filter.

3.2.4. Release kinetics of retinoic acid

AF750 scaffolds (average weight = 228 mg, n=3) were cut into pieces (4.71 cm by 2.5 cm each) and soaked in 5 ml of PBS. To account for possible dissolution of gelatin from the nanofibers, AF fibers (average weight = 220 mg, n=1,) were used as the control. All samples were protected from light and incubated under static conditions at 37 °C. At various time points, 2.5 ml of supernatant was retrieved, and replenished with 2.5 ml of fresh PBS. To analyze the amounts of RA that were released within day 1, early time points of 3 h, 6 h, 12 h and 24 h were chosen. The supernatants were 0.22 μ m filtered before

HPLC analyses. To obtain the standard curve for RA, the supernatant from AF fibers was doped with 0.31 to 10 $\mu\text{g/ml}$ of RA. Thereafter, the Agilent HPLC apparatus (Agilent 1100 series, consisted of G1379A Degasser, G1311A Quat Pump, G1313A ALS, G1316A COLCOM, G1364C Analyt-FC, G1362A 1260 RID, G1315B DAD) with a reverse-phase column (Lichrospher[®] 100 RP-18 column, $4 \times 25 \text{ cm}$, $5\mu\text{m}$) was used to measure the concentrations of the solutions. During the analyses, the mobile phase consisted of a mixture of acetonitrile and aqueous ammonium acetate at a volume ratio of 9:1, delivered at a flow rate of 1 ml/h. The HPLC DAD detection was carried out at 340 nm with a reference at 500 nm [231, 232]. The data was quantified using the external standard method. A computer was connected to the detector for data acquisition and retention time and peak area calculations.

After 14 days incubation, all supernatant was removed by centrifugation and analyzed. The remaining scaffolds were then lyophilized. Thereafter, the scaffolds were dissolved in 2 ml of DCM and extracted with 5 ml of ethanol. DCM was evaporated when the extraction was completed. The remaining solutions were $0.45 \mu\text{m}$ filtered and adjusted to a final volume of 5 ml. Finally, the concentrations of RA were determined by UV-spectrophotometry (Genesys 10UV, Thermoscientific) and the experimental loading efficiency of RA was computed using the following equation:

$$\text{Loading efficiency} = \frac{(\text{cumulative amount of retinoic acid released} + \text{extracted retinoic acid})}{\text{original retinoic acid loading}} \times 100\%$$

3.2.5. Human mesenchymal stem cell culture

Human mesenchymal stem cells (passage 6–9, Lonza) were cultured and expanded in MSCGM medium supplemented with 1% of antibiotic-antimycotic solution. Due to poor cell attachment on PCL film, tissue culture polystyrene (TCPS) was used as the two-dimensional control throughout this study.

Effects of nanofiber topographical cues on MSCs:

RF and AF scaffolds were cut to fit the wells of 12-well plates (average weight = 7.4 mg, Table 3.2). Thereafter, all scaffolds were sterilized for 30 min on each side using UV-irradiation. MSCs were then seeded at 1.5×10^4 cell/well in 1 ml of MSCGM. The cells were cultured with a change of medium every 3 days. At days 4, 7 and 14, the cells were fixed or lysed for subsequent analyses.

Effects of sustained release vs. bolus delivery of retinoic acid on MSCs:

Bolus delivery of retinoic acid: RF and AF scaffolds (average weight = 7.4 mg, Table 3.2) were cut and sterilized as indicated above. To induce neural differentiation, MSCs were seeded at 1.5×10^4 cell/well in 1 ml of MSCGM

supplemented with 5 μ l of RA solution (1.8 mg/ml concentration in DMSO, resulting in a final concentration of 9 μ g/ml during cell culture). The samples were denoted as RF+RA9 and AF+RA9 for random and aligned fibers respectively (where RA denotes retinoic acid, Table 3.2). The cells were cultured with a change of medium and supplementation of fresh RA every 3 days. Thereafter, cells were fixed or lysed at days 4, 7 and 14 for subsequent analyses.

Sustained availability of retinoic acid: AF250 and AF750 scaffolds (average weight = 7.7 mg, Table 3.2) were cut as indicated above. The scaffolds was used directly without sterilization. Thereafter, MSCs were seeded at 1.5×10^4 cell/well in 1 ml of MSCGM, with half of the medium changed every 3 days. At days 4, 7 and 14, cells were fixed or lysed for subsequent analyses.

Table 3.2. Culture conditions for MSCs. Cells were seeded at a density of 1.5×10^4 cells/well in 12-well plates.

Sample	Retinoic acid supplementation (μ g/ml)	Medium changing frequency (day)
TCPS	--	3
RF	--	3
AF	--	3
RF+RA9	9	3
AF+RA9	9	3
AF250	--	3 (half change)
AF750	--	3 (half change)

3.2.6. Evaluation of cell-substrate interactions

At days 7 and 14, MSCs were fixed in 2.5% buffered glutaraldehyde for 4 hours, and washed in PBS and DI water. Thereafter, the samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 100%), followed by treatment with 100% HMDS. All samples were then vacuum dried overnight and stored under vacuum prior to 90 seconds of gold coating (JEOL, JFC-1600 Auto Fine Coater) and analysis under the SEM (accelerating voltage of 15kv, JEOL, JSM-6390LA Analytical Scanning Electron Microscope).

3.2.7. Evaluation of cell morphology

At days 7 and 14, cells were fixed in 10% formalin prior to permeabilization in 0.05% Triton-X and 50 mM glycine solution for 20 min. Thereafter the samples were incubated in phalloidin-Oregon green dye (1:250 dilution) and DAPI (1:1500 dilution) for 30 min. The samples were washed three times with PBS in between each step and all incubation steps were carried out at room temperature. Samples were then examined under a Zeiss LSM710 META confocal microscope at 40× and 63× magnifications.

The nuclei elongation (E) factor and the degree of nuclei alignment were computed by imaging cells at day 14 under 40x magnification. Image

analyses were then carried out using ImageJ (NIH). E-factor, which describes the extent to which the nuclei (approximated as an equimomental eclipse) is lengthened or stretched [233, 234], was computed as:

$$\text{E-factor} = \text{Long axis} / \text{short axis} - 1$$

Nuclei alignment was defined as the extent to which the long axis of an elongated nucleus is oriented with respect to a reference axis. The percentage of nuclei alignment was then computed by counting the number of cells with nuclei aligned $<15^\circ$ with respect to the reference axis. For aligned fibers, the reference axis was the aligned fiber axis. For random fibers, the axis of target rotation that was used during electrospinning was selected as the reference axis. For TCPS, an arbitrary horizontal line was used as the reference axis. At least 100 cells were analyzed for each sample.

3.2.8. Evaluation of cell proliferation

At days 7 and 14, cell proliferation was assessed by EdU incorporation using the Click-iT[®] EdU Alexa Fluor[®] 488 HCS Assay according to the manufacturer's instructions. Cells were incubated in EdU solution (1:1000 dilution) for 24 h at 37 °C. The cells were then fixed in 10% formalin solution and permeabilized as stated above. Thereafter the samples were incubated in Click-iT reaction cocktail and DAPI (1:1500 dilution) for 30 min. Samples

were then examined under an Olympus IX71 microscope at 20× magnification. The percentage of cells with EdU incorporation was finally measured using ImageJ. At least 500 cells were counted for each sample.

3.2.9. Conventional PCR and Real-time PCR

At days 4, 7 and 14, RNA was isolated using a combination of TRIzol[®] reagent and Qiagen RNeasy[®] kit. For cells cultured on TCPS, 2 wells were pooled together to obtain sufficient mRNA. For cells cultured on nanofibers, 2 scaffolds were pooled. The quality and quantity of the extracted RNA were evaluated using UV-spectrophotometry. Reverse transcription was performed using the Sensiscript Reverse Transcription kit with a poly-T 15 oligomer following manufacturer's protocol. Twelve pairs of primers were tested and their sequences are listed in Table 3.3. Human 18s rRNA was used as the endogenous control.

Conventional PCR was performed using Qiagen HotStarTaq Master Mix Kit on a Bio-Rad C1000 Thermocycler with the following cycling conditions: 95 °C for 5 min; 35 repeated cycles of 95 °C for 30 s followed by 58-62 °C for 30 s and 72 °C for 1 min; and another 72 °C for 10 min. PCR reactions were finally resolved using a 3% agarose gel in TAE buffer.

Real-time PCR was performed using Fast SYBR Green MasterMix with

the same primers and mRNA samples. The real-time PCR was performed on an Applied Bioscience Real-Time PCR System (StepOnePlus 7500) using the following cycling conditions: 95 °C for 3 min; 40-45 repeated cycles of 95 °C for 15 s followed by 58-62 °C for 30 s. The amplification efficiency of the genes of interest and the housekeeping gene were verified as similar. Therefore, the $\Delta\Delta C_t$ method was adopted for fold change analyses. The gene expressions were normalized with respect to the expression of MSCs cultured on TCPS for 7 days. The entire experiment was repeated 5 times.

Table 3.3. PCR primers sequences.

Gene	Sequence
18S	F 5'-TCTTAGTTGGTGGAGCGATTTG-3' R 5'-CCTGT TCACC GCAAG TCGG-3'
ACAN	F 5'-TGCATTCCACGAAGCTAACCTT-3' R 5'-GACGCCCTCGCCTTCTTGAAAT-3'
COL1A1	F 5'-GTGCGATGACGTGATCTGTGA -3' R 5'-TGGTCGGTGGGTGACTCTG -3'
COL2A1	F 5'-AGAACTGGTGGAGCAGCAAGA -3' R 5'-AGCAGGCGTAGGAAGGTCAT -3'
GalC	F 5'-GGCTCCTTGACAGCGATGGC-3' R 5'-CAACATTGAAATCATCCTTA-3'
GFAP	F 5'-CACCGCAGCCCTGAAAGA-3' R 5'-GTTGCTGGACGCCATTGC-3'
MAP2	F 5'-CTGCTTTACAGGGTAGCACAA-3' R 5'-TTGAGTATGGCAAACGGTCTG-3'
NEFL	F 5'-TGAAGGTGAAGAAGGAGAGGAA-3' R 5'-CTGTTGGTTGGTTGGTGAT-3'
Nestin	F 5'-CCCTTCCAGACTCCACTCCC-3' R 5'-GACCT TTCCT CCCGACCC-3'
TH	F 5'-CTGAGGAGCCTGAGATTC-3' R 5'-AGACACGAAGTAGACTGAC-3'
Tuj-1	F 5'-GCAACTACGTGGGCGACT-3' R 5'-CGAGGCACGTACTTGTGAGA-3'

3.2.10. Immunostaining of neural markers

At days 7 and 14, cells were fixed in 10% formalin followed by permeabilization as stated above. Thereafter, samples were blocked with 3% BSA at 4°C overnight. The samples were then incubated in either rabbit anti- β -Tubulin III (Tuj1) polyclonal antibody (1:2000 dilution), mouse anti-MAP2 monoclonal antibody (1:1000 dilution), chicken anti-GFAP monoclonal antibody (1:500 dilution), rabbit anti-oligodendrocytes (RIP) monoclonal antibody (1:1000 dilution) or mouse anti-synaptophysin (1:80 dilution) for 1 h at 37 °C. Thereafter, the samples was incubated in either FITC goat anti-rabbit antibody (1:200), Alexa-Fluor488 goat anti-chicken antibody (1:500) or Alexa-Fluor633 goat anti-mouse antibody (1:1000) for 30 min at 37 °C . All samples were washed three times with PBS in between each step. DAPI was used to stain cell nuclei at 1:1000 dilution. All samples were then examined under a Zeiss LSM710 META confocal microscope at 40× and 63× magnifications. All images were acquired using the same settings and quantitative fluorescence analysis was performed by a modified protocol [235, 236]. Briefly, after threshold adjustment, the fluorescence intensity of the whole image was measured using ImageJ. The results were then obtained by calculating fluorescence intensity against DAPI-stained cells. 15 images (≥ 150

cells) were measured for each sample.

3.2.11. Statistical analyses

Data is presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were conducted using one-way ANOVA followed by *Tukey* or *Games-Howell* post-hoc tests where appropriate. $p < 0.05$ was considered statistically significant, and $p < 0.01$ was considered statistically highly significant.

3.3. RESULTS

3.3.1. Characterization of scaffolds

Figures 3.1a to c show the SEM micrographs of PCL/gelatin nanofiber scaffolds. Bead-free and uniform nanofibers were obtained for all samples. Regardless of the loading level of RA, no significant difference in average fiber diameter was observed between samples (Table 3.1). As indicated in Figure 3.1d, RA was distributed uniformly and continuously throughout PCL/gelatin nanofibers.

Figure 3.1. SEM images of PCL/gelatin nanofibers. (a) Randomly oriented plain fibers (RF), (b) aligned plain fibers (AF), (c) fibers encapsulating RA (AF250/AF750) and (d) fluorescent image of RA distribution in electrospun fibers.

