Three-dimensional scaffold platform for controlled therapeutic drug and non-viral gene delivery to enhance nerve regeneration and remyelination in spinal cord injuries

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**THREE-DIMENSIONAL SCAFFOLD PLATFORM
FOR CONTROLLED THERAPEUTIC DRUG AND
NON-VIRAL GENE DELIVERY TO ENHANCE
NERVE REGENERATION AND REMYELINATION
IN SPINAL CORD INJURIES**

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Doctor of Philosophy

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Abstract

Spinal cord injuries (SCI) are followed by a complex series of events that contribute to the failure of regeneration. To date, there is no repeatable treatment that can restore the injury-induced loss of function. Since damaged spinal axons do not regenerate in their native inhibitory microenvironment, a combined application of biomaterials and neurotrophic factors that induce nerve regeneration emerges as an attractive treatment for SCI. Moreover, damage of myelin membranes, that ensheath axons, and failure of remyelination after nerve injury can disrupt neural signals, leading to nerve degeneration. In this study, a scaffold-mediated RNA interference approach using nanofiber scaffolds incorporated with microRNAs (miRs) is proposed to direct and enhance nerve regeneration and remyelination after SCI.

We report the novel use of sparsely distributed aligned nanofibers to provide contact guidance for regrowth of axons *in vivo*. The scaffold comprises of aligned poly (ϵ -caprolactone-co-ethyl ethylene phosphate) (PCLEEP) nanofibers that are supported within a collagen hydrogel. To demonstrate the efficacy of our PCLEEP-collagen hybrid scaffold in achieving effective local drug/gene delivery *in vivo*, we incorporated neurotrophin-3 (NT-3) as the model protein and miR-222 as the model miR. The scaffolds were implanted into a hemi-incision injury, which was created at level C5 in the rat spinal cord. The results showed that our biofunctionalized scaffolding platform effectively provided bio-mimicking contact guidance and allowed the controlled delivery of various drugs and therapeutic biomolecules of drastically different nature and molecular weights. After 3 months of implantation, the results showed that the

nanofiber scaffold elicited the longest attainable neurite length of $1039.53 \pm 264.99 \mu\text{m}$, which was significantly longer as compared to the untreated group ($371.78 \pm 194.83 \mu\text{m}$, $p \leq 0.01$) and the isotropic hydrogel-treated group ($287.42 \pm 100.11 \mu\text{m}$, $p \leq 0.001$). Importantly, the regenerated axons and blood vessels formed along the direction of the aligned nanofibers, regardless of their orientation. Moreover, the presence of the nanofiber scaffolds did not affect tissue scarring and inflammatory reaction. Taken together, these findings demonstrate that our aligned nanofibers-hydrogel scaffold is a promising bio-functional platform for nerve injury treatment.

On the other hand, promoting remyelination after SCI is challenging since oligodendrocyte precursor cells (OPCs) often fail to differentiate and mature into oligodendrocytes (OLs), the myelin-forming cells of the CNS. We have previously demonstrated that miR-219 and miR-338 (miR-219/miR-338), when coupled with fiber topography, enhanced OPC differentiation and maturation *in vitro*. Therefore, our vision for future study is to incorporate miR-219/miR-338 into our scaffold platform to promote OPC maturation and differentiation, hence enhancing remyelination for SCI treatment. However, SCI is a complicated process that involves a vast number of cell types beside OPCs such as microglia and astrocytes. Non-specific uptake of miR-219/miR-338 by these cells may cause undesired side effects that potentially interfere with the regeneration process of the nervous system. Therefore, we also examined the effect of miR-219/miR-338 on microglia and astrocytes *in vitro*. In addition, we studied the influence of astrocytes on OPC maturation and

differentiation under the presence of these miRs in the injury mimicking condition *in vitro*.

Chapter 1. Introduction

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1.1. Statement of problems

The importance of neural tissue engineering has been recognized for decades due to the limited regeneration capacity of nerve tissues after injuries, especially within the CNS. Unlike the peripheral nervous system (PNS), where damaged axons can regenerate depending on the extent of the injuries, damaged CNS axons often do not regenerate in their native environment ^{1,2}. This poor regeneration is an outcome of the existence of a hostile microenvironment that is formed by a complex series of events after nerve injuries ³. This series of events has been studied comprehensively and reviewed in many articles ¹⁻³. Briefly, in the CNS, injuries trigger the activation of astrocytes. In particular, reactive astrocytes are recruited to the site of injury and form a barrier, termed the glial scar, to cover the lesion. Besides protecting the adjacent tissues from further damage, the glial scar also

physically blocks regenerating axons from entering the lesion, thereby inhibiting the regeneration of neuronal connections ². Reactive astrocytes also produce inhibitory molecules, such as chondroitin sulfate proteoglycans, which further prevent neuronal growth ². In addition, myelin of the CNS contains potent growth inhibitory molecules, such as Nogo, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein, which also deter neurite outgrowth ⁴⁻⁶. Therefore, an important approach in neural tissue engineering is to manipulate and neutralize the local environment to make it more permissive for nerve regeneration ^{1,3}. However, recreation of the growth-promoting environment after nerve injuries remains challenging.

Currently, neural tissue engineering focuses on biomaterial designs such as nerve grafts and polymeric scaffolds, or on biochemical therapy using cells and neurotrophic factors ^{1,3}. The introduction of biomaterial scaffolds into the injury sites is to bridge the injury gap and support neo-tissue formation. Hydrogels ⁷⁻¹⁷ and self-assembled peptide nanofibers ^{18,19} are often used as they are biocompatible with the host tissue and mimic the mechanical properties of the spinal cord. In addition, they can be synthesized in large quantities. However, the isotropic architecture of these materials cannot direct cellular and neuronal regeneration, resulting in limited axonal reconnections with the host tissue ^{13,20-22}. Hence, scaffolds with topography, such as microchannels, microfilaments, and conduits have been used to provide physical cues. Results to date show that these scaffolds could indeed direct axonal regeneration ²³⁻²⁵. However, the formation of thick fibrous scars around these scaffolds partially hindered the growth of the regenerated axons beyond

the lesion site to repenetrate the host spinal cords ^{23,24}. In addition, cell penetration into these scaffolds was also limited ²³⁻²⁵. On the other hand, direct administration of therapeutic drugs at the lesion site did not effectively treat SCI due to their rapid clearance from our bodies ²⁶⁻²⁹. As a result, utilization of a single strategy, either physical or biochemical therapy, is not always effective. We envision that their synergistic combination could better mimic the complex milieu of signals in the nerve tissue, thereby resulting in improved recovery. Yet challenges remain to implement this treatment clinically. Alternative treatments for nerve injuries are, therefore, subjects of further investigation.

OLs are the myelin-forming cells of the CNS ^{30,31}. The importance of myelination is highlighted by the presence of a broad range of neurological diseases following myelin failure or loss, such as leukodystrophies and multiple sclerosis ^{30,32}. After demyelination, axons can be spontaneously remyelinated by new mature OLs to restore the myelin sheaths ^{31,33}. During adulthood, these new mature OLs can be generated via the differentiation process of OPCs ³⁴. Thus, promoting OPC maturation, differentiation, and myelination is one of the approaches to enhance nerve regeneration in the CNS.

Recent studies demonstrated that the cellular responses triggered after nerve injuries are tightly regulated by various signaling pathways involving miRs ³⁵. Since their discovery, miRs together with their biogenesis and functions have been extensively studied and documented ^{36,37}. Specifically,

miRs do not encode proteins but function as post-transcriptional regulators to down-regulate protein expression by preventing translation or triggering mRNA degradation^{36,37}. As miRs are often only partially complementary to their target mRNAs, a single miR can concurrently regulate a broad network of related genes, giving rise to robust and widespread chains of responses. Hence, RNA interference using miRs is a promising method to repair the injured nervous system. Specifically, functional miRs have been shown to be required for OPC maturation, differentiation, and myelination in the mammalian CNS.

1.2. Hypothesis

In this study, we hypothesize that the combination of substrate topography and gene silencing therapy can direct cell fate to enhance tissue regeneration after SCI. In particular, aligned nanofibers can provide contact guiding cues to direct neurite regrowth along the longitudinal axis of the spinal cord. As compared to microstructures, nanofibers more closely mimic the size scale and architecture of the natural extracellular matrix. Correspondingly, these implants improved host-implant integration with reduced scar tissue formation^{25,38}. Importantly, the versatility of the scaffold fabrication process allows the incorporation of a wide variety of drugs ranging from proteins to low molecular weight lipophilic drugs and nucleic acids to further facilitate axonal regrowth and remyelination.

1.3. Specific aims

In order to test our hypothesis, we propose to pursue the following specific aims:

1. Fabricate and characterize aligned nanofiber scaffolds that are biofunctionalized through the incorporation of growth factors and miRs
 - 1.1. Establish a robust protocol to fabricate nanofiber scaffolds
 - 1.2. Characterize fiber morphology, fiber diameter and distribution
 - 1.3. Evaluate degradation rate of the scaffolds
 - 1.4. Establish a robust protocol to incorporate growth factors and miRs into the scaffolds
 - 1.5. Characterize the loading efficiency, release profile, and distribution of the incorporated growth factors and miRs
2. Evaluate host-implant integration, cellular responses, and axonal regeneration after scaffold implantation *in vivo*
 - 2.1. Establish a repeatable SCI model and scaffold implantation
 - 2.2. Optimize scaffold dimensions for *in vivo* studies
 - 2.3. Evaluate host-implant integration, cellular responses, and nerve regeneration
 - 2.4. Establish reliable behavioral tests to evaluate functional recovery
 - 2.5. Evaluate the efficacy of the scaffolds in promoting functional recovery via behavioral tests
3. Evaluate cellular responses of the glial cells towards miRs *in vitro*
 - 3.1. Establish microglia, astrocyte, and OPC cell cultures, and astrocyte/OPC co-culture
 - 3.2. Optimize miR transfection protocol in these cultures

3.3. Evaluate the effects of miRs on these cells viability, morphology, activation, gene expression, and OPC maturation and differentiation

Chapter 2. Literature review

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2.1. Topographical effects of scaffold in nerve regeneration

One of the current strategies for SCI treatment is the use of biochemical signaling, either in the form of cell transplantation or bioactive factor delivery into the injuries. Pre-clinically, functional recovery has been observed in rodent SCI models through the transplantation of various adult and stem cell types³⁹⁻⁴⁵. Furthermore, the transplantation of olfactory ensheathing cells recently resulted in partial functional recovery in a single SCI patient. However, more success cases are required to mark the significance of this method⁴⁶. In the

case of bioactive factor delivery, various classes of molecules have been attempted, including antibodies ⁴⁷⁻⁵⁰, enzymes ⁵¹⁻⁵⁴, corticosteroids ⁵⁵⁻⁵⁷, and neurotrophic factors ⁵⁸⁻⁶⁰. Although promising results have been obtained, the biochemical approach cannot resolve the issue of cyst formation after SCI, which partially contributes to the inhibition of nerve regeneration. Importantly, the direct injection of cells and bioactive factors often result in low cell engraftment/survival ^{61,62} and the lack of sustained availability of bioactive factors ^{27,63,64} over prolonged duration that is necessary for successful axonal regrowth.

Another SCI repair strategy is the introduction of biomaterial scaffolds to bridge the injury gap and support neo-tissue formation. Currently, hydrogels ⁷⁻¹⁷ and self-assembled peptide nanofibers ^{18,19} are often used as they mimic the mechanical properties of the spinal cord. Although morphological regeneration was observed in these constructs, functional recovery remains disappointing ^{16,21,61,65-67}. One probable reason is the isotropic nature of these materials that cannot direct cellular and neuronal regeneration in the rostral-to-caudal direction ^{13,18,20-22,61,65}, resulting in relatively few axons ultimately forming reconnections with the host tissue ^{13,20-22}. Moreover, due to the fast degradation rate of these materials, the problem of cyst formation remains poorly addressed ^{67,68}.

To provide physical cues, scaffolds with topography, such as microchannels, microfilaments, and electrospun fiber meshes have been used. In general, guidance effect was seen for axonal regeneration ²³⁻²⁵. However,

these regenerated axons did not repenetrate the host spinal cord beyond the lesion site partially due to the presence of thick fibrous scar formation around the scaffolds ^{23,24}. In addition, there was limited cell penetration into the scaffolds ²³⁻²⁵ ultimately resulting in regenerated tissue structures that are far from the physiological architecture of the spinal cord.

The unique properties of nanofibers, such as high surface area and permitting easier addition of surface properties, make them ideal candidate for application in tissue engineering ⁶⁹. As compared to microstructures, nanofibers more closely mimic the size scale and architecture of the natural extracellular matrix and might provide signaling cues to the cells ^{69,70}. Correspondingly, these implants not only supported axon regeneration but also improved host-implant integration with reduced scar tissue formation ^{25,38,71}. Liu *et al.*, in particular, reported extensive cellular penetration and axon ingrowth into the nanofibrous collagen conduit at 10 days post-implantation ¹⁷. Furthermore, this scaffold also helped lessen the extent of glial scar formation ¹⁷. On the other hand, a study from Liu and coworkers demonstrated that neural stem cell-seeded PLGA-PEG nanofiber scaffold improved functional recovery and reduced cavity formation post-SCI ⁷¹. Furthermore, nanofibers can also act as drug reservoir and carrier to release various types of active compounds at the injury sites for nerve treatment ^{69,72}.

Here, we demonstrate the effects of aligned PCLEEP nanofibers in axonal regrowth, host-implant integration, and blood vessel formation after *in vivo* SCI. Importantly, to facilitate cell penetration throughout the nanofiber

construct, the scaffold was fabricated with sparsely distributed aligned nanofibers that were dispersed throughout a collagen hydrogel.

2.2. Biochemical therapy for nerve regeneration

NT-3 is known to promote neuronal survival, axonal sprouting and regeneration^{58,73-75}. In addition, it facilitates the proliferation and differentiation of oligodendrocyte precursors cells^{73,76,77}. On the other hand, miR-222 is enriched in axons and participates in controlling local protein synthesis at distal axons⁷⁸⁻⁸⁰. Such controlled local protein synthesis plays critical roles in allowing severed axons to undergo regeneration within hours after injuries, independent of protein transport from the cell soma of neurons^{78,81}. Unfortunately, the expression of miR-222 is often significantly altered after nerve injuries⁸²⁻⁸⁸. *In vitro*, when miR-222 was deliberately over-expressed in injured adult neurons, enhanced regrowth was observed^{82,83,89,90}. However, the use of miR-222 to enhance nerve regeneration after SCI has not been attempted. We speculate that this is largely due to the lack of effective non-viral methods to deliver miRs to neurons *in vivo*. Thus, in this study, we incorporated NT-3 and miR-222 into the PCLEEP-collagen hybrid scaffold to evaluate the feasibility of a single scaffold in imparting synergistic biochemical and topographical signals to enhance nerve regeneration after SCI.

2.3. RNA interference for axonal remyelination

OPCs are progenitor cells in the CNS that are capable of self-renewal and OL differentiation⁹¹. OLs produce myelin sheaths that wrap around axons to physically protect them, electrically insulate them, and induce a clustering of

sodium channels along them for saltatory nerve conduction to support high-speed transmission of nerve impulses over long distances ^{30,92}. As such, without myelination, the newly regenerated axons cannot function and die soon after that.

miR-219 and miR-338 are critical regulators of OL differentiation and myelination in the vertebrate CNS that share many common targets, including Sox6, Hes5, and ZFP238 ^{93,94}. The overexpression of each of these miRs alone in OPCs was sufficient to elicit OL differentiation even without the need of specific differentiation condition. In particular, the transfection of miR-219 mimics into mouse OPCs resulted in 2.1-fold more RIP⁺ OLs and 3.0-fold more MBP⁺ OLs at four days post transfection in OL growth medium ⁹³. Similarly, the overexpression of miR-338 in mouse OPCs led to 3.4-fold and 4.6-fold increases in the number of RIP⁺ and MBP⁺ OLs under the same culture conditions ⁹³. Moreover, the overexpression of these two miRs is effective to promote OL differentiation not only *in vitro* but also *in vivo*. Specifically, utero electroporation of miR-219-expressing vectors into the mouse-developing cortex at E14.5 led to 47% more Olig2⁺ OPCs and 25% more PDGFR α ⁺ OPCs in comparison with the negative plasmid control ⁹³. Similarly, utero electroporation of miR-338-expressing vectors increased the number of Olig2⁺ and PDGFR α ⁺ OPCs by 47% and 56% ⁹³. Hence, given the potency of miR-219 and miR-338 in OL differentiation under non-specific *in vitro* and *in vivo* conditions, treatment of these miRs would be useful to promote OPC maturation and myelination for nerve regeneration in the CNS.

2.4. miR delivery methods

Although miRNAs hold many potential applications in nerve regeneration and remyelination, practical considerations must be made in terms of their delivery. MiRs are labile molecules. Specifically, the ribose sugar group of RNA molecule is chemically reactive; thereby RNAs are unstable and more prone to degradation^{95,96}. Moreover, besides the instability nature of RNAs, cellular uptake and endosomal escape are two main barriers for efficient miR delivery. First, the negative charge of miR molecules prevents them from crossing biological membranes, hence deterring their cellular uptake^{97,98}. Second, after taken up by cells, miRs have to leave the endosome and enter the cytoplasm to avoid lysosomal degradation⁹⁷. Therefore, miR treatment for nerve regeneration requires effective delivery methods for transporting miRs into the cytoplasm of the targeted cells. Since each method has its own advantages and drawbacks, direct comparison remains elusive. Thus, this section will focus on the effectiveness of some frequently used methods in neural tissue engineering based on available works.

Due to the high transfection efficiency, viral transduction has frequently been the first choice for delivering miRs and studying their functions^{99,100 101 102 103}. Furthermore, viral transduction offers sustained expression of miRs in the transfected cells due to the integration of viral genome into host genome¹⁰⁴. Although such sustained expressions of miRs are desirable for basic cell biological studies, the genomic integration can lead to uncontrolled insertional mutagenesis^{104,105}, therefore, limiting the use of viral-mediated miR delivery in translational therapeutic applications.

As compared to viral transduction, non-viral delivery methods emerged with their own advantages, such as the lack of mutagenesis, low immunogenicity, and the capacity of delivering a large number of therapeutic agents. Hence, for translational applications, non-viral approaches are preferred. Electroporation is a classic method for gene delivery by permeabilizing cell membrane using electric forces. This method is appropriate for both transient and stable transfection¹⁰⁶. As compared to commercialized liposomes and synthesized cationic polymers, electroporation can transport miRNAs more efficiently into virtually any cell types, even hard-to-transfect cells like NPCs^{104,107}. However, there remains a significant trade-off between transfection efficiency and cell viability¹⁰⁶. The resulting damages on cellular membranes due to electroporation often leads to the imbalanced ion gradient across cellular membrane, hence causing non-specific materials transport and ultimately cell death¹⁰⁴.