3.3.2. Release kinetics of retinoic acid

The drug release profile of AF750 scaffolds is shown in Figure 3.2a. After an initial burst release of 31.2 ± 1.5 % within day 1, RA was released at a fairly constant rate of 2.1 %/day for at least 14 days. A total of 60.1 ± 2.2 % was released by day 14. The experimental loading efficiency of RA in AF750 scaffolds was 35.1 ± 4.9 %.

Based on the RA release profile, Figure 3.2b shows the absolute amount of RA that was present in the culture medium during MSC differentiation.

Compared with a bolus supplementation of 9 $\mu\text{g}/\text{ml}$ of RA, the amount of drug that was released from AF750 scaffolds was at least 8 times lower (1.1 μg at 3 h from AF750 vs. 9 μg). As shown in Figure 3.2c, nanofiber scaffolds remained structurally intact throughout the period of study. This suggested that diffusion was likely to be the main mode of drug release from the nanofiber scaffolds.

Figure 3.2. (a) RA release profile of AF750 scaffolds, (b) the effective amount of RA in each well during cell culture and (c) SEM image of RA-encapsulated scaffold after 14 days of cell culture.

3.3.3. Evaluation of cell morphology

Effects of nanofiber topographical cues on MSCs:

As indicated in Figure 3.3, MSCs appeared polygonal and adopted random orientations when cultured on TCPS and randomly oriented nanofibers. In contrast, cells aligned and elongated along the axes of aligned fibers. Comparing Figures 3.3d to f, stress fiber formation was more prominent in cells on 2D controls (TCPS). Similar to SEM observations, actin cytoskeleton and nuclei staining revealed that cells responded to the underlying aligned nanofiber topography with enhanced nuclei elongation and alignment ($p < 0.01$ with

respect to TCPS and RF, Figures 3.4 a-b).

Effects of sustained release vs. bolus delivery of retinoic acid on MSCs:

As indicated in Figures 3.3 g-j and Supplementary Figures 3.1 c-d, in addition to cell alignment and elongation along aligned fiber axes, MSCs acquired elongated and thinner morphology with multipolar extensions when exposed to RA. As compared to 2D controls, nuclei elongation increased significantly even on randomly oriented fibers (Figure 3.4c). Furthermore, the sustained availability of RA elicited significantly higher degree of nuclei elongation as compared to bolus delivery (AF750 vs. AF+RA9, $p < 0.05$).

Figure 3.3. SEM and confocal images of MSCs cultured in (a-f) MSCGM only and (g-j) MSCGM with RA for 14 days. Cells were cultured on (a, d) TCPS, (b, e) RF, (c, f) AF, (g,i) RF+RA9 and (h, j) aligned fibers with RA. H inset: multipolar projections. Green: actin cytoskeleton. Blue: cell nuclei. White arrow: aligned fiber axes.

Figure 3.4. (a,c) Nuclei elongation and (b,d) nuclei alignment of MSCs cultured on different substrates for 14 days (a, b) without and (c, d) with RA. $n > 100$, mean $\pm$ SE, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA. Statistical comparisons were made with respect to MSCs on TCPS, unless otherwise indicated.

3.3.4. Evaluation of cell proliferation

Effects of nanofiber topographical cues on MSCs:

As shown in Figure 3.5a, nanofiber topography, regardless of orientation, significantly decreased cell proliferation as compared to the 2D control. Additionally, cell proliferation decreased by day 14 on all substrates, likely due to confluence.

Effects of sustained release vs. bolus delivery of retinoic acid on MSCs:

Figure 3.5b illustrates the significant decrease in MSC proliferation in response to the combined effects of nanofiber topography and RA. The extent of EdU incorporation was similar amongst cells on nanofibers, regardless of fiber orientation. No EdU incorporation was detected in cells on nanofibers by day 14.

Figure 3.5. (a) EdU (green color) incorporation by MSCs on scaffolds after 7 days and 14 days differentiation. And EdU incorporation rate of MSCs on scaffolds (a) without and (b) with RA. $n > 500$, mean $\pm$ SE, * $p < 0.05$, one-way ANOVA. Statistical comparisons were made with respect to MSCs on TCPS.

3.3.5. Gene expression analyses

Effects of nanofiber topographical cues on MSCs:

As shown in Figure 3.6a, nanofiber topography decreased MSC expression of COL1A1 (osteogenic marker) and COL2A1 (chondrogenic marker), with gene products detected only at day 14. Detailed analyses by real-time PCR revealed that the expressions of the two markers were significantly lower than basal level even at day 14 (Figure 3.6b). In contrast, up-regulation of neuronal markers, Tuj-1, tyrosine hydroxylase (TH) and neurofilament light (NEFL), and oligodendrocytes marker, galactosylceramidase (GalC), were observed as early as day 4. No significant difference in gene expression was detected for aggrecan (ACAN, chondrogenic marker), nestin (neural stem cell marker) and glial fibrillary acidic protein (GFAP, astrocyte marker).

As indicated in Figure 3.6c, quantitative analyses of gene expression by real-time PCR confirmed that nanofiber topography, particularly aligned architecture, significantly up-regulated the expression of MAP2 (mature neuronal marker) by day 7 and GalC by day 14. In contrast to conventional PCR results, cell alignment on AF substrates enhanced GFAP expression at day 14. However, the level of fold change was lower as compared to MAP2 and GalC.

Figure 3.6. (a) Conventional RT-PCR of MSCs cultured on TCPS, RF and AF. Gene expression analysis of MSCs cultured on TCPS, RF and AF as evaluated by real-time PCR. The expressions of (b) COL1A1 and COL2A1; and (c) MAP2, GalC and GFAP were normalized to the gene expression of MSCs cultured on TCPS day0 with 18s rRNA as the housekeeping gene. $n = 3$, mean $\pm$ SE, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA. Statistical comparisons were made with respect to MSCs on TCPS at day 7.

Effects of sustained release vs. bolus delivery of retinoic acid on MSCs:

Figure 3.7a shows the effects of nanofiber topography and RA on MSC gene expression. Similar to cells subjected to nanofiber topography only, COL1A1 expression decreased for the first 7 days and returned to near basal levels by day 14. In contrast, COL2A1 was not detected throughout the period of study. The presence of nanofibers and RA enhanced the expression of neural markers, Tuj-1, NEFL, TH and GalC. Additionally, GalC expression appeared to increase on aligned fibers at day 14 as compared to TCPS and random fibers.

On the contrary, no changes were detected for ACAN, nestin, and GFAP.

As shown in Figure 3.7b, further quantitative evaluation by real-time PCR confirmed that the presence of RA significantly enhanced MAP2 expression ($p < 0.01$ with respect to TCPS for all samples). Coupled with cell alignment, MAP2 expression was further enhanced (RF+RA9 vs. AF+RA9 and AF250, $p < 0.05$; and RF + RA9 vs. AF750, $p < 0.01$). Additionally, the sustained availability of RA significantly increased MAP2 expression (AF+RA9 vs. AF750, $p < 0.01$). Similar results were observed for GalC expression at day 14. The combined effects of cell alignment and sustained release of RA also increased GFAP expression (RF+RA9 vs. AF250 and AF750, $p < 0.05$). However, the fold change extent was generally lower as compared to MAP2 and GalC.

Figure 3.7. (a) Conventional RT-PCR of MSCs cultured on TCPS, RF+RA9, AF+RA9, AF250 and AF750. (b) Gene expression analysis of MSCs cultured on TCPS, RF+RA9, AF+RA9 and AF750 as evaluated by real-time PCR. The expressions of MAP2, GalC and GFAP were normalized to the gene expression of MSCs cultured on TCPS day0 with 18s rRNA as the housekeeping gene. n= 3, mean $\pm$ SE, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA. Statistical comparisons were made with respect to MSCs on TCPS at day 7.

3.3.6. Immunostaining of neural markers

Effects of nanofiber topographical cues on MSCs:

As shown in Figure 3.8, the expressions of Tuj-1 and MAP2 were enhanced on nanofiber substrates. GFAP was expressed at basal levels regardless of the underlying substrate topography. In contrast, mature oligodendrocyte marker, RIP, was very weakly expressed in all samples. Semi-quantitative analyses by fluorescence intensity measurements revealed

that nanofiber topography enhanced Tuj-1, MAP2 and RIP protein expressions ($p < 0.01$ with respect to TCPS, Figures 3.8c to e). In particular, cell alignment increased the expression of MAP2 and RIP proteins (RF vs. AF, $p < 0.01$, Figures 3.8d and e). On the contrary, GFAP protein expression decreased in the presence of nanofibers. Aligned fibers, in particular, decreased GFAP expression significantly (AF vs. TCPS, $p < 0.01$, Figure 3.8f).

Figure 3.8. Immunofluorescent staining of MSCs cultured on TCPS, RF and AF for 14 days. (a) Tuj-1 (green) and MAP2 (red), (b) RIP (red) and GFAP (green). Blue: nuclei, white arrow: aligned fiber axes. (c to f) Mean fluorescence intensities per cell for (c) Tuj-1, (d) MAP2, (e) RIP and (f) GFAP. $n = 15$, mean $\pm$ SE, ** $p < 0.01$, one-way ANOVA. Statistical comparisons were

made with respect to MSCs on TCPS.

Effects of sustained release vs. bolus delivery of retinoic acid on MSCs:

Figure 3.9 shows the effects of nanofiber topography and RA on MSC protein expression. The presence of RA enhanced the expression of Tuj-1, MAP2 and RIP ($p < 0.01$ with respect to TCPS, Figure 3.9c to e). Coupled with cell alignment, neuronal markers were further enhanced (RF+RA9 vs. AF+RA9, AF250 and AF750, $p < 0.01$, Figures 3.9c and d). The effects of sustained availability of RA, although at significantly lower amounts than bolus delivery, led to similar levels of neuronal protein markers expression and an increase in RIP expression (AF250 and AF750 vs. AF+RA9 and RF+RA9, $p < 0.01$, Figure 3.9e). In contrast, GFAP expression remained at basal level for all samples and cell alignment appeared to decrease GFAP expression levels. As indicated in Figure 3.10, the presence of aligned nanofibers and RA enhanced the expression of synaptophysin, which indicated the possibility of synapse formation (AF+RA9, AF250 and AF750).

Figure 3.9. Immunofluorescent staining of MSCs cultured on TCPS, RF+RA9, AF+RA9, RA250 and RA750 for 14 days. (a) Tuj-1 (green) and MAP2 (red), (b) RIP (red) and GFAP (green). Blue: nuclei, white arrow: aligned fiber axes. (c to f) Mean fluorescence intensities per cell for (c) Tuj-1, (d) MAP2, (e) RIP and (f) GFAP. n = 15, mean $\pm$ SE, * p < 0.05, ** p < 0.01, one-way ANOVA. Statistical comparisons were made with respect to MSCs on TCPS.

3.4. DISCUSSION

Directing stem cell differentiation, regardless of the origin of the cells, remains a challenge. Currently, the most common approach to understand and direct stem cell differentiation involves 2D cultures on polystyrene plates with bolus supplementation of cocktails of induction factors. This approach neglects the critical role of the ECM in determining cell fate. While several studies are beginning to introduce plain nanostructured scaffolds as alternative supporting

substrates to direct stem cell differentiation [40, 46, 237], due to the short half-lives of the induction factors, these platforms still require the frequent supplementation of drugs throughout the period of culture. Therefore, the ability to combine biochemicals within a single scaffold construct for sustained delivery and to provide synergistic topographical signaling to seeded cells would be advantageous.

Figure 3.10. Immunofluorescent staining of Tuj-1 (red) and synaptophysin (green) in MSCs cultured on different substrates for 14 days with RA. Blue: cell nuclei, white arrow: aligned fiber axes. Dotted circles: synaptophysin signals.

Electrospun nanofibers can enhance tissue-implant integration [238] and can easily be functionalized by drug encapsulation [79, 239, 240] or surface modification [230]. Introduced to stem cell engineering, such biofunctional

matrices may permit the possibility of subsequent direct stem cell transplantation or facilitate control over differentiation of endogenously recruited stem cells. In recent works, Cho et al. [230] cultivated rat MSCs on nerve growth factor (NGF)-immobilized or NGF-absorbed nanofibers with random and aligned topography. They demonstrated more prominent up-regulation of neuronal markers in cells that were cultured on NGF-immobilized nanofibers after 5 days. The authors attributed these observations to the prolonged availability of conjugated NGF as compared to surface absorbed NGF. Furthermore, similar to our results, aligned nanofibers promoted neuronal marker expression more effectively as compared to random fibers. However, besides the involvement of additional chemistry, surface immobilization of proteins can also compromise the bioactivity of the molecules. In addition, the extent of MSC neural differentiation and maturation remains in question due to the short evaluation time point of 5 days. In contrary, by making use of the versatility of the electrospinning technique, we encapsulated RA directly into nanofibers for sustained release to induce hMSCs neural differentiation over an extended time period.