Like electroporation, the use of lipid-based reagents results in transient transfection¹⁰⁸. The main advantages of this system are its biocompatibility, reproducibility, and ease of large-scale production¹⁰⁹. As such, many conventional reagents are available to date, for example Lipofectamine® 2000, Lipofectamine® RNAiMAX, siPORT™, HiPerFect, and siLentFect™. Despite their variation in structure, these reagents share some common features. In particular, they contain positively charged groups, which interact with the negatively charged sugar-phosphate backbone of RNA molecules⁹⁸. This interaction aids the contact between RNA/reagent complex and cell

membrane, thereby facilitating subsequent cellular uptake. However, the major shortcoming of lipid-based transfection reagents is their low transfection efficiency as compared to viral transduction and electroporation ¹⁰⁸. Another drawback of lipid-based transfection reagents is their potential cytotoxicity by lipofection. This cytotoxicity, accompanied by gene changes, could in turn hinder the desired outcome of gene-based therapy ¹¹⁰.

Cationic polymer is another class of non-viral vectors. In general, the positively charged amine groups in cationic polymers interact with the negatively charged phosphate groups of RNA molecules ^{105,111}. Like lipid-based reagents, transfection efficiency and toxicity of cationic polymer are major issues that must be considered for translational applications of cationic polymers ^{111,112}. Currently, polyethylenimine (PEI) is the most promising cationic polymer due to its high transfection efficiency ¹¹¹. Specifically, it effectively protects oligonucleotides from lysosomal degradation and delivers them into the cytoplasm ^{105,113}. Besides PEI, chitosan is also extensively studied for gene delivery since it is biocompatible and able to effectively bind and compact oligonucleotides ¹¹⁴. However, its transfection efficiency is generally limited in many cell types ¹⁰⁵.

Compared to the aforementioned methods, scaffold-mediated delivery is more advantageous for RNA interference in neural tissue engineering. The incorporation of RNAs within a scaffold protects these labile molecules from biodegradation. In addition, scaffold-mediated delivery allows the localized and sustained availability of gene silencing molecules, thus enhancing cellular

uptake and potentially minimizing systemic off-target side effects ^{115,116}. Currently, scaffold-mediated delivery of small non-coding RNAs remains at a nascent stage, with most reports being restricted to siRNA delivery. However, being chemically similar in nature (double stranded RNA molecules), the scaffold incorporation and delivery mechanisms are generally similar and applicable between siRNAs and miR mimics. In this regard, the encapsulation of siRNAs within PCLEEP nanofibers significantly enhanced the *in vitro* cellular uptake of gene silencing molecules into human dermal fibroblasts (HDFs) as compared to bolus delivery ¹¹⁷. In addition, a sustained release of these encapsulated siRNAs for up to one month *in vitro* was reported, which prolonged *in vitro* silencing of collagen type I (COL1A1) by at least 2-3 times as compared to conventional bolus delivery ¹¹⁷.

Besides acting as a reservoir for sustained delivery of gene silencing molecules, topographical features of scaffolds may also serve as additional physical signals to modulate cellular behavior and gene uptake and silencing efficiencies. Using MSC as a model stem cell type, cells cultured on aligned fiber scaffolds demonstrated increased gene knockdown efficiencies when RE-1 silencing transcription factor (REST) and green fluorescent protein (GFP) were targeted (1.6 and 1.5-folds increase respectively, as compared to random fibers ¹¹⁸. On the other hand, using HDF as a model adult somatic cell type, gene-silencing efficiency was found to be dependent on fiber diameter. Specifically, smaller nanofiber diameters promoted the silencing of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Collagen-I by 3.8

and 4.4-folds respectively as compared to scaffolds with an average diameter of 1.3 μm ¹¹⁸.

Apart from affecting gene-silencing extents, scaffold architecture also modulates cell phenotype. For example, fiber diameter affects NSPC differentiation ¹¹⁹ and oligodendrocyte myelination ¹²⁰. In terms of PNS myelination, fiber scaffolds, especially aligned fibers, promoted human Schwann cell maturation ¹²¹. Moreover, three-dimensional nature of nanofibers affected differentiation outcomes of both mouse NPCs and rat OPCs. Specifically, the silencing of REST together with the use of nanofibers significantly enhanced neuronal commitment of mouse NPCs by 32% as compared to 2D film ¹²². Meanwhile, higher proportion of OPCs was differentiated on nanofiber than on 2D conventional culture either with or without the presence of miR-219/miR-338 (3-fold versus 2-fold increase in RIP⁺ cells) ¹²³. Taken together, by providing mechanical support and developmental guidance for axonal regrowth, neotissue deposition ^{104,124}, sustained gene silencing and topographical features to direct cell fate, scaffold-mediated gene silencing approach is a promising strategy for RNA interference in neural tissue engineering.

Chapter 3. Three-dimensional nanofiber hybrid scaffold directs and enhances axonal regeneration after spinal cord injury

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3.1 Introduction

Spinal cord injuries (SCI) often result in the extensive loss of nerve connections. As damaged spinal axons do not spontaneously regenerate in their native inhibitory microenvironment, SCI lead to permanent functional impairment below the injury sites. Unfortunately, to date, there has not been any robust treatment that can promote tissue regeneration and restore the injury-induced loss of function to prevent life-long disability^{125,126}.

One of the current strategies for SCI repair is the introduction of biomaterial scaffolds to bridge the injury gap and support neo-tissue formation. Currently, hydrogels⁷⁻¹⁷ and self-assembled peptide nanofibers^{68,127} are often used as they mimic the mechanical properties of the spinal cord. However, the isotropic architecture of these materials cannot direct cellular and neuronal regeneration in the rostral-to-caudal direction^{13,21,22,24,61,65,127}, resulting in relatively few axons ultimately forming reconnections with the host tissue

^{13,21,22,24}. Moreover, due to the fast degradation rate of these materials, the problem of cyst formation remains poorly addressed ^{67,68}.

To provide physical contact guiding cues, scaffolds with topography, such as microchannels, microfilaments, and conduits made of electrospun fiber meshes, have been used. In general, guidance effect was seen for axonal regeneration ^{13,24,25}. However, these regenerated axons did not re-penetrate the host spinal cord beyond the lesion site partially due to the formation of thick fibrous scars around the scaffolds ^{13,24}. In addition, there was limited cell penetration into the walls of the scaffolds ^{13,24,25}, ultimately resulting in regenerated tissue structures that are far from the physiological architecture of the spinal cord.

Given the limitations of previous scaffold designs, we hypothesized that a combination of hydrogel and aligned nanofibers would be favorable to SCI treatment. The hydrogel gel component provides a three-dimensional structure that mimics the mechanical environment of the spinal cord to facilitate cell penetration. More importantly, its three-dimensional isotropic architecture could be exploited to disperse aligned nanofibers throughout the hydrogel matrix to guide nerve regeneration along the longitudinal axis of the spinal cord. On the other hand, aligned electrospun fibers have been shown to promote axonal regeneration and improve functional recovery after injuries in the peripheral nervous system ¹²⁸⁻¹³⁰. In addition, as compared to the microchannels and microfibers that were used previously, nanofibers more closely mimic the size scale and architecture of the natural extracellular matrix.

Correspondingly, these constructs are expected to improve host-implant integration with reduced scar tissue formation^{25,38}.

In this study, we demonstrate the effects of a three-dimensional hydrogel-nanofiber hybrid scaffold on axonal regrowth, host-implant integration, and blood vessel formation after SCI (Fig. 3.1). Specifically, the scaffold was fabricated with sparsely distributed aligned PCLEEP nanofibers that were dispersed throughout a collagen hydrogel. PCLEEP, a biodegradable copolymer of ϵ -caprolactone (PCL) and ethyl ethylene phosphate (EEP), is an ideal material for sustained drug and protein delivery^{115,117,130-132}. This scaffold design is robust and easy to handle *in vivo*, thus allowing us to explore the effects of fiber orientation on axonal regrowth. Furthermore, neurotrophin-3 (NT-3) could be easily incorporated into the scaffold as an additional biochemical signal for nerve regeneration. NT-3 promotes neuronal survival, axonal sprouting, and regeneration^{58,73,74}. It also facilitates the proliferation and differentiation of OPCs^{73,76,77}. Thus, a combined treatment that comprises of scaffold topography and neurotrophic factor delivery would be promising in enhancing nerve regeneration after SCI.

Figure 3.1. Experimental set-up.

PCLEEP aligned nanofiber-collagen hydrogel hybrid scaffolds were implanted into rat spinal cords. WM, white matter; GM, grey matter

3.2 Materials and Methods

3.2.1 Materials

2, 2, 2-trifluoroethanol (TFE, $\geq 99.0\%$), bovine serum albumin (BSA, A9649), Triton X-100, sodium hydroxide (NaOH), NaCl, normal goat serum (NGS), rabbit anti-neurofilament 200 (NF, 1:700 dilution), and rabbit anti-laminin (LN, 1:200 dilution) were purchased from Sigma–Aldrich. Rat-tail Collagen type I (10.08 mg/ml, LOT 4041009) was purchased from Corning. Human NT-3 was purchased from Peprotech. DAPI (1:1000 dilution), 1x PBS (pH 7.4), and 10x PBS (pH 7.4) were purchased from Life Technologies. Paraformaldehyde (PFA), mouse anti-glial fibrillary acidic protein (GFAP, clone GA5, 1:500 dilution), and mouse anti-macrophages/monocytes (clone ED-1, 1:200 dilution) were purchased from Millipore. Alexa Fluor 488-conjugated Goat Anti-Mouse/Rabbit IgG (1:700 dilution) and Alexa Fluor 555-conjugated Goat Anti-Mouse/Rabbit IgG (1:1000 dilution) were purchased from Invitrogen. Tris-EDTA buffer (TE buffer, pH 8.0) was purchased from 1st BASE. Mouse anti-CD11b/c (anti-Ox42, 1:200 dilution) was purchased from Abcam. Mouse anti-Rat RECA-1 (clone HIS52, 1:800 dilution) was purchased from AbD Serotec.