RA-encapsulated PCL/gelatin nanofibers were successfully fabricated by electrospinning. The resulting fibers possessed similar fiber diameter regardless of drug loading levels. This is likely due to the much lower amounts

of RA that was used as compared to PCL and gelatin. Fiber size affects cell fate and differentiation [46, 241]. Therefore, having similar fiber diameters throughout this study helped eliminate fiber size effect on cellular response.

RA plays important roles in many aspects of neural development. Besides being used commonly as an inducible reagent for *in vitro* stem cell neuronal differentiation, it is also used as a therapeutic agent for the treatment of neurodegeneration [242]. However, high amounts of retinoid can induce cytotoxicity [243]. Therefore, initial optimization studies were carried out to deduce the appropriate loading levels of RA for sustained release applications. Based on these results and the fact that aligned fibers more favorably promoted MSC neural commitment, aligned nanofibers with loading levels of 0.1 wt% (AF250) and 0.3 wt% (AF750) were chosen. As shown in Figure 3.2a, RA was released at a fairly constant rate for at least 14 days after an initial burst release. The initial burst releases are likely attributed to RA that was located near the surface of the nanofibers, since uniform PCL/gelatin/RA mixtures were electrosun. The fairly constant release rate is likely due to the fact that RA is a small hydrophobic molecule (Mw: 300.4) and the release of RA was mainly mediated by diffusion. A concentration of 9 $\mu\text{g/ml}$ of RA was supplemented in a bolus format to MSCs according to reported works [40]. From our preliminary studies and other reported works [244-246], RA possess short half-lives in the

presence of serum (~ 4-6 h). Therefore, the bolus supplementation of RA served as a short-term biochemical cue to seeded cells and was represented accordingly in Figure 3.2b. In contrast, the effective amount of RA as derived from the sustained release profile was at least 8 times lower (Figure 3.2b). This sustained and localized release of RA was maintained for at least 14 days and worked as a long-term biochemical signal to seeded cells.

Topographical signaling affects cell elongation and nuclei distortion through actin-intermediate filament system and may correlate with changes in gene and protein expression and MSC differentiation [40, 247, 248]. Correspondingly, we observed that MSCs adopted the typical fibroblast-like morphology in 2D culture [38, 225] but demonstrated enhanced nuclei elongation and cytoskeleton rearrangement on nanofibers. Reduced actin stress fiber formation was also seen on nanofibers. Such behavior has been observed in cells in 3D culture on natural ECM components [249, 250] and suggested that nanofibers provided a more biomimicking architecture for MSCs as compared to TCPS. The more prominent stress fibers within cells on 2D culture is likely due to the stiffer nature of TCPS, which could promote more extensive focal adhesion formation and higher level of cytoskeletal stress [249]. In the presence of RA, more pronounced cellular protrusions were observed. While the breakdown of actin cytoskeleton and retraction of cell edge in the presence

of DMSO have been suggested [225], we believe that the cell shape changes observed in this study are more likely associated with nanofiber topography and the presence of RA and may correlate with MSC differentiation. This is because the amount of DMSO used in this study is significantly lower than reported (0.5 % v/v vs. 2% v/v [38, 225]).

Similar to previous observations, along with morphological changes, cell proliferation decreased on 3D fibrous scaffolds as compared to TCPS. The presence of RA further decreased cell proliferation. These observations are likely due to the cell-growth arrest that is associated with stem cell differentiation [40, 46].

Studies thus far have suggested the potential of MSCs to differentiate along the neural lineage under *in vitro* and *in vivo* conditions [22, 251]. Our results appear to support this notion. The down-regulated expressions of mesenchymal markers, COL1A1 and COL2A1, suggested that our fibrous scaffolds hindered MSCs differentiation along the mesenchymal lineage. In contrast, the expression of neural markers was enhanced at mRNA and protein levels. Although real-time PCR analyses revealed that MAP2, GalC and GFAP mRNA expressions were enhanced in response to topographical changes, the observed fold changes were higher for MAP2 and GalC (Figure 3.6c). Compared to MAP2, the much weaker immunoreactivity of RIP suggested that

MSCs did not differentiate into mature oligodendrocytes. In addition, GFAP protein expression decreased in response to nanofiber topography. Taken together, nanofiber topography alone, particularly aligned fibers, appeared to enhance the potential of MSCs to differentiate along the neuronal lineage. A detailed analyses of global gene and protein expression changes in MSC would more strongly substantiate these observations and will be conducted in future studies.

The synergistic effects of nanofiber topography and RA further enhanced the potential of MSCs to undergo neural differentiation. This was evident from mRNA and protein expression analyses. A similar down-regulation of COL2A1 expression was detected. On the other hand, COL1A1 was not detected in the presence of RA throughout the period of study. Furthermore, a direct comparison between AF and AF+RA9 samples revealed that MAP2 mRNA and protein expressions were enhanced by 2- and 1.5-fold respectively in the presence of RA. GalC also showed a 1.8-fold enhancement in mRNA level when RA was supplemented. In contrast, GFAP mRNA and protein expressions were not significantly altered.

The most important observation from this study is perhaps the fact that the manner in which induction factor was presented to MSCs appeared to have significant effects on stem cell differentiation. Although RA was presented at

much lower concentrations, (at least 8 times lower), AF750 scaffolds induced more significant up-regulation of neural markers as compared to a bolus delivery of 9 $\mu\text{g/ml}$ of RA. Specifically, MAP2 mRNA expression was enhanced by 2-2.5 fold on AF750 scaffolds and protein expression levels were similar to AF+RA9 samples. While the exact reason behind this observation remains to be elucidated, it is possible that the RA-encapsulated fibers provided a local concentration of RA to allow more efficient cellular uptake. Such phenomenon has been reported in the cellular uptake of nucleic acids for gene transfer and knockdown [239, 252]. Altogether, these results highlight the advantage of drug-encapsulated nanofibers as biomimicking platforms for directing stem cell fate.

Although the combination of RA and topography enhanced the mRNA expressions of neural markers, the fold change intensity was again highest for MAP2, suggesting that MSCs were driven towards the neuronal lineage. Similarly, at protein level, a much weaker RIP immunoreactivity was detected and GFAP expression decreased to below basal levels on aligned fibers. However, the co-expression of neuronal markers (Tuj-1 and MAP2), oligodendrocyte markers (GalC and RIP) and astrocyte marker (GFAP) indicated the necessity of using other factors to further commit MSCs into neuronal lineage. It is possible that similar to induced pluripotent stem cells

[253], reprogramming of MSCs might be required.

Synaptophysin staining was carried out to investigate the maturation of the differentiated MSCs. Synapse formation is critical in the functional development of the nervous system. In mature neurons, synaptophysin often concentrates and remains at sites where axons contact neuronal cell bodies or dendrites [254, 255]. Under the influence of topography alone, MSCs were immunonegative towards synaptophysin. In contrast, the presence of RA enhanced the expression of synaptophysin. However, there was no obvious concentrated synaptophysin punctuate appearance at cell-cell contacts. This indicated that although the synergistic effect of RA and nanofiber topographical signaling promoted the potential of MSC neuronal differentiation, the cells are likely to be neuronal precursors and are still in the process of maturation.

3.5. CONCLUSIONS

In this study, we introduced a scaffold-based approach to direct stem cell fate. By using a nanofiber construct endowed with sustained release of induction factor, RA, the potential of MSC neuronal differentiation was enhanced. The sustained and localized delivery of RA promoted MSC differentiation more significantly as compared to the typical bolus delivery. These results highlight the importance of drug delivery approach in directing

stem cell differentiation and the potential of such biofunctional scaffolds for cell transplantation applications in nerve injury repair.

CHAPTER FOUR:

POLYSACCHARIDE NANOFIBERS WITH VARIABLE COMPLIANCE FOR DIRECTING CELL FATE

(Partially published with Mui Hoon Nai, Chwee Teck Lim, Catherine Le Visage, Jerry K.Y. Chan, and Sing Yian Chew. J Biomed Mater Res Part A 2014, Article in Press)

SUMMARY

Cells perceive their microenvironment through physical and mechanical cues, such as extracellular matrix (ECM) topography or stiffness. In this study, we developed a polysaccharide scaffold that can provide combined substrate topography and matrix compliance signals to direct cell fate. Pullulan/dextran (P/D) nanofibers were fabricated with variable stiffness by in-situ crosslinking during electrospinning. By varying the chemical crosslinking content between 10, 12, 14 and 16%, (denoted as STMP10, STMP12, STMP14 and STMP16 respectively), scaffold mechanical stiffness was altered. We characterized substrate stiffness by various methods. Under hydrated conditions, atomic force microscopy (AFM) and tensile tests of bulk scaffolds were conducted. Under dry conditions, tensile tests of scaffolds and single nanofibers were examined. In addition, we evaluated the efficacy of the scaffolds in directing stem cell

differentiation. Using human first trimester mesenchymal stem cells (fMSCs) cultured on STMP14 P/D scaffolds (*Young's* modulus: 7.84 kPa) in serum-free neuronal differentiation medium exhibited greatest extent of differentiation. Cells showed morphological changes and significantly higher expression of motor neuron markers. Further analyses by western blotting also revealed the enhanced expression of ChAT on STMP14 (7.84 kPa) and STMP16 (11.08 kPa) samples as compared to STMP12 (7.19 kPa). Taken together, this study demonstrates that the stiffness of P/D nanofibers can be altered by differential *in situ* crosslinking during electrospinning and suggests the feasibility of using such polysaccharide nanofibers in supporting fMSC neuronal commitment.

4.1. INTRODUCTION

Cells respond to signals from their microenvironment. While the responses of cells to biochemical signals have been well characterized, the effects of physical cues on cell fate remains less understood [24]. Extracellular matrix (ECM) compliance and architecture play important roles in influencing stem cell differentiation and function [23, 24, 34]. However, most studies to date have focused mainly on mechanical [34, 60] and topographical [40, 43] properties separately. Therefore, a deeper understanding of stem cell differentiation in response to substrate topographical and mechanical properties may be an attractive strategy to improve the differentiation efficiency.

Here, we developed pullulan/dextran (P/D) scaffolds that can provide combined substrate topography and matrix compliance signals to direct stem cell differentiation. We analyzed the potential of nanofiber scaffolds with variable compliance in directing mesenchymal stem cells (MSCs) neuronal differentiation. Motor neuron degeneration may lead to muscle atrophy and death. However, there is currently no effective treatment for these devastating diseases [256-258]. In attempt to treat motor neuron degeneration, stem cell-derived motor neurons have been introduced as potential cell sources for cell therapy and high throughput drug screens [259-261]. While human embryonic stem cells and induced pluripotent stem cells have been reported to

differentiate into motor neurons in the presence of retinoic acid (RA) and sonic hedgehog (SHH) [262-264], concerns with potential teratoma formation remain. On the other hand, MSCs have shown potential for neuronal differentiation, and has not been encumbered by risks associated with the use of pluripotent stem cell types. In addition, recent studies have indicated the capacity of MSCs to adopt “astrocyte like” and “neuronal like” cell fates [223, 265], along with the potential of using MSCs to treat various diseases in the central nervous system and spinal cord injuries in animal models [266, 267]. Moreover, MSCs can be induced to differentiate into specific lineages depending on ECM stiffness [34]. Therefore, a deeper understanding on motor neuron differentiation in response to substrate topographical and mechanical properties may be attractive towards improving the differentiation efficiency of these stem cells.

In this study, human first trimester MSCs (fetal MSCs, fMSCs) were cultured on pullulan/dextran (P/D) scaffolds of varying stiffness and induced to differentiate into motor neuron lineage in the presence of RA and SHH. Fetal MSCs obtained from human first trimester fetal blood have longer telomeres and greater telomerase activity as compared to adult MSCs [268]. They are highly proliferative with greater plasticity than adult MSCs, which is an advantage for regenerative cell therapy [268, 269]. Pullulan and dextran are naturally derived polysaccharides. They have been widely investigated for

biomedical applications due to their biodegradability, hemocompatibility, and non-immunogenicity [270]. In our previous studies, we developed a P/D nanofibrous scaffold for cell culture [271]. The nanofibers were *in situ* cross-linked during electrospinning and the nanofibrous architecture remained intact with negligible mass loss for at least 28 days under hydrated conditions *in vitro*. Compared to P/D hydrogels, nanofibers significantly enhanced human dermal fibroblast attachment and proliferation. In this study, we hypothesized that the stiffness of P/D nanofibers can be altered by differential *in situ* crosslinking during electrospinning and such variation in nanofiber stiffness may play synergistic roles in directing fMSC neuronal commitment.