3.2.2 Scaffold fabrication

3.2.2.1 Aligned PCLEEP fibers

The PCLEEP copolymer ($M_w = 40,509$, $M_n = 17,288$) was synthesized by previously reported methods^{133,134}. PCLEEP (22 % wt.%) was dissolved in TFE. One-hour prior to electrospinning, TE buffer was added to the polymer

solution at a 1:10 (TE: TFE) volume ratio to form the spinning solution. Sparsely distributed aligned PCLEEP fibers were fabricated using the two-pole air gap electrospinning technique¹³⁵. In brief, the spinning solution was loaded into a 1 ml syringe that was capped with a 21-gauge blunt-tipped needle. This solution was then charged at + 10 kV and released at a flow rate of 1.9 ml/h. The PCLEEP fibers were deposited within a 5.0 cm air gap area that was between two stationary collector poles (- 5.0 kV). Each set of fiber was obtained after 1.0 minute of spinning. These fiber bunches were then sterilized under UV light for 30 minutes.

3.2.2.2 *Aligned PCLEEP fibers in collagen hydrogel*

Rat-tail Collagen type I was used to fabricate hydrogel according to the manufacturer's protocol (BD Biosciences). Briefly, 10x PBS, collagen type I, 1.0 N NaOH, and de-ionized (DI) water were added into a sterile microtube and mixed properly to get a final collagen concentration of 6.0 mg/ml. The collagen mixture was kept on ice until used. 50 μ l of the mixture was then added into a sterilized cylinder mold of 6.0 mm in length and 3.0 mm in inner diameter to obtain isotropic hydrogel scaffolds. To obtain scaffolds with aligned nanofibers, the mold was pre-set with 4 sets of fibers in the core region prior to the addition of the collagen mixture. For NT-3-incorporation, 1.0 μ l of 10x PBS and 9.0 μ l of DI water in the collagen mixture was substituted by 10 μ l of 0.1 % BSA containing 5.0 μ g of human NT-3. The mixture was then added into the PCLEEP fiber-embedded mold. Hydrogel formation took place at 37 °C in the incubator for 45 minutes. After gelation, the scaffolds were rinsed with 1x and

0.1x PBS sequentially. Thereafter, the scaffolds were lyophilized and kept at -20 °C until used.

3.2.3 Scaffold characterization

3.2.3.1 *Fiber diameter*

The PCLEEP aligned fibers and scaffolds were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 120 s and 140 s, respectively. Thereafter, these samples were observed under the scanning electron microscope (SEM; JEOL, JSM-6390 LA) at an accelerating voltage of 10 kV. The fiber diameters were measured using ImageJ software (<http://imagej.nih.gov/ij/>) on SEM images. At least 100 fibers were measured.

3.2.3.2 *Fiber distribution*

Scaffolds were fixed with 10 % formalin for 45 minutes at room temperature. Thereafter, the scaffolds were rinsed with PBS and then placed at -80 °C overnight. These scaffolds were then cross-sectioned in liquid nitrogen. Subsequently, they were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 150 s and observed under the SEM at an accelerating voltage of 10 kV. The ratio of fiber area to scaffold area was measured using ImageJ software. Three scaffolds were used for this measurement.

For fiber density, 3 different cross-sectioned areas (50 μm x 50 μm) of a scaffold were imaged and the number of fibers in each area was counted using ImageJ software. Three scaffolds were used for this measurement.

3.2.4 Animals

Adult female Sprague Dawley rats (6 - 8 weeks, 200 - 250 g) were obtained from In Vivos Pte Ltd (Singapore). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC, NTU).

Animals were randomly divided into five groups. Specifically, the experimental groups comprised of scaffolds with aligned fibers that were implanted parallel (denoted as Parallel or NT-3 Parallel, where NT-3 was incorporated) or at an angle (Angled) to the longitudinal axis of the spinal cord. The control groups comprised of plain collagen hydrogels without aligned nanofibers (Gel) and SCI animals without any treatment (SCI). Table 3.1 summarizes the experimental groups and test conditions.

Time point	Group name	Description	Sample size
4 weeks	Parallel	Plain nanofiber-hydrogel scaffolds, with nanofibers aligned parallel to spinal cord longitudinal axis	3
12 weeks	Parallel	Plain nanofiber-hydrogel scaffolds, with nanofibers aligned parallel to spinal cord longitudinal axis	8
	Angled	Plain nanofiber-hydrogel scaffolds, with nanofibers aligned at an angle to spinal cord longitudinal axis	8
	NT-3	NT-3 incorporated nanofiber-hydrogel	4

	Parallel	scaffolds, with nanofibers aligned parallel to spinal cord longitudinal axis	
	Gel	Plain collagen hydrogel scaffolds	6
	SCI	Injured, untreated animals	6

Table 3.1. Experimental groups and test conditions.

3.2.5 Implantation Surgeries

Rats were injected with buprenorphine subcutaneously (0.05 mg/kg) 30 minutes before surgery. Right before surgery, they were anesthetized using a combination of ketamine (73 mg/kg) and xylazine (7.3 mg/kg). Thereafter, the surgical field was shaved and cleaned with Betadine. The skin was cut open and the muscle was cut, peeled back to the sides, and held in place. The C5 vertebra was located and its right side was broken open to gain access to the C5 spinal cord. A 1/3 incision was then made on the right side (Fig. 3.1 and 3.2A) using a pair of iridectomy scissors. Thereafter, scaffolds (1.0 mm in length) were implanted into the incision site such that the fibers were either parallel to the long axis of the spinal cord (Parallel and NT-3 Parallel groups, Fig. 3.2B, C), or at an angle (Angled group, Fig. 3.2D, E). For Gel group, a plain collagen hydrogel (1.0 mm in length) was implanted into the site of injury (Fig. 3.2F, G). For SCI group, the incision was left untreated. Thereafter, in all the groups, a layer of fat was used to cover the injury, the muscle was then sutured, and the skin was closed with auto clips. All animals were injected with buprenorphine subcutaneously (0.05 mg/kg) twice a day for 3 days post-operation. Subsequently, they were fed with acetaminophen (200 mg/kg) three times a day and meloxicam (5.0 mg/ml, 1.0 ml in 500 ml drinking water) continuously for the next 7 days. If forepaw biting was noticed, the rats were

fed with acetaminophen (200 mg/kg) three times a day for another 3 days and injected with Baytril (5.0 %, 0.1 ml/kg) subcutaneously once a day until the symptom ceased.

Figure 3.2. Injury model.

(A) 1/3 incision was performed at C5. For the scaffold-treated groups, 1mm scaffold was implanted into the injury site at the moment of the surgery. (B, D, F) Schematic representation of a horizontal section containing an implant with parallel fibers (B), angled fibers (D), and

isotropic hydrogel (F). (C, E, G) Bright-field images of the scaffold with aligned PCLEEP fibers (C), angled fibers (E), and isotropic hydrogel (G). X: left side of the body; Y: right side of the body. Rostral, left; caudal, right. WM, white matter; GM, grey matter.

3.2.6 Immunohistochemistry and Histology

At 4 weeks and 3 months post-operation, animals were perfused with 0.9 % saline followed by 4.0 % ice-cold PFA. After perfusion, spinal cords were post-fixed for 2 h at 4 °C and then sequentially transferred to 15 % sucrose and 30 % sucrose after 24 h and 48 h, respectively. All samples were stored at 4 °C until sectioned. Spinal cord samples of 1.5 cm containing the lesion site were sectioned horizontally on a cryostat set at 20 µm thickness. Immunohistochemistry and histology were performed to evaluate axonal regrowth, scar formation, host-implant reaction, and blood vessel reformation.

3.2.6.1. *Immunohistochemistry*

For immunohistochemistry, all the steps were done at room temperature. Spinal cord sections were treated with 0.3 % Triton X-100 for 5 minutes and then incubated in 10 % NGS blocking solution for 1 h at room temperature. Thereafter, the sections were incubated with primary antibodies diluted in 5.0 % NGS overnight followed by secondary antibodies diluted in PBS for 1.5 h. The following primary antibodies were used: rabbit anti-neurofilament 200 (NF, 1:700 dilution), mouse anti-glial fibrillary acidic protein (GFAP, clone GA5, 1:500 dilution), mouse anti-CD11b/c (anti-Ox42, 1:200 dilution), mouse anti-macrophages/monocytes (clone ED-1, 1: 200 dilution), rabbit anti-laminin (LN, 1:200 dilution), mouse anti-Rat RECA-1 (clone HIS52, 1:800 dilution). Secondary antibodies were Alexa Fluor 488-conjugated Goat Anti-Mouse/Rabbit IgG (1:700 dilution) and Alexa Fluor 555-conjugated Goat

Anti-Mouse/Rabbit IgG (1:1000 dilution). Nuclear staining was performed by incubating the sections with DAPI (1:1000 dilution) for 10 minutes after the secondary antibodies. Thereafter, all samples were examined using an Olympus IX71 inverted microscope and a Zeiss LSM710 META confocal microscope.

3.2.6.2. Histology

Masson's trichrome staining was performed at room temperature following the standard protocol (Sigma-Aldrich). All samples were then examined using an Olympus IX71 inverted microscope.

3.2.7 Quantification of cystic cavity size

Only samples that exhibited cystic cavity were evaluated (n = 5) (Table 2). The length and width of the cystic cavity were measured using the cellSens Dimension 1.11 software (Olympus). Each cavity had one measurement for length and 2 - 11 measurements for width depending on its extent.

Measurement	Sample size (animals)				
	SCI	Gel	Parallel	Angled	NT-3 Parallel
Cystic cavity	n = 5	-	-	-	-
Neurite ingrowth	n = 6	n = 6	n = 3 (4 weeks) n = 8	n = 8	n = 4
Glial scar	n = 6	n = 6	n = 7	n = 8	n = 4

Table 3.2. Sample sizes in each experiment. All animals were examined at 12 weeks post-injury, unless otherwise stated.

3.2.8 Quantification of axonal length and glial scar thickness

3.2.8.1 *Axonal length*

Axonal regrowth was examined at 4 and 12 weeks post-injury. NF signal was traced as individual neurites and measured using the ImageJ software. Specifically, for the analysis of the average axonal ingrowth length, all NF signals in two to three tissue sections of each animal were measured. While for the longest neurite length, only the longest NF signal of each animal was taken into account. The sample pool is stated in Table 3.2.

3.2.8.2 *Glial scar thickness*

For quantification of glial scar formation, the GFAP-positive area in the grey matter surrounding the injury sites or the scaffolds at 12 weeks post-injury was measured. The sample pool is stated in Table 3.2. Briefly, a horizontal plane cutting through the middle of the injury site or the scaffold was defined on each tissue section. The side facing the spinal cord was then divided by nine lines, which were 20° apart from each other (Fig. 3.3). The lengths of these lines that passed through the glial scar area were measured using the ImageJ software.

Figure 3.3. Illustration of glial scar measurement.

A horizontal plane cutting through the middle of the injured site or the scaffold was defined on each tissue section. The side facing the spinal cord was then divided by nine lines (red line), which were 20° apart from each other.

3.2.9 Evaluation of laminin expression

Laminin expression at 12 weeks post-injury was analyzed qualitatively. The grades were given from “0” indicating the absence of LN signal or alignment of LN-positive structures to “++++” indicating the strongest LN signal or the highest alignment of LN-positive structures.

3.2.10 Evaluation of functional recovery

Two behavioral tests, the cylinder test and the grid walk test, were performed. The extent of recovery was described by the recovery index where a higher value indicated better recovery. For the cylinder test, after placing each rat into a cylinder (13 cm in diameter, and 24 cm in height), the rat was monitored until its left paw has touched the wall of the cylinder for 50 times. During this period, the number of times the right paw touched the wall in an open configuration was recorded (Fig. 3.4A). The recovery index at each

designated time point was then obtained using the right paw values normalized to the value at day 3 post-injury. For those day 3 results that happen to be zero, a value of one was used instead.

For the grid walk test, each rat was allowed to walk freely on a grid (32 x 50 cm) elevated 60 cm above the ground for 3 minutes (Fig. 3.4C). The struts of the grid were 0.2 cm in thickness and formed 3.5 x 3.5 cm squares. The right paw normal step was computed as the percentage of the number of times the right paw did not miss the grid over the total steps. The recovery index at each designated time point was then obtained using the right paw normal step normalized to the value at day 3 post-injury. For those day 3 results that happen to be zero, a value of one was used instead.

Figure 3.4. Motor performance at the cylinder test (A, B) and the grid walk test (C, D). (A) image depicting a typical open right paw in the cylinder test. (C) image depicting a typical right paw slip in the grid walk test. (B, D) the recovery index score of the right paw. Scaffold implanted groups showed faster functional recovery as compared to the SCI group. However, there was no significant difference among the groups. Insets: results at 21 days post-injury. Data are shown as mean $\pm$ SEM.

3.2.11 Statistical Analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). For the analysis of average NF ingrowth length, the differences between experimental groups were examined using the Kruskal Wallis test followed by the Mann-Whitney U test. All other experimental results were verified to have equal variances and were analyzed using one-way ANOVA followed by Turkey post hoc test.

3.3 Results

3.3.1 Scaffold characterization

3.3.1.1 *Aligned PCLEEP nanofibers*

Fig. 3.5A and B show the morphology and appearance of electrospun PCLEEP fibers. Bead-free and uniformly aligned nanofibers with an average diameter of 818 ± 11 nm were obtained.

Figure 3.5. SEM images of the aligned PCLEEP nanofibers.

3.3.1.2 *Aligned PCLEEP nanofibers in collagen hydrogel*

Regardless of the presence of aligned PCLEEP fibers, all collagen hydrogels appeared similar macroscopically with three-dimensional interconnected macroporous structures (Fig. 3.6A, B, E, F). Aligned PCLEEP fibers were distributed uniformly in the middle of the scaffold with a density of $1.37 \times 10^5 \pm 755$ fibers/mm² (Fig. 3.6E - H). The fibers occupied 46.3 ± 3.9 % of the scaffold cross-sectional area. The presence of collagen hydrogel within the scaffold supported and maintained fiber orientation and alignment without inducing their fusion (Fig. 3.6F, H).

Figure 3.6. SEM images of the aligned PCLEEP nanofibers and the scaffolds.

(A, B) Longitudinal section of the isotropic hydrogel scaffold. (C, D) Side view of the isotropic hydrogel scaffold. (E, F) Longitudinal section of the scaffold containing parallel PCLEEP nanofibers. (G, H) Cross-section of the scaffold containing parallel PCLEEP fibers. (B, D, F, and H) The high magnifications of the insets in (A, C, E, and G, respectively).

3.3.2 Host-implant integration of the scaffolds

As indicated in Fig. 3.2C and E, the orientation and alignment of the nanofibers were maintained inside the rat spinal cords at 12 weeks post-implantation. Moreover, this scaffold design allowed one to easily introduce different fiber orientations *in vivo* (Fig. 3.2B - E).

As indicated in Fig. 3.7, all scaffolds remained intact after 12 weeks *in vivo*. In addition, there was no difference in collagen density within the scaffolds between 4 and 12 weeks post-implantation (data not shown). The Gel group exhibited highly compacted collagen hydrogel with limited cell penetration (Fig. 3.7D). On the other hand, the Parallel, Angled, and NT-3 Parallel groups showed less compact collagen with good cell penetration throughout the scaffolds (Fig. 3.7A - C).

As shown in Fig. 3.7A - D, fibrous scars were rarely detected in the scaffold-treated groups and they did not exceed 15 μm in thickness. Moreover, fibrotic scars surrounding the scaffolds were more extensive in the Gel group than in all the fiber-containing groups.

For the SCI group, the outer white matter (WM) was always densely filled with cells. Due to the contraction of the scar tissue around the lesion, the injury site in the SCI group appeared smaller as compared to the other groups with scaffold implantation. However, this healing process was only partial and resulted in cystic cavity formation. We observed that 5 out of 6 animals developed a cystic cavity in the gray matter (GM) (Fig. 3.7E). For these five

animals, the average cavity length was $440.1 \pm 74.9 \mu\text{m}$ and the average cavity width was $91.5 \pm 9.9 \mu\text{m}$. In contrast, the presence of scaffolds prevented the contraction process and bridged the two ends of the host tissue. As a result, cavity formation was not observed in all the scaffold-treated groups (Fig. 3.7A - D).

Figure 3.7. Masson's Trichrome staining of spinal cord sections at 12 weeks post-injury. (A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. Rostral, right; caudal, left. Arrowheads: fibrous scar.

3.3.3 Neurite ingrowth

For the Parallel group, neurites extended along the aligned PCLEEP nanofibers at 4 weeks post-implantation (Fig. 3.8A, B). The average axonal ingrowth length was $268.7 \pm 13.0 \mu\text{m}$ while the longest neurite reached $856.0 \pm 92.2 \mu\text{m}$ in length (Fig. 3.8K, L). At 12 weeks post-implantation, more neurofilaments were observed inside the scaffolds as compared to 4 weeks (Fig. 3.8C, D). Furthermore, the average axonal ingrowth length was also significantly longer ($326.1 \pm 15.6 \mu\text{m}$) (Fig. 3.8K).

Similarly, neurite extension was also observed along the aligned PCLEEP nanofibers in the Angled group (Fig. 3.8E, F). In addition, the angled orientation of the fibers decreased the average neurite length significantly as compared to the parallel fibers ($326.1 \pm 15.6 \mu\text{m}$ vs. $271.7 \pm 13.0 \mu\text{m}$ for Parallel vs. Angled, Fig. 3.8K). However, fiber orientation did not significantly affect the longest attainable neurite length ($731.4 \pm 125.3 \mu\text{m}$ vs. $788.6 \pm 78.2 \mu\text{m}$ for Parallel vs. Angled, Fig. 3.8G, H).

The NT-3 Parallel group (Fig. 3.8G, H) showed the highest average neurite length as compared to all the other experimental groups ($391.9 \pm 12.9 \mu\text{m}$) (Fig. 3.8K). However, the longest attainable neurite length was not significantly different between all groups that received hybrid nanofiber-hydrogel scaffolds (Fig. 3.8L, NT-3 Parallel vs. Parallel and Angled).

In general, at 12 weeks post-implantation, the longest neurite lengths of the Parallel, Angled, and NT-3 Parallel groups were significantly longer as

compared to the Gel group (Fig. 3.8L). Furthermore, no orientation of regrown neurites was observed in the Gel group (Fig. 3.8I). The SCI group also exhibited regrown neurites inside the injury site. However, similar to the Gel group, their growth was highly disorganized, losing the distinct rostro-caudal alignment within the spinal cord (Fig. 3.8J). Moreover, when comparing the length of the longest neurites in all groups, the SCI and Gel groups showed the lowest recorded lengths (Fig. 3.8L).

Figure 3.8. Immunofluorescent staining of neurite ingrowth (NF⁺, red) in spinal cord sections.