4.2. MATERIALS AND METHODS

4.2.1. Materials

All-trans retinoic acid (> 98%, RA, gradient grade for HPLC), ascorbic acid, bovine gelatin type B, bovine serum albumin (BSA), cyclic AMP (cAMP), dextran (*M_w* 500 000, Pharmacia), dimethyl sulfoxide (biotechnology performance certified, DMSO), dimethylformamide (DMF), 10% formalin, fluoromount™ aqueous mounting medium, heparin, methanol, RIPA buffer, sodium hydroxide (NaOH), Triton-X, Tween 20, rabbit anti-microtubule-associated protein 2 (MAP2) monoclonal antibody, goat

anti-rabbit IgG-FITC, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from Sigma Aldrich, Singapore. B27 supplement without vitamin A 50x, Dulbecco's modified eagle medium (DMEM, GlutaMAX™), Dulbecco's modified eagle medium/Nutrient mixture F-12 (DMEM/F12 GlutaMAX™), MEM non-essential amino acids solution (MEM NEAA), N2 supplement 100x, phosphate buffered saline (PBS, pH 7.4) were purchased from Gibco. Fetal bovine serum (FBS) was purchased from Hyclone. Recombinant human fibroblast growth factor 2 (FGF2) was purchased from R&D Systems. Recombinant human brain derived neurotrophic factor (BDNF), recombinant human glial cell-derived neurotrophic factor (GDNF), recombinant human insulin-like growth factor-binding protein 1 (IGF-1), and recombinant human sonic hedgehog (SHH) were purchased from Gene-ethics (Asia) Pte Ltd. Alexa Fluor® 555 goat anti-mouse antibody , Click-iT® EdU Alexa Fluor® 633 Imaging kit, DAPI stock solution (5 mg/ml) and Oregon green® 488 phalloidin were purchased from Invitrogen. RNeasy® Mini kit was purchased from Qiagen. Fast SYBR® Green Master Mix was purchased from Applied Biosystems by Life Technologies. Rabbit anti- β -Tubulin III (Tuj1) polyclonal antibody was purchased from Covance. Mouse anti-choline acetyltransferase (ChAT) monoclonal antibody, mouse anti-oligodendrocyte lineage transcription factor 2

(Olig2) monoclonal antibody, and mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody were purchased from Millipore. Goat anti-HB9 polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc.. Micro BCA protein assay kit was purchased from Thermo Scientific-Pierce. Amersham ECL Plus Western Blotting Detection System was purchased from GE Healthcare. 10x Tris/Glycine/SDS buffer, 10x Tris/Glycine buffer, extra thick blot paper, Immun-Blot[®] PVDF membrane were purchased from BioRad. 10x Tris Buffered Saline (TBS) and all primers were ordered from 1st base, Singapore. All materials were used as received without further purification unless otherwise noted.

4.2.2. Fabrication of pullulan/dextran scaffolds

Pullulan (20% w/w) and dextran (5% w/w) were dissolved in deionized water at room temperature. Thereafter, sodium trimetaphosphate (crosslinker, *SMTP*) was added into the polysaccharide solution at concentrations of 10, 12, 14 and 16 wt% (with respect to P/D, denoted as STMP10, STMP12, STMP14 and STMP16 respectively, Table 4.1) and vortexed for 4 hours. Four ml of 0.1% gelatin solution was then added into 12 ml of polysaccharide solution and mixed well. The polysaccharide solution was then mixed with DMF at a volume ratio of 10:1 (polysaccharide solution: DMF). Before electrospinning,

10 wt% NaOH aqueous solution was mixed with polysaccharide solution at 1:10 (NaOH: polysaccharide solution) volume ratio to provide an alkaline condition to activate cross-linking. The resulting solution was then charged at 18 kV and fed at a flow rate of 3-3.5 ml/h. All fibers were finally collected on the negatively charged rotating drum (-4 kV, 150 rpm) that was set at 10 cm away from the polymer supply. Following electrospinning, all scaffolds were dried at 37 °C for 3 days before rinsing with water to remove unreacted reagents and freeze-dried by lyophilisation.

In order to compare the effects of substrate topography on cellular behavior, P/D hydrogels with the same chemical content were prepared by mixing pullulan/dextran/gelatin (0.2/0.05/ 0.025 g in 1mL of DI water) solution with STMP and NaOH at room temperature. The resulting solution was poured into a 40 mm Petri dish and then incubated 6 h for crosslinking. Thereafter, all hydrogels were washed with sterile PBS for 2 days with a change of fresh PBS every 12 h. After preparation and equilibration in PBS, the hydrogels were cut to the same size as the fibrous scaffolds and sterilized under UV for 1 h on each side prior to cell culture.

Table 4.1. Characterization of pullulan/dextran fibers.

Sample	Fiber Φ (nm)	Swelling ratio (%)	Mean Pore Diameter (μm)
STMP10	409 $\pm$ 43	369 $\pm$ 26 ^{a, b}	1.83
STMP12	487 $\pm$ 31	338 $\pm$ 2	1.63
STMP14	477 $\pm$ 37	320 $\pm$ 12 ^a	2.39
STMP16	470 $\pm$ 33	319 $\pm$ 8 ^b	2.11

* The same letter indicates significant difference between the two samples ($p < 0.05$).

4.2.3. Characterization of pullulan/dextran scaffolds

4.2.3.1. Scaffold morphology

The morphology of the electrospun fibers was investigated using a scanning electron microscope (SEM, JEOL, JSM-6390LA) at an accelerating voltage of 10 kV after 60 seconds of gold coating. The fiber diameters were measured using Image J (NIH). Five images at 10,000 $\times$ magnification were used and at least 100 fibers were measured for each sample.

4.2.3.2. Scaffold swelling behavior

Three separately electrospun scaffolds (diameter 20 mm, average weight = 19.5 mg) were tested for each sample group. The weights of dried scaffolds were measured and denoted as M_d . Thereafter, all dried scaffolds were incubated in PBS at 37 °C for 4 hours. The PBS on the scaffolds was then removed with filter paper and the scaffolds were weighed in wet condition

(denoted as M_w). Scaffold swelling ratio was finally computed as $((M_w - M_d)/M_d) \times 100\%$.

4.2.3.3. Nanofibers scaffold stability

Dried scaffolds were incubated in PBS at 37 °C for 14 and 28 days. At each time point three scaffolds were retrieved and washed with deionized water. The scaffold morphology was then checked using the SEM or an environmental scanning electron microscope (ESEM).

4.2.3.4. Pore size distribution

One piece of electrospun scaffold (diameter 20 mm) was tested for each sample group. The pore size distribution of scaffolds was tested using a capillary flow porometer (Porous Media Inc., USA).

4.2.3.5. Measurement of mechanical properties

Tensile test of bulk scaffolds

Tensile tests were performed using a 5848 microtester (Instron, Canton, MA, USA) at a strain rate of 5 mm/min with a 20 mm gauge length. The P/D scaffolds were cut to a rectangular shape with dimension of $5 \times 40 \text{ mm}^2$. The thickness of each sample was measured by vernier caliper and determined by an

average of measurements at three locations. Three scaffolds were tested for each group.

To test the mechanical property of hydrated P/D scaffolds, samples with dimension of $5 \times 40 \text{ mm}^2$ were incubated in PBS (pH 7.4) for 12 hours. The width and thickness of each sample was measured by vernier caliper and determined by an average of measurements at three locations. The tests were performed using the same settings as indicated above. Finally the *Young's* moduli were calculated as the slope of the “linear” region of each recorded curve [272]. Three scaffolds were tested for each group.

Tensile test of single nanofibers

The tensile test on single nanofibers was performed according to our previous protocol with slight variations [273]. The fibers were first deposited onto a grounded aluminum frame. A single fiber was then selected and mounted onto a cardboard with the gauge length of 20 mm. After vacuum dried over night, the tensile test was performed using MTS Nanomechanical Testing System (Nano UTM™, MTS Systems Corporation). To ensure that the failure strain was 0.6-1.0 mm/mm, the strain rate was set as 0.001 mm/s. At least 25 samples were tested for each group.

Atomic Force Microscopy (AFM) measurements of bulk scaffolds

Stiffness measurements were performed using Nanowizard II BioAFM

(JPK instruments AG, Germany). The scaffold samples were mounted on a petri dish and immersed in the liquid media (PBS with 0.1% BSA) for half an hour prior to force measurements. Force measurements were performed in liquid media. The probe consisted of a 25 μm diameter polystyrene bead attached to a single beam cantilever. The spring constants of the cantilever used was determined by the thermal tune method [274] and was typically in the range of 0.03 ± 0.003 N/m. A maximum force of 5 nN and loading rate of 6 $\mu\text{m/s}$ were applied. A hundred force-distance curves in an area of $10 \mu\text{m} \times 10 \mu\text{m}$ and an average of 5 reference areas from each sample were used to estimate the modulus values. *Young's* modulus values were calculated for each recorded curve using JPK Data Processing Software (JPK instruments AG, Germany), which employs a Hertz's contact model for spherical indenters (diameter 25 μm ; Poisson's ratio 0.5) fitted to the extend curves [275]. At least three samples were tested for each group.

4.2.4. Human fetal mesenchymal stem cell culture and differentiation

P/D scaffolds (diameter 20 mm) were sterilized for 30 min on each side using UV-irradiation. Prior to cell seeding, all scaffolds were equilibrated in culture medium (DMEM with 10% FBS) at 37 °C for 1 h. Human fMSCs were derived from clinically-indicated termination of pregnancy under institutional

review board at NUH, Singapore. All women gave separate written consent to the use of fetal tissue for the derivation of human fMSCs. Fetal MSCs (passage 4-6) derived from human fetal bone marrow [276] were then seeded at 3×10^4 cells/well in 1 ml of culture medium. Fetal MSCs were induced to differentiate into motor neurons based on reported protocols [263, 264] with slight modifications. After 2 days, cells were incubated in DMEM/F12 containing 1% FBS and 20 ng/ml FGF2 for 1 day. Thereafter, cells were maintained in neural differentiation medium (489 ml of DMEM/F12, 5 ml of N2 supplement, 5 ml of MEM NEAA solution and 1ml of 1 mg/ml heparin, NDM) supplemented with 1 μ M of RA and 200 ng/ml SHH. The media was changed every 2 days. After an additional 7 days of culture (denoted as Day 7), the cells were induced in neural differentiation medium supplemented with BDNF, GDNF, IGF1 (each at 10 ng/ml), cAMP (1 mM) and ascorbic acid (200 ng/ml) for another 7 days (denoted as Day 14). RA and SHH concentrations were reduced to 0.5 μ M and 100 ng/ml, respectively. P/D hydrogels with the same chemical content were prepared according to previous protocols [277] in order to understand the effects of scaffold topography on fMSC response. Cells cultured on glass coverslips were used as 2D control. All growth factors and chemicals were prepared and stored according to published works [263].

4.2.5. Evaluation of cell viability and proliferation

At day 3, cell viability was assessed by using the Live/dead[®] viability assay kit according to the manufacturer's instructions. After briefly washed by PBS, the samples were incubated in Calcein AM and Ethidium homodimer-1 cocktail for 20 min. Samples were then briefly washed by PBS and examined under an Olympus IX71 microscope at 10× magnification.

At days 7 and 14, cell proliferation was assessed by EdU incorporation using the Click-iT[®] EdU Alexa Fluor[®] 633 HCS Assay according to the manufacturer's instructions. Cells were incubated in EdU solution (1:1000 dilution) for 24 h at 37 °C. The cells were then fixed in 10% formalin solution and permeabilized in 0.25% Triton-X for 20 min. Thereafter, the samples were incubated in Click-iT reaction cocktail and DAPI (1:1000 dilution) for 20 min. Samples were then examined using Zeiss LSM710 META confocal microscope at 20× magnification. The percentage of cells with EdU incorporation (shown as pink in fluorescent images) was finally measured using ImageJ. At least 500 cells were counted for each sample.

4.2.6. Evaluation of cell morphology

At days 7 and 14, cells were fixed in 10% formalin prior to permeabilization in 0.25% Triton-X for 20 min. Thereafter the samples were

washed three times with PBS and then incubated in phalloidin-Oregon green dye (1:250 dilution) and DAPI (1:1000 dilution) for 30 min. All steps were carried out at room temperature. Samples were then examined under a Zeiss LSM710 META confocal microscope at 63× magnification.

4.2.7. Real-time PCR analysis of gene expression

At days 7 and 14, RNA from 2 wells was isolated using Qiagen RNeasy kit and pooled together. The quality and quantity of the extracted RNA were evaluated by NanoDrop Reader (Thermo fisher scientific). Reverse transcription was performed using the reverse transcription kit (M-MLV Rnase H (-) Mutant) with random primers following manufacturer's protocol. Eight pairs of primers were tested and their sequences are listed in Table 4.2. Human GAPDH was used as the endogenous control. Real-time PCR was performed on an Applied Biosystems Real-Time PCR System (StepOnePlus 7500) with Fast SYBR Green MasterMix. The amplification efficiency of the genes of interest and the housekeeping gene were verified as similar. Therefore, the $\Delta\Delta C_t$ method was adopted for fold change analyses. The gene expressions were normalized with respect to the expression of undifferentiated fMSCs cultured on TCPS with proliferation medium (DMEM + 10% FBS). The entire experiment was repeated three times.

Table 4.2. PCR primers sequences.