(A, B) The Parallel group at 4 weeks post-injury. (C, D) The Parallel group, (E, F) The Angled group, (G, H) The NT-3 Parallel group, (I) The Gel group, and (J) The SCI group at 12 weeks

post-injury. (A, C, E, G, I, J) Fluorescent images of NF⁺ staining. (B, D, F, H) Merged images of the fluorescent images (NF⁺ staining) and bright-field images (fibers). (K) Quantification of the average neurite ingrowth length and (L) the longest neurites of different experimental groups. Rostral, left; caudal, right. NS non-significant; * $p \leq 0.05$. ** $p \leq 0.01$; *** $p \leq 0.001$. Data are shown as mean $\pm$ SEM.

3.3.4 Glial scar

As illustrated in Fig. 3.9, there was no significant difference in glial scar thickness among all experimental groups at 12 weeks post-injury.

Figure 3.9. GFAP⁺ scar formation around the scaffolds and injury sites at 12 weeks post-injury.

(A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. (F) Quantification of the scar thickness around the injury site. Rostral, left; caudal, right. Data are shown as mean $\pm$ SEM.

3.3.5 Inflammatory response

Inflammatory reaction towards the scaffolds was evaluated by the appearance of microglia and macrophages at the injured sites.

In general, microglial cells reacted well towards the hybrid scaffolds as they remained in a quiescent state with a highly ramified morphology within host-tissues that were surrounding the implants (Fig. 3.10A - D). The cells stayed activated, displaying cellular hypertrophy, only within the implant, where the aligned PCLEEP fibers remained present at 12 weeks post-implantation (Fig. 3.10A - C). The Gel group showed restricted microglial penetration (Fig. 3.10D). However, there was almost no microglia in the lesion cavities of the SCI group (Fig. 3.10E).

Figure 3.10. Immunofluorescent staining of microglia (Ox-42⁺, red) in spinal cord sections at 12 weeks post-injury.

(A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. Rostral, left; caudal, right.

Contrary to the microglial reaction, no clearly distinguishable macrophages could be found along the aligned PCLEEP fibers in all the fiber-containing groups (Fig. 3.11A - C). The fluorescence signals within the implants, as seen in the low magnification images (Fig. 3.11A - C), were mainly attributed to the auto-fluorescence of the scaffolds. In addition, macrophages were only found at the outer border of the scaffold but not inside in the Gel group (Fig. 3.11D). Moreover, the number of macrophages around

all the scaffolds was limited and not significantly different from the SCI group (Fig. 3.11).

Figure 3.11. Immunofluorescent staining of macrophages (ED1⁺, red) in spinal cord sections at 12 weeks post-injury.

(A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. Rostral, left; caudal, right.

3.3.6 Laminin expression and blood vessel formation

In all animals that received the hybrid scaffolds, laminin expression was found extensively along the aligned PCLEEP fibers regardless of the fiber orientation (Fig. 3.12A - C). Moreover, the amount of laminin inside the

scaffolds of the NT-3 Parallel group was the highest as compared to the other hybrid scaffold groups (Fig. 3.12C, F).

Figure 3.12. Immunofluorescent staining of laminin (LN⁺, green) at 12 weeks post-injury. (A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. (F) Qualitative analysis of the LN⁺ structures inside the scaffolds/ injury

site. Rostral, left; caudal, right. 0, absence of the orientation/no signal; +, the lowest score; +++, the highest score.

Similarly, newly regenerated blood vessels, as indicated by RECA-1⁺-tube like structures, were found along the aligned PCLEEP fibers in all the fiber-containing groups (Fig. 3.13A, B). On the other hand, limited blood vessel formation with no orientation was found inside the isotropic hydrogels in the Gel group (Fig. 3.13C). Thus, the compaction of the collagen hydrogel seemed to restrict cell penetration and blood vessel formation. Meanwhile, the SCI group displayed typical hypertrophied blood vessels at the lesion site (Fig. 3.13D). However, these newly formed blood vessels were tortuous and disorganized.

Figure 3.13. Immunofluorescent staining of endothelial cells (RECA-1⁺, red) and Laminin (LN⁺, green) at 12 weeks post-injury.

(A) The Parallel group. (B) The NT-3 Parallel groups. (C) The Gel group. (D) The SCI group. Rostral, right; caudal, left. Arrowheads: RECA-1/LN co-localization.

3.3.7 Functional recovery

Both of the cylinder (Fig. 3.4B) and the grid-walk tests (Fig. 3.4D) revealed that the scaffold-implanted groups had faster functional recovery as compared to the SCI group. Importantly, the NT-3 Parallel group appeared to give the best functional improvement, especially in the cylinder test. On the other hand, the fiber-containing groups (Parallel, Angled, and Nt-3 Parallel groups) clearly showed improved functional recovery in the grid-walk test as compared to the Gel and SCI groups, suggesting the supporting role of the aligned nanofibers. However, there was no significant difference among these groups.

3.4. Discussion

Despite the intrinsic regeneration capacity of neurons, restoration of neuronal functions following SCI is still generally poor due to the complex inhibitory microenvironment that exists after injuries. Hence, methods that provide support for spinal nerve regrowth without causing host rejection are attractive.

In this study, we hypothesized that by implanting a hybrid scaffold with three-dimensionally distributed aligned nanofibers into the injured spinal cord, contact guiding cues can be provided to direct neurite regrowth along the longitudinal axis, thereby facilitating tissue regeneration. In our previous work, a sheet of aligned nanofibers was rolled into a spiral conduit and implanted into the spinal cord. Cell penetration was only observed throughout the spaces in between the layers of conduit, but not within the nanofiber sheet ²⁵. This

poor cell penetration was attributed to the small pore sizes within the nanofiber mesh. Hence, in this study, to enhance cell penetration, we introduced the use of three-dimensionally, sparsely distributed aligned nanofibers. Correspondingly, robust cell penetration was observed throughout the scaffold (Fig. 3.7A - C, Fig. 3.10A - C, and Fig. 3.11A - C) without compromising contact guidance effect.

PCLEEP is a biodegradable copolymer. Our previous study showed that after three months of incubation in distilled water at 37 °C, the mass loss of PCLEEP fibers was less than 8 %¹³¹. *In vivo*, the presence of PCLEEP fibers was still detected at 3 months post-implantation into the rat's sciatic nerve¹³⁰. Importantly, the incorporation of EEP into the poly (ϵ -caprolactone) backbone enhanced the hydrophilicity of the resulting polymer, making it an ideal material for sustained drug and protein delivery^{115,117,130-132}. Incorporated with glial cell-derived neurotrophic factor, aligned PCLEEP fibers enhanced peripheral nerve regeneration across a 15-mm critical defect gap in the rat model at 3 months post-implantation¹³⁰. Together with the results from this study, PCLEEP fibers are useful in enhancing nerve regeneration in the peripheral and central nervous systems.

Compared to the conventional way of obtaining meshes of fibers by using a rotating target, the air-gap electrospinning provided sparsely distributed aligned fibers. In addition, electrospun nanofibers are at least 1 - 3 orders of magnitude smaller in diameter than other micron-sized features that have been explored for contact guidance in SCI treatment¹³⁶⁻¹³⁸. This

reduction in size of fibers clearly hinders their handling process. Therefore, the implantation of sparsely distributed aligned nanofibers into the injured spinal cord has not been achieved prior to this study. Here, the scaffold is fabricated by suspending the aligned nanofibers within a collagen hydrogel. This scaffold design allows us to implant aligned nanofibers *in vivo* without compromising fiber alignment. Thus, the effects of fiber orientation on axonal regrowth can be studied. Importantly, the fact that regenerated neurites extended along the fibers regardless of their orientation clearly confirmed the significant role of aligned nanofibers as topographical signals that directed the alignment of regenerated axons *in vivo*. The current fiber density was chosen through an initial optimization process. With a lower fiber density, the impact of physical guidance on axonal regrowth was insignificant; while cell and neurite penetrations were largely hindered with higher fiber densities.

An ideal scaffold for SCI treatment should bridge the injury gap and integrate well into host tissues. However, most of the scaffolds developed to date have yet fulfilled these requirements since they are unable to prevent the formation of lesion cavities^{127,139}. These cystic cavities formed after SCI is one of the factors that impede axonal ingrowth and nerve reconnection¹⁴⁰⁻¹⁴⁴. We observed that 5 out of 6 animals in the SCI group developed cystic cavity (Fig. 3.7). In contrast, cystic cavity formation was absent in all animals that received scaffold implantation at 12 weeks post-injury (Fig. 3.7). These results suggest that the collagen-based scaffolds integrated well into adjacent tissues.

Besides cystic cavity formation, SCI also leads to the formation of scar tissues around the lesion. These scar tissues, consisting of glial and fibrotic scars, act as physical barriers that prevent regenerated axons from entering/exiting the lesion, thereby inhibiting neuronal reconnections^{2,145-148}. As indicated in Fig. 3.9, there was no significant difference in glial scar thickness among all the experimental groups. These results demonstrated that the scaffolds did not enhance the extent of astrocytic activation. On the other hand, there were thicker fibrotic capsules in the Gel group than in all the fiber-containing groups (Fig. 3.7). One possible reason may be the presence of nanofibers, which prevented the hydrogel from collapsing or becoming compacted after implantation. Consequently, robust cell penetration and enhanced host-implant integration were observed in the nanofiber-hydrogel scaffolds as compared to the isotropic hydrogel constructs.