Gene	Sequence
ChAT	F 5'-GAGGAGCAGTTCAGGAAGAGC C-3' R 5'-AGATGAGGCTGGCTGCAAACC-3'
GAPDH	F 5'-AGGGCTGCTTTAACTCTGGT-3' R 5'-CCCCACTTGATTTTGGAGGGA-3'
GFAP	F 5'-CACCGCAGCCCTGAAAGA-3' R 5'-GTTGCTGGACGCCATTGC-3'
HB9	F 5'-ACAACCTCCCGTACAGCAAT-3' R 5'-CTTCCGCCCTGGAGGCAA-3'
MAP2	F 5'-CTGCTTTACAGGGTAGCACAA-3' R 5'-TTGAGTATGGCAAACGGTCTG-3'
Tuj-1	F 5'-GCAACTACGTGGGCGACT-3' R 5'-CGAGGCACGTACTTGTGAGA-3'
Olig2	F 5'-ATGCACGACCTCAACATCGC-3' R 5'-CTCACCAGTCGCTTCATCTCCT-3'

4.2.8. Immunostaining and western blotting of neural markers

At day 14, cells were fixed in 10% formalin followed by permeabilization as stated above. Thereafter, samples were blocked with 5%FBS + 1%BSA at 4°C overnight. The samples were then incubated with primary antibodies at 37 °C for 1 hour. Thereafter, the samples were incubated in either FITC goat anti-rabbit antibody (1:200), or Alexa-Fluor555 goat anti-mouse antibody (1:1000) for 30 min at 37 °C. All samples were washed three times with PBS in between each step. DAPI was used to stain cell nuclei at 1:1000 dilution. Primary antibodies used for immunostaining were rabbit anti-β-Tubulin III (Tuj1) polyclonal antibody (1:1000 dilution), rabbit anti-MAP2 monoclonal antibody (1:1000 dilution), mouse anti-GFAP

monoclonal antibody (1:500 dilution), and mouse anti-ChAT (1:200 dilution). All samples were then examined under a Zeiss LSM710 META confocal microscope at 63× magnification.

At day 14, cells were lysed by RIPA buffer supplemented with protease inhibitor. The protein concentrations were measured by Micro BCA Protein Assay Kit following manufacturer's protocol. Ten µg of cell lysates were separated by 10% SDS-PAGE and transferred to a PVDF membrane (BioRad Trans-Blot® SD semi-dry transfer cell). The membrane was blocked with TBST (1× TBS with 0.1% Tween-20) containing 5% skim milk at room temperature for 1 hour. The membrane was then incubated with primary antibody at 4 °C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Signals were detected with Amersham ECL Plus Western Blotting Detection System and Kodak x-ray film. The densitometry protein quantification was measured by ImageJ and normalized with respect to the expression of undifferentiated fMSCs cultured on TCPS with proliferation medium (DMEM + 10% FBS).

4.2.9. Statistical analyses

Data is presented as mean ± standard deviation (Std.). Analysis of

variance (ANOVA) and *Tukey* post-hoc tests were used to compare more than two groups when variances were equal. Otherwise, *Kruskal-Wallis* test was used. $p < 0.05$ was considered statistically significant, and $p < 0.01$ was considered statistically highly significant.

4.3. RESULTS

4.3.1. Characterization of pullulan/dextran scaffolds

As indicated in Figure 4.1a, bead-free and uniform P/D nanofibers were obtained for all samples. The fiber diameters ranged from 400 to 500 nm (Table 4.1). In addition, as STMP content increased from 10 to 16 wt%, a decreasing trend in swelling ratio was observed (Table 4.1). Meanwhile, scaffolds with low STMP levels (STMP10 and STMP12) possessed smaller pore sizes than high STMP levels (STMP14 and STMP16), which was likely due to the swelling of the fibers during measurements. After 14 days incubation in PBS, all scaffolds maintained their fibrous structures under hydrated conditions (Figure 4.1b, ESEM image).

Figure 4.1. (a) Representative SEM image of P/D scaffolds, (b) ESEM image of wet P/D scaffolds. (c) The schematic of mechanism for P/D crosslinking. Scale bars: 10 μm .

Figure 4.2 presents the mechanical properties of electrospun P/D scaffolds under various mechanical test conditions. Figures 4.2a and c show the representative stress-strain responses of P/D scaffolds under tensile testing. In general, the stiffness of P/D scaffolds increased with higher concentrations of STMP (except STMP10). However, the hydrated scaffolds showed lower *Young's* moduli (Figure 4.2b vs. 4.2d) with larger strain values (Figures 4.2a vs. 4.2c). Meanwhile, tensile tests of dry single nanofibers (Figure 4.2e and f)

showed a same trend as the bulk scaffolds. However, consistent with literature [273], the *Young's* moduli of single fibers were found to be 400 - 600 times larger than bulk scaffolds. To further understand the local stiffness of the scaffolds, the *Young's* modulus was also obtained by AFM testing. The mechanical stiffness of the hydrated scaffolds measured using AFM showed a similar trend as the results from tensile tests. The *Young's* modulus changes from 7.7 ± 4.6 to 7.2 ± 4.6 , 7.8 ± 4.7 and 11.1 ± 6.4 kPa for STMP10, STMP12, STMP14 and STMP16 respectively (Figure 4.2g). Taken together, in order to observe a general trend of motor neuron commitment with respect to increasing nanofiber stiffness, all subsequent analyses were focused on STMP12, 14 and 16.

4.3.2. Effects of substrate on fMSC viability and proliferation

The cell viability was evaluated by LIVE/DEAD[®] assay with P/D hydrogels and glass coverslips as 2D controls (Figure 4.3). On Day 3, fMSC attached and proliferated well on P/D fibrous scaffolds. In contrast, few cells remained on P/D hydrogels. This was likely due to poor cell attachment on P/D hydrogels which eventually led to poorly attached cells being washed off during the staining process. Taken together, the results implied that nanofiber topography had a positive effect over fMSCs attachment and proliferation.

Figure 4.2. (a) Representative stress-strain curves of dry scaffolds from tensile tests. (b) *Young's* modulus of dry P/D scaffolds with different STMP content (n=3, mean $\pm$ Std.). (c) Representative stress-strain curves of mechanical tensile test of hydrated scaffolds. (d) *Young's* modulus of hydrated P/D scaffolds with different STMP content (n=3, mean $\pm$ Std.). (e) Representative stress-strain curves of dry single electrospun nanofiber. (f) *Young's* modulus of dry single nanofibers with different STMP content. (STMP10, n=23; STMP12, n=27; STMP14 n=28; STMP16 n=25, mean $\pm$ Std.). (g) *Young's* modulus of hydrated P/D scaffolds with different STMP content from AFM measurements. Mean $\pm$ Std., n=3, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA or Kruskal-Wallis test.

The extent of cell proliferation as evaluated by EdU incorporation assay was similar amongst cells on P/D scaffolds, regardless of STMP concentration (Figure 4.4). In general, nanofibers decreased cell proliferation as compared to glass coverslips. Additionally, EdU incorporation rate was decreased by day 14 on all substrates, which is likely due to confluence and fMSC differentiation.

Figure 4.3. Live/dead[®] assay of fMSCs differentiated on glass coverslips and P/D scaffolds for 3 days.

Figure 4.4. a) EdU (bright) incorporation by undifferentiated human fMSCs, and cells on the glass coverslip and P/D scaffolds after 7 days and 14 days differentiation. b) Percentage of EdU positive cells after 7 days and 14 days differentiation. $n > 200$, mean $\pm$ Std., ** $p < 0.01$, indicates significant difference with undifferentiated fMSCs, one-way ANOVA.

4.3.3. Effects of substrate topographical and mechanical properties on fMSC differentiation to motor neuron-like cells

As shown in Figure 4.5a, undifferentiated fMSCs exhibited a flattened

and fibroblast-like morphology. After 7 days differentiation, most of the cells on glass coverslips exhibited elongated cell shape. A few cells on glass coverslips even adopted a neuron-like structure with long thin processes when exposed to neural differentiation medium for 14 days (Figure 4.5a, with arrow). However, no cell survived on P/D hydrogels. The presence of actin stress fibers revealed good cell attachment on P/D nanofiber scaffolds 14 days post differentiation (Figure 4.6).

To monitor the differentiation of fMSCs, realtime-PCR was adopted to evaluate gene expression changes. After 14 days of differentiation, the mRNA level of neuronal markers, Tuj-1, MAP2 and motor neuron related markers, Olig2, HB9 and ChAT [263, 264] were significantly increased in all groups ($p < 0.05$) when compared with undifferentiated fMSCs, and cells cultured on glass coverslips (Figure 4.5b). Specifically, MAP2 and ChAT mRNA expressions were enhanced by 5-6.5 folds on STMP14 and 16 scaffolds, as compared to undifferentiated fMSCs. Among the P/D scaffolds, STMP14 and STMP16 showed higher MAP2 and ChAT expressions, although it was not statistically significant. Meanwhile, the expression of astrocyte marker, GFAP, was kept at a basal level.

Figure 4.5. (a) Bright field images of undifferentiated human fMSCs, and cells on the glass coverslips after 7 days and 14 days differentiation. (b) Gene expression of human fMSCs differentiated on glass coverslips and P/D scaffolds after 14 days as evaluated by real-time PCR. The expressions of Tuj-1, MAP2, GFAP, Olig2, HB9 and ChAT were normalized to the gene expression of undifferentiated human fMSCs cultured on glass coverslip with GAPDH as the housekeeping gene. $n=6$, mean $\pm$ Std., # indicates significant difference as compared to undifferentiated fMSCs, * $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test.

Protein expression was also detected by immunostaining and western blotting. As shown in Figure 4.7a, the expressions of MAP2 and ChAT were enhanced after 14 days of differentiation, as compared to glass coverslips. In particular, cells on STMP14 and 16 showed more prominent staining of MAP2 and ChAT as compared to STMP12. The enhanced neuronal commitment on STMP 14 and 16 scaffolds (vs. STMP12) was further supported by increased

expression levels of MAP2 and ChAT as revealed by western blotting (Figure 4.7b and Figure 4.8). Meanwhile, astrocyte marker, GFAP and oligodendrocyte marker, O4 were only weakly expressed at day 14 (Figure 5a). This indicated that the fMSCs were likely not undergoing astrocyte or oligodendrocyte lineage differentiation.

Figure 4.6. Cytoskeleton staining of undifferentiated human fMSCs, and cells on the glass coverslip and P/D scaffolds after 7 days and 14 days differentiation.

4.4. DISCUSSION

Stem cell behaviors can be regulated by many microenvironmental signals. While recent studies have separately highlighted the importance of substrate topography and compliance in regulating cell fate [24, 34, 278], analysis on the synergistic effects of mechanical and topographical properties of the ECM remains limited. Accordingly, we hypothesized that the stiffness of

P/D nanofibers can be altered by crosslink degree during the electrospinning process, and that such nanofibers could provide combined effects of substrate topography and matrix compliance signals to regulate fMSC differentiation.

Figure 4.7. (a) Immunofluorescent staining of human fMSCs differentiated on glass coverslips and P/D scaffolds after 14 days. (b) Western blotting of human fMSCs differentiated on P/D scaffolds after 14 days. Motor neuron marker (ChAT) was analyzed. Beta-actin was used as housekeeping control. (c) Densitometry protein quantification of ChAT. The results were normalized to the expression of fMSCs cultured on STMP12 scaffolds with Beta-actin as the housekeeping control.

Figure 4.8. Western blotting of human fMSCs differentiated on glass coverslips and pullulan scaffolds after 14 days.

In this study, glass coverslips was used as 2D control instead of TCPS. TCPS is commonly used 2D control. However, we need to know the substrate topographical and mechanical properties of 2D control as compared with P/D

scaffolds. Some studies reported that the stiffness of glass coverslips is over 600 kPa with smooth surface and use it as a control group to study the effect of substrate stiffness on cell behavior. Moreover, cells on glass coverslips were easier to operate for subsequent testing, such as EdU staining and immunostaining.

The P/D fibers possessed similar average fiber diameters, which helped eliminate fiber size effect on cellular response. Scaffolds prepared with different STMP concentrations possessed different mechanical properties. To stabilize the electrospinning process and obtain similar average fiber diameters, the STMP content could only be varied between 10 to 16%. Our previous study also demonstrated the retention of scaffold fibrous morphology and negligible weight loss in all samples after 28 days incubation in PBS [271]. To characterize the mechanical behavior of P/D scaffolds, tensile testing of bulk scaffolds and single nanofibers, and AFM were used. The local stiffness of the scaffolds was obtained from AFM measurements while the bulk properties were obtained from tensile tests.

In the tensile test of bulk scaffolds, the *Young's* modulus of hydrated scaffolds changed from 1.9 ± 0.2 to 1.3 ± 0.1 , 2.5 ± 0.5 and 2.8 ± 0.7 kPa, as STMP level increased from 10% to 12%, 14% and 16% respectively. This range of stiffness is comparable to electrospun collagen fibers and

decellularised porcine urinary bladder [272, 279], which were effective in supporting stem cell attachment and growth. Bulk scaffold properties from tensile testing were most commonly reported in literature. However, tensile tests do not exactly reflect the local stiffness of scaffolds, which the cells will sense. Hence, characterization of single fibers and AFM were used to provide an estimation of extracellular microenvironment which directly influences cell behaviour [273]. AFM has become a powerful tool for measuring material properties in biological studies. The sensitivity of the AFM probes allows considerably precise stiffness measurements to be obtained, which would be relevant for cellular studies. Moreover, the AFM technique would yield consistent values for the scaffolds and most accurately describe the local stiffness that a cell would experience on a substrate [280, 281]. Even though the AFM technique may be susceptible to variations in pore and fiber diameters in electrospun scaffolds, it is still a good method for studying the local substrate properties at the micron length scale. Regardless of measurement techniques, it was observed that the stiffness of STMP12 was lower than STMP10 and 14. This is likely due to the fact that the mechanical behavior of P/D is dependent on the balance between the degree of crosslinking and charge repulsions between polysaccharide chains. Monolinked phosphorus contributes negatively charged species, which have a positive effect over swelling but counteracts

crosslinking [282, 283]. The increase in STMP content would induce an increase in crosslinking and an increase in overall charge repulsions, thereby affecting the mechanical behavior of P/D scaffolds. It is likely that charge repulsions dominated at low STMP levels (10% and 12%), such that the increase in STMP content to 12% decreased substrate stiffness. In contrast, at higher STMP levels (14 and 16%), the crosslinking effect became critical, thus leading to the increase in substrate stiffness.

P/D scaffolds provided mechanical cues with stiffness ranging from 7.71 to 11.08 kPa according to AFM measurements (corresponding to 1.32 to 2.76 kPa in tensile test of hydrated scaffolds). Despite the narrow range of stiffness variation (less than 1 order of magnitude), we believed that this range could offer a finer resolution to understand the roles of mechanical cues and topography on neuronal differentiation in fMSCs. This is because recent works have shown that slight mechanical variations in soft tissues can elicit drastic physiological responses. For example, neural cells like oligodendrocyte progenitor cells changed from migratory and proliferative phenotype in areas of intermediate stiffness (0.4-0.7 kPa) to more differentiated phenotype in response to 1 kPa stiffness [284]. In the case of angiogenesis, Mammoto *et al.* demonstrated significant difference in angiogenesis as hydrogel matrix stiffness changed from 150 to 4000 Pa (particularly from 700-900 Pa) [285].

Overall, besides the significant differences observed between cells on soft P/D nanofibers vs. stiff glass coverslips, only a general trend of increasing motor neuron commitment was observed as nanofiber stiffness increased. Under our test conditions, the effect of nanofiber matrix compliance appeared less prominent as compared to changes in substrate topography (nanofibers vs. hydrogels). It is possible that within the range of stiffness adopted in this study, substrate topography is the dominant factor in controlling cell fate. Therefore, future works should involve evaluations of nanofiber stiffness effects over a wider range of mechanical stiffness to confirm our observations.

MSCs have the potential to differentiate into cells with an oligodendrocyte or motor neuron-like phenotype [286, 287]. In this study, differentiation of fMSC into motor neuron-like cells was induced by neuron differentiation medium containing SHH and RA [263, 264]. After 7 days differentiation, most of the cells exhibited elongated cell shapes with significant up-regulation of motor neuron markers, such as, Olig2, HB9 and ChAT (Figure 4.5b, scaffolds vs. glass, $p < 0.05$). The results indicated that the nanofiber topography appeared to enhance the potential of MSCs to differentiate along the motor neuron lineage. Specifically, STMP14 and 16 (7.84 kPa and 11.08 kPa) scaffolds induced higher up-regulation of motor neuron markers as compared to STMP12 (7.19 kPa) (Figure 4.5b). Furthermore, a direct comparison between

P/D nanofibers samples revealed that ChAT protein expression was enhanced on STMP14 and 16 as compared to STMP12 (Figure 4.7c). These observations suggest that fMSCs were driven towards the motor neuron lineage. Further induction of fMSCs into mature motor neurons might be enhanced by prolonging the differentiation process. Additionally, gene transfer or knockdown might be used to enhance neuronal commitment of the cells [288, 289]. Overall, the above results suggested that the stiffness of electrospun P/D scaffolds can be altered by crosslink degree. Fetal MSCs cultured on STMP14 P/D scaffolds (*Young's* modulus: 7.84 kPa) in serum-free neuronal differentiation medium exhibited greatest extent of differentiation.

4.5. CONCLUSIONS

Substrate compliance has effects on influencing stem cell differentiation and function. In this study, we evaluated the efficacy of the scaffolds in combining topography and compliance signals to direct fMSCs differentiation. The stiffness of P/D scaffolds can be altered by varying crosslink degree during electrospinning. After 14 days of differentiation, the motor neuron related markers were up-regulated more significantly on nanofibers than on glass coverslips. This suggested that fMSCs on P/D scaffolds were driven towards the motor neuron lineage. Moreover, fMSCs cultured on STMP14 P/D scaffolds

(7.84 kPa) exhibited greatest extent of differentiation. These results highlight the combined effects of substrate topography and matrix compliance signals in enhancing the probability of fMSC motor neural commitment and may help us understand the influence of topography and matrix stiffness on fMSC differentiation.

CHAPTER FIVE:

NANOFIBROUS NERVE CONDUIT-ENHANCED PERIPHERAL NERVE REGENERATION

(Partially published with Ruifa Mi, Ahmet Hoke, and Sing Yian Chew. J Tissue Eng Regenerative Med 2014;8:377-85)

SUMMARY

Fiber structures represent a potential class of materials for the formation of synthetic nerve conduits due to their biomimicking architecture. Although the advantages of fibers in enhancing nerve regeneration have been demonstrated, *in vivo* evaluation of fiber size effect on nerve regeneration remains limited. In this study, we analyzed the effects of fiber diameter of electrospun conduits on peripheral nerve regeneration across a 15-mm critical defect gap in a rat sciatic nerve injury model. By using the electrospinning technique, fibrous conduits that comprised of aligned electrospun poly (ϵ -caprolactone) (PCL) microfibers (981 ± 83 nm, Microfiber) or nanofibers (251 ± 32 nm, Nanofiber) were obtained. At three months post implantation, axons regenerated across the defect gap in all animals that received fibrous conduits. In contrast, complete nerve regeneration was not observed in the control group that received empty, non-porous PCL film conduits (Film).

Nanofiber conduits resulted in significantly higher total number of myelinated axons and thicker myelin sheaths as compared to Microfiber and Film conduits. Retrograde labeling revealed a significant increase in number of regenerated dorsal root ganglion sensory neurons in the presence of Nanofiber conduits ($1.93 \pm 0.71 \times 10^3$ vs. $0.98 \pm 0.30 \times 10^3$ in Microfiber, $p < 0.01$). In addition, the CMAP amplitudes were higher and distal motor latency values were lower in the Nanofiber conduit group as compared to the Microfiber group. This study has demonstrated an impact of fiber size on peripheral nerve regeneration. Such information may provide useful insights for future nerve guide designs.

5.1. INTRODUCTION

Peripheral nerve damage is a common problem associated with traumatic injuries and most patients require reconstructive surgery. However, functional recovery across large-gap lesions is often sub-optimal, particularly when empty synthetic nerve conduits are implanted. Therefore, despite the well-documented drawbacks related with autologous nerve grafts [1], these implants remain the gold standard for peripheral nerve injury treatments. In an attempt to enhance the performance of synthetic conduits, pores [2-4] and lumen fillers [5-8] have been introduced as potential modifications. Pores in the range of 1- 20 μm can enhance nerve regeneration by promoting nutrient transport and blood vessel infiltration [2-4, 9, 10] while reducing fibrous tissue invasion [11]. Lumen fillers, on the other hand, provide contact guidance and enhanced surface area for cell attachment and growth [1, 12].

In this context, fiber structures represent a potential class of materials for synthetic nerve guides due to their biomimicking architecture and have been implemented either as fillers within the lumens of nerve conduits [5-8]; or as the sole component of nerve guides to form fibrous porous conduits [13-18]. Much of the earlier related works have focused on evaluating the functionality of microfilaments and microfibers (diameter $\sim 10 - 250 \mu\text{m}$) in enhancing peripheral nerve regeneration [6, 19]. With the development of new techniques

such as electrospinning, later works presented promising results involving fibers with diameters that are in the sub-micron to nanometer range [7, 8, 290]. Part of the motivation to decrease fiber size stems from the fact that the natural extracellular matrix (ECM) comprises of fibers in the nano-scale and that *in vitro* studies have suggested advantages of nano-size in promoting more physiologically relevant cellular behaviors [20]. Some researchers have used electrospun collagen nanofibrous scaffolds to repair centre nerve system injuries and evaluated the effect of fiber origination on nerve regeneration. However, *in vivo* analysis on fiber size effect and nerve regeneration remains poorly evaluated.

In this study, we analyzed the effects of fiber diameter on peripheral nerve regeneration across a critical defect gap in a rat sciatic nerve injury model. Using the electrospinning technique, fibrous conduits comprising of aligned micro- or nano-sized fibers were obtained. Such fibrous conduits offer the advantage of combining permeability for nutrient transport with contact guidance and high surface area for cell attachment and growth [12, 291-293]. The versatility of the electrospinning technique also permits future expansion to include biochemical signals to enhance nerve regeneration [8]. We hypothesize that fiber size effect would influence peripheral nerve regeneration. Such details would provide useful information for future nerve guide designs.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Poly (ϵ -caprolactone) (PCL, Mn 65,000), 2,2,2-trifluoroethanol (TFE) with purity 99.0%, chloroform, methanol, sulphuric acid, phenol, paraformaldehyde, glutaraldehyde, sucrose, toluidine blue and Fluoro-goldTM were purchased from Sigma Aldrich, USA. Teflon microtubings (outer diameter = 1.63 mm) were purchased from Scientific Commodities Inc.. Isoflurane was purchased from Atlantic Biomedical. Wound clips were purchased from Autoclips. 10–0 nylon monofilament and 6–0 silk filament were purchased from Surgical Specialties Corporation and Ethicon Inc. respectively. All materials were used as received without further purification.

5.2.2. Fabrication of Nerve Guide Conduits

PCL films were fabricated by subjecting 0.6 g of PCL polymer to a uniaxial compression load of 8×10^3 kg for 5 min at 65 °C. Following that, the films were cut into sheets of 2 cm $\times$ 0.6 cm, rolled around a Teflon microtubing and sealed with PCL-chloroform solution (15 wt%) to form Film conduits, which served as the controls (denoted as Film).

In order to fabricate electrospun fibrous nerve conduits that comprised

of aligned PCL micron-sized fibers within the inner lumen (denoted as Microfiber), a layer of randomly-oriented fibers was first electrospun as the underlying support. Briefly, 15 wt% of PCL was dissolved in a solvent mixture of chloroform and methanol at a volume ratio of 4:1 to form solution A. Thereafter, to obtain randomly-oriented fibers, 3 ml of solution A was dispensed at 1.5 ml/h and electrospun at a voltage of 8 kV onto a negatively charged rotating target (-2 kV, 500 rpm, 8 cm from polymer supply). Following that, the rotational speed of the target was increased to 2,200 rpm and an additional 1 ml of solution A was electrospun using the same parameters to obtain the layer of aligned microfibers. The resulting mesh was then cut into sheets of 2 cm $\times$ 0.6 cm and rolled around a Teflon microtubing to form nerve conduits. The axis of fiber alignment was orientated parallel to the longitudinal direction of the microtubing. In order to seal the conduits, 0.3 ml of solution A was electrospun uniformly over the rolled conduits using the same parameters as stated above.

In order to fabricate electrospun fibrous nerve conduits that comprised of aligned PCL nano-sized fibers within the lumen (denoted as Nanofibers), the rotating target was first precoated with a layer of randomly-oriented PCL microfibers using 2.5 ml of solution A and the electrospinning parameters stated above. This approach ensured that nerve conduits with similar thickness as Microfiber conduits were obtained using a reasonable amount of time and

polymer. In order to electrospin aligned nanofibers, 16 wt% of PCL was dissolved in TFE. Thereafter, PBS was added into the resulting solution at 1:5 (PBS:TFE) volume ratio to form solution B. Following that, 1.5 ml of solution B was dispensed at 0.8 ml/h and electrospun at a voltage of 16 kV onto the microfiber-coated rotating target (-3 kV, 2,900 rpm). Finally, the sheets of PCL fibers were cut, rolled and sealed by electrospinning according to the method described above. All PCL Film, Microfiber and Nanofiber conduits were cut to a length of 16 mm for *in vivo* experiments.

5.2.3. Characterization of Nerve Conduits

5.2.3.1. Morphology Observation

In order to ensure a clean sectioning of the nerve conduits, the samples were frozen in liquid nitrogen and cut along the cross-section using a surgical blade. Thereafter, the samples were dried overnight under vacuum prior to 90 seconds of gold coating and investigation under the scanning electron microscope (SEM, JSM-6390LA, JEOL Ltd., Japan, accelerating voltage of 15 kV). The fiber diameters were measured using the Image J software (Version 1.41q). Ten images at 10,000× magnification were used and at least 100 fibers were measured for each sample.