Good host-implant integration was also observed in terms of inflammatory response. Activated microglia and macrophages are mainly responsible for the inflammatory reactions after SCI^{2,149}. Hence, Ox42 and ED-1 were used as markers for microglia and macrophages, respectively. Quiescent microglia were seen within the host tissue surrounding our implants. These cells remained activated only within the implants in all experimental groups that contained PCLEEP aligned nanofibers. This observation is similar with the implantation of the RADA16-I scaffolds for SCI treatment in rats⁶⁸. The presence of the nanofibers within the injury is likely the reason for the prolonged activation of microglial cells within the implants. In contrast to microglial infiltration, the number of macrophages found inside and around the

nanofiber-hydrogel scaffolds was similar with the SCI group at 12 weeks post-injury. Comparatively, Nomura *et al.* reported a massive aggregation of macrophages inside chitosan scaffolds and near-by spinal cord tissues even after 14 weeks post-implantation ¹⁵⁰. Similarly, a large number of macrophages was also found surrounding the RADA16-I scaffolds ⁶⁸. Taken together, these results indicate that our scaffolds were well accepted by the host tissue and did not induce extensive chronic inflammatory reaction in comparison with other scaffold models.

Laminin, a major component of the extracellular matrix in the CNS ^{151,152}, has been widely known to promote neurite outgrowth as well as to direct axon development ¹⁵²⁻¹⁵⁵. As such, the extensive presence of laminin within our scaffolds at 3 months post-implantation indicates a favorable microenvironment for axonal regeneration. Moreover, the existence of laminin-positive structures in the injured area is also an indicator of blood vessel reformation ^{156,157}. Indeed, the NT-3 Parallel group possessed greater amounts of both laminin expression and aligned blood vessels within the scaffold as compared to the Gel group (Fig. 3.12C, D, F and Fig. 3.13B, C). This result is in agreement with Cristofaro *et al.*'s study, which showed that NT-3 promoted neovascularization in both rat mesenteric angiogenesis assay and mouse limb ischemia ¹⁵⁸. Revascularization of the injured spinal cord is a critical tissue repair process as newly formed blood vessels provide trophic and metabolic support, which is essential for wound healing ¹⁵⁹. Under normal physiological conditions, blood vessels create a favorable environment for axonal growth ^{160,161}. Thus, axons can be frequently found in close association

with the walls of newly formed blood vessels ¹⁶⁰. Correspondingly, our hybrid scaffold, especially with the inclusion of NT-3, provided a beneficial local habitat for axonal regrowth and tissue regeneration.

Although a general trend of enhanced neurite length was observed in the presence of NT-3, there was no significant effect on the longest attainable neurite length as compared to the other hybrid scaffold groups. This minor improved neurite regeneration outcome suggests that either a higher loading dose of NT-3 or the synergistic effect of NT-3 and other factors, is required. The release of NT-3 from our scaffold has been evaluated (data not shown) to reveal that after an initial burst release at day 1, a steady release of NT-3 was obtained for at least 1 month with a cumulative release of 48.3 ± 13.2 ng/mg of scaffold. On the other hand, a combination of NT-3 and BDNF might be considered to achieve the synergistic effect. This combination was shown to generate the longest neurite elongation from hippocampal progenitor HiB5 cells, as compared to the over-expression of either NT-3 or BDNF alone ¹⁶². Therefore, future works should expand along these lines. Similarly, the incorporation of factors that minimize neurite inhibition, such as antibodies against myelin inhibitor ⁴⁷⁻⁵⁰ or enzymes against chondroitin sulfate proteoglycans ⁵¹⁻⁵⁴, may also be explored for additional synergistic effects to further promote nerve regeneration and functional recovery. The versatility of our scaffold fabrication process will allow the easy extension to incorporate multiple factors within the hydrogel and nanofibers ^{132,163}.

In contrast, the SCI group displayed typical hypertrophied and disorganized blood vessels at the lesion site (Fig. 3.13D), which were in sync with the lowest score for average axonal ingrowth length (Fig. 3.8). These results are consistent with the literature showing that after SCI, newly formed blood vessels do not guide regenerated axons into and beyond the injury site because of their tortuous form ^{160,161}. Similarly, nerve regeneration also remained sub-optimal with limited neurite extension in the Gel group, which also demonstrated correspondingly limited and disorganized revascularization (Fig. 3.8 and Fig. 3.13C). These results are in agreement with the study by Joosten *et al.*, which demonstrated that nerves did not penetrate a solid collagen implant ¹⁶⁴.

The incision SCI model was chosen in this study as it is repeatable, thus allowing us to carry out preliminary morphometric evaluation and focus on critical factors affecting nerve regeneration, such as neurite extension, cell penetration, revascularization, host responses, and host-implant integration. Correspondingly, our behavioral tests, the cylinder and the grid-walk tests (Fig. 3.4), only revealed that the scaffold-implanted groups had faster functional recovery as compared to the SCI group. In addition, aligned nanofibers possessed supporting role in enhancing functional recovery. However, there was no significant difference among the groups. It is possible that this model was not sufficiently severe to result in significant differences in rat locomotion among different groups. We believe that this may be due to the spontaneous recovery processes following incomplete injuries in the CNS ^{165,166}. Specifically, after partial SCI, a new intraspinal axonal circuit may form

spontaneously, bypassing the lesion and contributes to the recovery of function^{167,168}. Furthermore, it is known that intensive locomotor training can improve functional recovery after an incomplete SCI, especially when it is initiated immediately post-injury^{169,170}. As such, the exercise we introduced while doing the grid walk test assessment might possibly enhance functional recovery, thus masking our results. Hence, future studies should involve a complete transection SCI model to examine the effectiveness of the hybrid scaffolds in promoting functional recovery.

3.5. Conclusions

In this study, we introduced the three-dimensional hybrid scaffold as a potential platform for SCI treatment. *In vivo*, aligned nanofiber topography guided neurite extensions and neovascularization into the lesion site in an organized and aligned manner. In addition, the scaffold provided good host-implant integration, as it did not trigger additional inflammatory response and scar tissue formation. These findings clearly demonstrate the advantages of our scaffold design for SCI repair in terms of axonal regeneration guidance and host-implant integration.

Chapter 4. Three-dimensional aligned nanofibers-hydrogel scaffold for controlled therapeutic drug and non-viral gene delivery to direct axon regeneration in spinal cord injury treatment

* Reproduced from [Nguyen, L. H. *et al.* Three-dimensional aligned nanofibers-hydrogel scaffold for controlled non-viral drug/gene delivery to direct axon regeneration in spinal cord injury treatment. *Sci. Rep.* **7**, 42212; doi: 10.1038/srep42212 (2017).] with permission from Nature Research. Copyright [2017].

4.1 Introduction

Spinal cord injuries (SCI) irreversibly disrupt the spinal tracts and ultimately lead to permanent functional impairment. Although the direct administration of biological factors to injury sites is frequently applied, such an approach often does not lead to robust tissue regeneration and reformation due to the rapid biological clearance of these agents from our bodies²⁶⁻²⁹. Given this limitation, biodegradable scaffolds are increasingly employed as temporary frameworks for sustained delivery of biomolecules and to support neo-tissue formation. To mimic the mechanical properties of the spinal cord, hydrogels and self-assembled peptide nanofibers are commonly used^{7,8,15,66,68,127}. Unfortunately, these scaffolds are often isotropic in architecture and hence lack the ability to direct the growth of regenerated axons through the extensively disorganized injured tissues for proper neuronal reconnections.

We introduce herein a biodegradable, three-dimensional aligned nanofibers-hydrogel scaffold as a biofunctionalized platform to provide contact guidance and sustained therapeutic drug and non-viral gene delivery for nerve injury treatment. The scaffold comprises of aligned PCLEEP electrospun nanofibers that were distributed in a three-dimensional configuration within a collagen hydrogel. Comparing to the microchannels and microfibers that were previously used for SCI treatments^{23,24,171-173}, nanofibers more closely imitate the size scale and architecture of the natural extracellular matrix that is present in our body. More importantly, our scaffold design allows robust drug/gene encapsulation. Our previous works have shown that electrospinning enabled the incorporation of a wide variety of drugs ranging from proteins to low molecular weight lipophilic drugs and nucleic acids within nanofiber matrices^{115,117,130,131,174-180}. Collagen hydrogel, on the other hand, is well-established in its ability to enable sustained drug/gene delivery¹⁸¹⁻¹⁸³.

The cellular responses triggered after nerve injuries are tightly regulated by various signalling pathways involving miRs³⁵. Specifically, miRs are small non-coding RNAs that function as post-transcriptional regulators^{36,37}. They down-regulate protein expression by preventing translation or triggering mRNA degradation^{36,37}. Since miRs are only partially complementary to their target mRNAs, a single miR can inhibit the translation of hundreds of different mRNAs, giving rise to robust and diverse chains of responses. Hence, RNA interference using miRs is a promising method for nerve repair.

To demonstrate the efficacy of our PCLEEP-collagen hybrid scaffold in achieving effective local drug/gene delivery *in vivo*, we incorporated neurotrophin-3 (NT-3) as the model protein and miR-222 as the model miR. NT-3 is known to promote neuronal survival, axonal sprouting and regeneration.^{58,73-75} In addition, it facilitates the proliferation and differentiation of oligodendrocyte precursors cells^{73,76,77}. On the other hand, miR-222 is enriched in axons and participates in controlling local protein synthesis at distal axons⁷⁸⁻⁸⁰. Such controlled local protein synthesis plays critical roles in allowing severed axons to undergo regeneration within hours after injuries, independent of protein transport from the cell soma of neurons^{78,81}. Unfortunately, the expression of miR-222 is often significantly altered after nerve injuries⁸²⁻⁸⁸. *In vitro*, when miR-222 was deliberately over-expressed in injured adult neurons, enhanced regrowth was observed^{82,83,89,90}. However, the use of miR-222 to enhance nerve regeneration after SCI has not been attempted. We speculate that this is largely due to the lack of effective non-viral methods to deliver miRs to neurons *in vivo*. Thus, in this study, we incorporated NT-3 and miR-222 into the PCLEEP-collagen hybrid scaffold, as shown in Fig. 4.1, to evaluate the feasibility of a single scaffold in imparting synergistic biochemical and topographical signals to enhance nerve regeneration after SCI. The results showed that our biofunctionalized scaffolding platform effectively provided bio-mimicking contact guidance and allowed the controlled delivery of various drugs and therapeutic biomolecules of drastically different nature and molecular weights.