5.2.3.2. Porosity Measurement

The apparent porosity of Microfiber and Nanofiber constructs was calculated based on the following equations, where the bulk density of PCL = 1.145 g/cm³:

$$\begin{aligned} \text{Apparent density of scaffold } \left(\frac{g}{cm^3}\right) \\ = \frac{\text{mass of scaffold (g)}}{\text{scaffold thickness(cm)} \times \text{scaffold area (cm}^2\text{)}} \times 100\% \end{aligned}$$

$$\begin{aligned} \text{Apparent porosity (\%)} \\ = \left(1 - \frac{\text{apparent density of PCL scaffold } \left(\frac{g}{cm^3}\right)}{\text{bulk density of PCL } \left(\frac{g}{cm^3}\right)} \right) \times 100\% \end{aligned}$$

The pore size distribution of Microfiber and Nanofiber conduits were tested using a capillary flow porometer (Porous Media Inc., USA).

5.2.3.3. Mechanical Test

The compressive behavior of the conduits was investigated using an Instron5543 machine (Instron Pte. Ltd., USA). Conduits (length = 16 mm) were placed between two parallel plates, and a force was applied perpendicularly to the longitudinal axis of the conduits at a compression rate of 1mm/min. The compression load was recorded at 10%, 20%, 30%, 40%, and 50% deformation [294]. Five conduits were measured for each sample.

5.2.4. *In Vivo* Experiments: Surgical Procedure

All experiments and animal care procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Johns Hopkins University Animal Care and Use Committee. Twenty-six adult female Sprague–Dawley rats were deeply anesthetized using isoflurane (flow rate: 2 L/min) throughout the surgical procedure. Surgery was performed on the rat's left leg under aseptic conditions. For conduit implantation, the sciatic nerve was exposed by an incision in the mid-thigh, overlying muscles were separated by traction and 10 mm of the nerve was resected out to result in 15 mm defect gap. The nerve defects were then repaired with Film conduits (control, n = 9), Microfiber conduits (n = 8), or Nanofiber conduits (n = 9). All nerve conduits (length = 16 mm) were filled with 10 μ l of PBS prior to implantation. Following that, the proximal and distal stumps were sutured to the conduit using a 10–0 nylon monofilament. For autograft implantation (n = 7), a 15 mm sciatic nerve was transected and reverse transposed into the gap. Both ends were then sutured using 10-0 strings. The wound was then closed using stainless steel wound clips. The animals were kept in temperature (28°C) and humidity (45%) controlled rooms with 12 h light cycles and allowed access to food and water.

5.2.5. Electrophysiology—Motor-Evoked Responses

At 3 months after surgery, motor-evoked responses were used to evaluate electrophysiological recovery prior to sacrificing the rats. After anesthetization under isoflurane (flow rate 2 L/min), the compound motor action potential (CMAP) recordings in the tibial nerve-innervated intrinsic foot muscles were recorded with a pair of recording needle electrodes [188]. The stimulating electrodes were placed at the sciatic notch near the sciatic nerve, while the recording electrodes were placed in the distal foot muscles to record the CMAP values. CMAP readings from the left and right sciatic nerves were recorded for each rat.

5.2.6. Retrograde Labeling With Fluorogold

At the end of three months, 3 wt% of Fluorogold (FG) was prepared for retrograde labeling. 1 μ l of Fluorogold was directly injected at 4-5mm away from the crushed distal tibial nerve after electrophysiological recordings were completed. After allowing 2 days for retrograde transport, the animals were deeply anesthetized, perfused with saline and then 4 wt% paraformaldehyde and the L3-S2 segments of the spinal cord and L4 and L5 dorsal root ganglia (DRGs) were harvested. Tissues were further post-fixed with 4 wt%

paraformaldehyde overnight and then transferred to 30% sucrose solution for storage until sectioning [295]. Serial cross-sections (50 μm) of DRGs and longitudinal sections (50 μm) of the spinal cords were obtained by cryostat sectioning. Thereafter, the sections were viewed under an Olympus IX71 microscope (Olympus Pte. Ltd., Japan). The total number of FG-labeled cells in each sample was counted in every alternate section.

5.2.7. Morphometric Evaluation

Three months post-implantation, after the animals were perfused, all nerve conduits were retrieved and the segment at 5 - 8 mm from the proximal end was cut out for morphological evaluation. The samples were fixed in a solution of 4 wt% paraformaldehyde and 3 wt% glutaraldehyde for 2 days, followed by soaking into Sorrensons phosphate buffer (0.2M) for further processing. After mounting in embedding resin, the samples were sectioned using an Ultracut E microtome (Reichert Inc., USA) at 1 μm thickness and stained with 1% toluidine blue for light microscopy. Samples were then examined under an Olympus IX71 microscope at 400 $\times$ magnification. The total number of myelinated axons per cross section of each regenerated nerve was quantified from consecutive non-overlapping images using ImageJ. The G ratio, is the ratio of axon circumference to myelin circumference, which is defined as

the ratio of dividing the diameter of the axon (D1) by the diameter of the entire myelinated fiber (D2), (D1/D2). It was evaluated from randomly selected fields of each sciatic nerve cross-section at 1,000× magnification using ImageJ. For each sample, at least 200 myelinated axons were measured.

5.2.8. Statistical Analyses

Data is presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were conducted using unpaired student t-test and one-way ANOVA followed by *Tukey* or *Games-Howell* post-hoc tests where appropriate. $p < 0.05$ was considered statistically significant, and $p < 0.01$ was considered statistically highly significant.

5.3. RESULTS

5.3.1. Characterization of Nerve Guide Conduits

Electrospun nerve conduits with an average inner diameter of 1.7 – 1.8 mm were fabricated. The average diameter of electrospun fibers that were deposited on the inner lumen of the conduits, pore size and apparent porosity of the conduits are summarized in Table 5.1. Nanofiber conduits possessed smaller fiber diameter and pore size as compared to Microfiber conduits. However, the apparent porosity was similar between both samples. As shown in Figure 5.1a,

Microfiber and Nanofiber conduits comprised of longitudinally aligned fibers and the average conduit wall thickness was $206 \pm 37 \mu\text{m}$. As indicated in Figures 5.1b and c, the inner lumen of the Microfiber and Nanofiber conduits comprised of aligned micron- and nano-sized electrospun fibers respectively. In contrast, Film conduits comprised of generally smooth and non-porous surfaces as shown in Figure 5.1d. Figure 5.1e illustrates the mechanical behavior of the conduits under compression. PCL Film conduits presented better pressure resistance ($15.69 \pm 1.69 \text{ N}$, 50% deformation, $p < 0.01$). In contrast, electrospun conduits appeared less resistant towards compression ($1.97 \pm 0.14 \text{ N}$ vs. $1.37 \pm 0.23 \text{ N}$, at 50% deformation for Nanofiber and Microfiber conduits respectively).

Table 5.1. Characterization of Microfiber and Nanofiber nerve conduits

Sample	PCL concentration (w/w) %	Solvent	Fiber Φ (nm)	Apparent porosity (%)	Pore radius, R (μm)
Microfiber	15	4:1 (chloroform: methanol)	981 ± 83	71.14 ± 1.42	1.33
Nanofiber	16	5:1 (TFE: PBS)	251 ± 32	70.53 ± 0.86	0.28

5.3.2. Morphometric evaluation

At three months post-implantation, all rats that received electrospun nerve conduits demonstrated nerve regeneration across the critical defect gap. In contrast, nerve regeneration did not occur across the lesion gap in all animals

that received the Film conduit.

Figure 5.1. SEM images of PCL nerve conduits. (a) Cross-sectional view of fibrous nerve conduit, (b) aligned microfibers (average diameter, $\phi = 0.98 \pm 0.08 \mu\text{m}$) within luminal surface of Microfiber nerve conduits, (c) aligned nanofibers ($\Phi = 251 \pm 32 \text{ nm}$) within luminal surface of Nanofiber nerve conduits and (d) inner surface of Film conduits. (e) Compressive behavior of nerve guide conduits. PCL Film conduits presented better pressure resistance at 50% deformation, compared to electrospun conduits. **: $p < 0.01$, one-way ANOVA, mean $\pm$ S.E.M..

As observed from the cross-sections of the regenerated nerves (Figures 5.2a and b), a portion of electrospun conduits (6 of 8 in Microfiber group, and 8 of 9 in Nanofiber group) ended up as spiral structures, with cells penetrating at the region where the tubes were sealed. The spiral structures were likely due to the compression of nerve conduits *in vivo*. Microfiber conduits allowed cell penetration through the walls of the conduit (Figure 5.2b). In contrast, the layer of nanofibers within the inner surface of Nanofiber conduits prevented cells that may have penetrated through the walls of the conduits from contacting the regenerated nerve (Figure 5.2d).

Figure 5.2. Light micrographs revealing nerve cross-sections within (a, b) Microfiber and (c, d) Nanofiber nerve conduits at 3 months post-implantation. (b, d) Higher magnification images of tissue locations highlighted in (a) and (c) respectively. (b) illustrates cell penetration into microfiber walls and (d) demonstrates lack of cellular penetration through the layer of nanofibers. White Arrow: layer of nanofibers.

Figure 5.3. (a to c) Light micrographs and (d, e) morphometric analysis of the cross-sections of regenerated sciatic nerves at 5–8 mm from the proximal end within (a) Film, (b) Microfiber and (c) Nanofiber conduits. (d) Total number of myelinated axons and (e) G ratio, defined as the ratio of diameter of the axon (D1) to the diameter of the entire myelinated fiber (D2), (D1/D2). Dashed circle: regenerated blood vessel. *: $p < 0.05$, **: $p < 0.01$, one-way ANOVA, mean $\pm$ S.E.M..

5.3.3. Retrograde Labeling With Fluorogold

No labeled neuron was detected in the DRGs and spinal cords of rats that received Film conduits. On the other hand, a significantly higher number of regenerated DRG sensory neurons were detected in rats that received Nanofiber conduits at 3 months post-implantation ($p < 0.01$ vs Microfiber, Figure 5.4a). No significant difference in the number of regenerated spinal motor neurons was observed between the electrospun conduits.

Figure 5.4. (a) Fluorescent micrographs of retrograde axonal tracing with fluorogold. Scale bar = 100 μm . (b) Counting of fluorogold labeled cells in every alternate section of DRGs and spinal cords. All DRGs and spinal cords were sectioned completely at 50 μm thickness. **: $p < 0.01$, two-tailed unpaired t-test, mean $\pm$ S.E.M..

5.3.4. Electrophysiological Assay of Nerve Functional Recovery

At three months post-implantation, no functional recovery was observed in rats that received Film conduits. However, all rats and six out of eight rats showed partial functional recovery when Nanofiber and Microfiber nerve conduits were used respectively. The CMAP amplitude and latency values are shown in Figure 5.5. Nanofiber conduits appeared to promote better functional recovery with significantly higher CMAP amplitude than Microfiber samples (0.326 ± 0.095 mV vs. 0.059 ± 0.016 mV, $p < 0.01$). The corresponding distal motor latency values also appeared to be lower than Microfiber nerve conduits. However, as compared to autografts, functional recovery through electrospun conduits remained inferior.

Figure 5.5. (a) CMAP amplitude and (b) CMAP latency. Film: $n = 9$, Microfiber: $n = 8$, Nanofiber: $n = 9$, Autograft: $n = 7$. **: $p < 0.01$, two-tailed unpaired t-test, mean $\pm$ S.E.M..

5.4. DISCUSSION

Peripheral nerve regeneration across critical defect gaps bridged by empty synthetic nerve conduits is often poor, due in part to the inadequate formation of the extracellular matrix during early stages of recovery and the lack of neurotrophic factors to enhance nerve regeneration [296, 297]. Consequently, fibrous nerve conduits have been introduced to provide contact guidance and enhanced surface area for cell attachment and growth; and to facilitate nutrient transport at the injury site [13-18]. In general, the presence of

fibers within the lumen of nerve conduits enhanced nerve regeneration [6, 8, 17, 19]. Although several *in vitro* studies have advocated the advantages of nanosized features in promoting more physiologically relevant cellular phenotypes as compared to larger feature sizes [20], *in vivo* outcomes in response to feature size variation remain poorly evaluated. In this study, we attempted to elucidate fiber size effect on peripheral nerve regeneration. Such understanding would provide relevant information for future nerve guide designs.

Electrospun fibrous conduits comprising of longitudinally aligned micron- or nano-sized fibers were fabricated in this study. Corresponding to the change in fiber diameter, a difference in average pore radius within the constructs was observed. However, the pore sizes remained sufficiently large to allow diffusion transport of nutrients and other molecules [298, 299]. Therefore, combined with the similar apparent porosities, the degree of nutrient transport through the Microfiber and Nanofiber scaffolds was anticipated to be similar. Besides the porosity and permeability, compressive resistance of the conduits is also critical to prevent *in vivo* nerve compression. In this study, the *in vitro* compressive behavior of Microfiber and Nanofiber conduits appeared similar to polyurethane–collagen conduits [11, 300]. However, *in vivo* tissue compression occurred, resulting in the formation of spiral conduit structures at three months

post implantation (Figure 5.2). Increasing the wall thickness of the electrospun conduits may help prevent future tissue compression problems as demonstrated by Zhu *et al.* [17].

Fiber alignment within the lumen of the conduits was introduced in attempt to provide topographical signals to promote Schwann cell maturation [301] and contact guidance for cell migration and axonal outgrowth during nerve regeneration [7, 17]. The supporting layer of micron-sized random fibers was incorporated to impart isotropic mechanical properties to the conduits for sufficient suture strength. Consequently, despite the small average pore radius, a limited degree of cellular penetration through the microfiber conduit walls was observed at 3 months post-implantation. Compared to our previous observations where randomly oriented nanofibers completely abrogated cell infiltration into electrospun scaffolds after 4 weeks of sub-cutaneous implantation [238], the results in this study suggested that given sufficient time, cellular penetration can occur, particularly through scaffolds comprising of micron-sized fibers. These observations also agreed with previous studies where pore sizes of less than 1 μm prevented cell infiltration and microfiber scaffolds were better for cellular infiltration than nanofiber substrates due to the corresponding decrease in pore sizes of the constructs [298, 302]. Correspondingly, we also observed a lack of cellular penetration through the

layer of aligned nanofibers within Nanofiber conduits.

Approximately $6.8 \pm 2.8 \times 10^3$ myelinated axons were found in the nerve cross-sections that were bridged with Nanofiber conduits at 3 months post implantation. This was significantly higher than Microfiber and Film conduits ($\sim 3.2 \pm 1.8 \times 10^3$ and $\sim 1.5 \pm 0.3 \times 10^3$ respectively). The ideal G-ratio for normal nerve conduction is ~ 0.7 [303] and it ranges about 0.6-0.7 in normal uninjured nerves [304]. The significant decrease in G-ratio observed with the introduction of a porous nanofiber conduit indicated the formation of larger nerve fibers with thicker myelin, and is an indication of better maturation of myelinated axons. Moreover, the G-ratio of Nanofiber group was comparable to Autograft group, which also indicated the advantage of nanofibers in supporting peripheral nerve regeneration. Compared with fibrous conduits with random fibers on the lumen surface [13], aligned fibers resulted in larger cross-sectional areas of regenerated sciatic nerves (Figure 5a, $p < 0.01$). Meanwhile, the myelinated axon diameter in Nanofiber conduits was also comparable to autografts and fiber conduits that were fabricated by a one-step electrospinning process [17] (Figure 5b). Even it remained inferior to autograft, the results still suggested the advantages of aligned nanofibers in supporting nerve regeneration.

Figure 5.6. (a) Cross-sectional areas of regenerated sciatic nerves at 5–8 mm from the proximal end within Film, Microfiber and Nanofiber conduits and (b) myelinated axon diameter in Film, Microfiber and Nanofiber conduits at 3 months. *: $p < 0.05$, **: $p < 0.01$, one-way ANOVA, mean $\pm$ S.E.M..

Similar to the morphometric analyses, the fiber size effect was also observed in the number of Fluorogold labeled DRG sensory neurons. The regeneration of sensory neurons typically precedes motor neurons [305]. Consequently, we only saw the significant enhancement in number of labeled DRG neurons at 3 months post implantation in the presence of nanofibers. The CMAP measurements were conducted by measuring the muscle action potential at the most distal foot muscles after sciatic nerve stimulation. This was adopted as a stringent test to examine the efficacy of the electrospun nerve conduits. The Film group showed bundles of axons in morphometric analysis but without any response in electrophysiological assay. This illustrated the lack of nerve regeneration across the lesion gap in all animals that received Film conduits. Whilst retrograde tracing revealed similar numbers of motor neurons between

the Microfiber and Nanofiber groups, the difference in CMAP latency was significant. This discrepancy may be due to the fact that even though there may be equal number of axons that regenerated, the Nanofiber group possessed axons that were more mature with better myelination and therefore demonstrated shorter distal motor latency. As compared to previous studies involving the bridging of shorter defect gaps with electrospun conduits [290], the fact that functional recovery was observed in our experimental samples across a critical defect gap demonstrated the promise of the scaffolds. As compared to autografts, functional recovery in electrospun fibrous conduits remained inferior at 3 months post implantation. One possible reason may be the lack of neurotrophic factors within the synthetic conduits. The incorporation of neurotrophic factors or stem cells within Nanofiber conduits may enhance nerve regeneration and will be evaluated in future studies.

Altogether, the results suggested that as compared to Microfiber conduits, Nanofiber conduits more effectively supported neurite outgrowth, and enabled the reinnervation of distal fiber tracts by regenerating axons. It is possible that as compared to microfibers, nanofiber scaffolds presented a larger surface area for cell attachment [292], while limiting cellular penetration into the site of injury and decreased fibrous tissue formation. While fiber size effect on nerve regeneration was clearly observed, the exact mechanisms involved

remains to be elucidated. Comparatively, Wang *et al.* [306] demonstrated *in vitro* that nanofibers (293 ± 65 nm) decreased S100⁺ cell migration distance and neurite extension lengths as compared to aligned microfibers (1325 ± 383 nm). It is possible that the difference in experimental time points (5 days *in vitro* vs 3 months *in vivo*) contributed to the differences between our studies and that cell migration speed may be critical only during the initial phase of recovery. Future experiments involving earlier time points would shed light in this area. Furthermore, the fabrication of electrospun fiber tubes using the one-step process introduced recently by Zhu *et al.* [290] may also be adopted in future studies to help preserve the original tubular configuration of Nanofiber nerve conduits.

5.5. CONCLUSIONS

Electrospun fibrous nerve guide conduits with longitudinally aligned fibers within the luminal surface enhanced peripheral nerve regeneration across a 15 mm critical defect gap in the rat sciatic nerve injury model. Compared to Microfiber conduits, enhanced nerve regeneration and functional recovery were observed in Nanofiber conduits. Our results demonstrated fiber size effect on nerve regeneration. Such information may provide useful insights to future scaffold designs for enhancing peripheral nerve regeneration.

CHAPTER SIX:

CONCLUSIONS AND FUTURE PERSPECTIVES

6.1. CONCLUDING REMARKS

Electrospun fibrous scaffolds serve as ideal biomimetic scaffolds for tissue engineering due to their unique physical properties that closely resemble that of the ECM. While advances in the field of electrospinning have been rapid, the full potential of the technique in tissue engineering remains to be utilized and evaluated using improved processing methods and in-depth assessment of cellular interactions with electrospun scaffolds.

In order to optimize the artificial environment, various materials have been explored. The synthetic polymers have the advantage in that their specific properties such as degradation time can be tailored by altering their molecular weights or compositions. On the other hand, biopolymeric materials, such as polysaccharides, are appealing since they closely recapitulate the biochemical nature of the extracellular matrix (ECM) and often elicit improved cell/substrate interactions. Additionally, nanofibers hold great potential as tissue engineered scaffolds since they closely mimic the architecture of native ECM. Derived from the versatile electrospinning technique, fiber orientation and size may be modulated to provide contact guidance to seeded cells. Furthermore, it is easy

to biofunctionalize electrospun nanofibers through the incorporation of drugs for a sustained delivery of biochemical signals to direct cell fate.

In this thesis, we aimed to develop biomimetic scaffolds capable of delivering topographical and biochemical signals to enhance tissue regeneration via electrospinning; and to understand the phenotypic changes of cells interacting with such biofunctional scaffolds. First, to evaluate the effect of substrate topography on directing stem cell fate, MSCs were cultured on TCPS and electrospun PCL nanofibers in neuronal differentiation medium. Compared with TCPS, nanofiber topography, especially aligned nanofibers, significantly up-regulated the expressions of neural markers. Moreover, to demonstrate the combined effects of nanofiber topography and sustained drug release on stem cell fate, a scaffold-based drug delivery system was developed by encapsulating RA within aligned PCL nanofibers. Analysis of the RA release profile revealed a sustained released of RA for at least 14 days (~ 60 % released). As indicated by MSCs differentiation, scaffold-based controlled delivery of RA enhanced MAP2 and RIP expressions as compared to bolus delivery despite lower amounts of drug (> 8 times lower). It is possible that the RA-encapsulated fibers provided a local concentration of RA to allow more efficient cellular uptake. Furthermore, more significant changes in cell morphology and enhancement in neural markers expressions were observed when combined

with sustained drug availability. These results suggested that the biomimicking drug-encapsulated scaffolds may permit subsequent direct cell transplantation and provide guidance cues to control the fate of endogenously recruited stem cells.

Meanwhile, we also developed a polysaccharide scaffold to investigate the synergistic effect of variation in nanofiber stiffness and topography in directing fMSCs neuronal commitment. P/D nanofibers were fabricated with variable stiffness by in-situ crosslinking during electrospinning. Under AFM testing, the Young's modulus changes from 7.19 to 11.08 kPa, which indicated that the stiffness of electrospun P/D scaffolds can be altered by crosslink degree. Physical studies revealed that all scaffolds maintained their fibrous structures for at least 14 days. Following cellular studies showed that fMSCs cultured on STMP14 P/D scaffolds (7.84 kPa) exhibited greatest extent of differentiation. These results highlight the combined effects of substrate topography and matrix compliance signals in enhancing the probability of fMSC motor neural commitment and may help us understand the influence of topography and matrix stiffness on fMSC differentiation.

Finally, we evaluated the feasibility of implanting the electrospun scaffolds *in vivo* and demonstrated the effects of substrate topography on nerve regeneration *in vivo*. Electrospun fibrous nerve guide conduits with

longitudinally aligned microfibers or nanofibers within the luminal surface were used peripheral nerve regeneration. Compared to two-dimensional Film conduits, both Microfibers and Nanofibers conduits demonstrated better nerve regeneration and functional recovery, especially Nanofibers conduits. These results demonstrated fiber size effect on nerve regeneration. Together with the feasibility of biomimicking drug-encapsulated scaffolds to direct stem cell behaviors, our works may provide useful insights to future bio-functionalized scaffold designs for enhancing peripheral nerve regeneration.

6.2. FUTURE DIRECTIONS

The biofunctional scaffolds fabricated by electrospinning showed promising efficacy on directing MSCs neural commitment and enhancing nerve regeneration *in vivo*. However, the ultimate goal of this study is to understand stem cell differentiation in response to substrate topographical and mechanical properties and develop biomimetic scaffolds capable of delivering topographical and biochemical signals to enhance nerve regeneration. Therefore, to optimize the design of fibrous scaffolds for tissue engineering, deep understanding of the knowledge on basic cell biology involving fibrous scaffolds is required.

In our works, topographical signaling affects cell elongation and nuclei

distortion through cell adhesion and actin-intermediate filament system and may correlate with changes in gene and protein expression and stem cell differentiation [40, 247, 248]. Cells perceive their microenvironment not only through soluble signals but also through physical and mechanical cues. By mechanotransduction systems, cells translate these stimuli into biochemical signals controlling multiple aspects of cell behavior. To deeper understand the exact mechanisms responsible for the substrate topographical and mechanical properties mediated cellular behaviors and how mechanical forces are transduced into biochemical signals, the role of the actin cytoskeleton and the activity and expression of transcription factors and chromatin remodeling enzymes directly involved in gene expression may be investigated, such as focal adhesion, RhoA-ROCK pathway and YAP/TAZ pathway.

Second, the P/D nanofibers could provide combined effects of substrate topography and matrix compliance signals to regulate fMSC differentiation, which has effects on analysis the synergistic effects of mechanical and topographical properties of the substrate compliance. Under our test conditions, the effect of nanofiber matrix compliance appeared less prominent as compared to changes in substrate topography (nanofibers vs. hydrogels). It is possible that within the range of stiffness adopted in this study, substrate topography is the dominant factor in controlling cell fate. Recent studies showed MSCs

differentiated into neural, myogenic or osteogenic phenotypes depending on the elastic moduli of substrate [34, 307]. Therefore, future works should involve evaluations of nanofiber stiffness effects over a wider range (0.1 – 40 kPa) of mechanical stiffness to confirm our observations.

Lastly, the exact effect of the drug-incorporated electrospun nanofibrous scaffolds on peripheral nerve regeneration should be elucidated. As discussed in previous Chapter, aligned nanofiber topography significantly up-regulated the expressions of neural markers. Sustained drug availability induced more significant changes in cell morphology and enhancement in neural markers expressions. Furthermore, electrospun fibrous nerve guide conduits with longitudinally aligned nanofibers demonstrated better nerve regeneration and functional recovery. It would be of great interest to fabricate biochemical signal-incorporated nanofibrous nerve conduit, such as NGF, GDNF and siRNA, and evaluate their effects on nerve regeneration *in vivo*.

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2. 9th Biomaterials world congress, 2012
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5. International Conference on Cellular & Molecular Bioengineering (*ICCMB2*), 2010

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