Figure 4.1. Schematic illustration of nanofibers-hydrogel scaffold fabrication process and implantation into the spinal cord.

Drugs may be loaded into the scaffolds by direct encapsulation (Step 1) into electrospun nanofibers or (Step 2) within collagen hydrogel.

4.2 Materials and Methods

4.2.1 Materials

TFE ($\geq 99.0\%$), BSA (A9649), Triton X-100, NGS, rabbit anti-neurofilament 200 (NF, N4142, 1:700 dilution), heparin, NaOH, sucrose, and

fluoromount were purchased from Sigma–Aldrich. Rat-tail Collagen type I (10.08 mg/ml, LOT 4041009) was purchased from Corning. 1x PBS (10010-023) was purchased from Gibco. Human NT-3 was purchased from Peprotech. 10x PBS (pH 7.4), DAPI (1:1000 dilution), collagenase type 1, Cy5-labeled oligonucleotides (Cy5-ODN), Alexa Fluor 488-labeled oligonucleotides (488-ODN), *miRVana*[™] miRNA mimic negative control #1 (NEG-miR, 4464060), and miR-222 mimic were purchased from Life Technologies. Paraformaldehyde (PFA), rabbit anti-gial fibrillary acidic protein (GFAP, clone GA5, 1:1000 dilution), and mouse anti-myelin associated glycoprotein (MAG, clone 513, 1:200 dilution) were purchased from Millipore. Chicken anti-NF (1:1000) was purchased from Biolegend. Quant-iT[™] RiboGreen® RNA reagent kit, Alexa Fluor 488-conjugated Goat Anti-Mouse/Rabbit IgG (1:700 dilution), and Alexa Fluor 555-conjugated Goat Anti-Mouse/Rabbit IgG (1:1000 dilution) were purchased from Invitrogen. Mouse anti-CD11b/c (anti-Ox42, ab1211, 1:200 dilution) was purchased from Abcam. Human NT-3 DuoSet ELISA kit (DY267) was purchased from RnD Systems. 1x Tris-EDTA buffer (TE buffer, pH 7.4) was purchased from 1st BASE.

4.2.2 Scaffold fabrication

4.2.1.1 Preparation of micellar nanoparticles

In this study, the micellar nanoparticles (MNP), which consist of poly(ϵ -caprolactone)-block-polyethylene glycol (PCL-PEG) and poly(ϵ -caprolactone)-block-poly (2-aminoethyl ethylene phosphate) (PCL-PPEEA), were used as transfection reagent. MNP were prepared according to the literature ¹⁸⁴. Briefly, 10 mg of PCL_{4.8k}-PEG_{3.4k} and PCL₂₉-PPEEA₂₁ (molar ratio = 1.5/1)

were dissolved in 500 μ l of solvent mixture, which comprised of methanol and acetonitrile (volume ratio = 1/1). Ultrapure water (5.0 ml) was added drop by drop into the polymer solution during stirring. After stirring at room temperature for 2 h, the chemical solvent was removed by vacuum. The MNP concentration was then adjusted to 2 mg/mL by adding extra ultrapure water. This MNP solution was kept at 4 °C until used.

4.2.1.2 *Aligned PCLEEP fibers*

The PCLEEP copolymer ($M_w = 59,102$, $M_n = 25,542$) was synthesized by previously reported methods^{85,133}. To fabricate aligned PCLEEP nanofibers, 35 wt.% of PCLEEP was dissolved in TFE and electrospun using the two-pole air gap electrospinning technique¹³⁵. Specifically, the spinning solution was loaded into a 1 ml syringe that was capped with a 22-gauge blunt-tipped needle. This solution was then charged at + 10 kV and released at a flow rate of 1.9 ml/h. The PCLEEP fibers were then deposited within a 5.0 cm air gap area that was between two stationary collector poles (- 4.0 kV). Each set of fibers was obtained after 90 seconds of spinning. These fiber bundles were then sterilized under UV light for 30 minutes.

To fabricate PCLEEP nanofibers that encapsulated nucleic acids, 40 wt.% of PCLEEP in TFE was used. Cy5-ODN (5'-Cy5-CCTACGCCACCAATTTCGTTT-3' (sense), 5'-ACGAAATTGGTGGCGTAGGTT-3' (antisense)) and 488-ODN (5'-488-CCTACGCCACCAATTTCGTTT-3' (sense); 5'-ACGAAATTGGTGGCGTAGGTT-3' (antisense)) were used to visualize miR

distribution in nanofibers and miR release *in vivo*, respectively. Only Cy5-ODN was used in *in vivo* experiment since it possessed better photostability as compared to 488-ODN in our preliminary study. First, Cy5-ODN, 488-ODN, NEG-miR (100 μ M) or miR-222 was complexed with MNP (2.0 mg/ml) at room temperature for 10 minutes. Thereafter, the nucleic acid complexes were added into the PCLEEP solution at an RNA/MNP/TFE ratio of 35 μ l/296 μ l/1 ml. Following that, this polymer solution was dispensed at a flow rate of 0.5 ml/h and electrospun at an electrical voltage of 13 kV. Each set of fibers was obtained after 60 seconds of electrospinning.

4.2.1.3 Scaffolds

Rat-tail Collagen type I was used to fabricate hydrogel according to the manufacturer's protocol (BD Biosciences). Briefly, 10x PBS, collagen type I, 1.0 N NaOH, and de-ionized (DI) water were added into a sterile microtube and mixed properly to get a final collagen concentration of 6.0 mg/ml. The collagen mixture was then kept on ice until used. For NT-3-incorporation, 0.5 μ l of 10x PBS and 4.5 μ l of DI water in the collagen mixture were substituted by 5 μ l of 0.1% BSA containing human NT-3 (4.0 μ g/ μ l) and heparin (400 μ g/ μ l) (1:1 v/v). The theoretical loading of NT-3 was 2 μ g/animal. For Cy5-ODN, 488-ODN, or miR-incorporation within collagen matrices, 2.6 μ l of Cy5-ODN, 488-ODN, or miR (100 μ M) were complexed with 22 μ l of MNP (2.0 mg/ml) for 10 minutes at room temperature. Following that, the miR complex was added into the collagen mixture in place of 24.6 μ l of DI water. The theoretical loading of miR was 0.76 μ g/animal. To obtain scaffolds with aligned nanofibers, a sterilized cylinder mold (5.0 mm in length and 4.5 mm in inner

diameter) was pre-set with 4 sets of fibers in the core region prior to the addition of the collagen mixture. Hydrogel formation took place at 37 °C in the incubator for 45 minutes. Thereafter, the scaffolds were frozen at - 80 °C then lyophilized and kept at - 20 °C until used.

4.2.3 Scaffold characterization

4.2.2.1 *Fiber diameter*

The nanofibers and the scaffolds were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 120 s and 140 s, respectively. Thereafter, the samples were observed under the scanning electron microscope (SEM; JEOL, JSM-6390 LA) at an accelerating voltage of 10 kV. The fiber diameters were measured using ImageJ software (<http://imagej.nih.gov/ij/>) on the SEM images. At least 100 fibers were measured.

4.2.2.2 *Scaffold degradation*

Each scaffold (5.0 mm in length, 1.8 ± 0.11 mg) was immersed in 1.2 ml of PBS in a microtube and incubated at 37 °C. To quantify the total scaffold degradation rate, five samples were used. At each designated time point, each scaffold was retrieved, slightly taped against the inner wall of the microtube for 10 s to remove excess water. Thereafter, its weight was recorded. The percentage mass loss was then determined as the ratio of the change in weight to the original weight of the scaffold. Results are presented as mean $\pm$ standard deviation (S.D.).

4.2.2.3 NT-3 release kinetic

The release kinetics of NT-3 from the scaffold was evaluated under static conditions. Briefly, three NT-3 encapsulated scaffolds (2.14 ± 0.15 mg) were immersed in PBS (1.2 ml of PBS per scaffold) and incubated at 37 °C. At each time point, 600 µl of PBS was collected and replaced by an equal volume of fresh PBS. The supernatant was then used to measure the amount of NT-3 that was released from the scaffolds over time using the ELISA assay, following manufacturer's protocol. After 3 months, the scaffolds were retrieved and fully dissolved in 300 µl of collagenase type 1 at 37 °C for 30 minutes. Thereafter, the supernatant was used to measure the remaining amount of NT-3 within the scaffolds by ELISA assay. Thus, the total amount of NT-3 retrieved from the scaffold was the NT-3 cumulative release amount plus the remaining amount of NT-3 retrieved after dissolving the scaffold. The experimental loading efficiency of NT-3 was then computed using the following equation:

$$\text{Loading efficiency} = \frac{\text{Total amount of NT-3 retrieved}}{\text{Original theoretical loading of NT-3}} \times 100\% \quad (\text{Equation 1})$$

The cumulative release profile of NT-3 (%), after taking into account the loading efficiency, is finally presented as mean $\pm$ standard deviation (S.D.).

4.2.2.4 miR release kinetics

The experimental loading efficiency of miR within the collagen hydrogel was evaluated after lyophilization. Briefly, NEG-miR-incorporated scaffolds ($n = 3$) were fully dissolved in 300 µl of collagenase type 1 at 37 °C for 30 minutes. The amount of NEG-miR within the extracted solution was

determined by Quant-iT™ RiboGreen® RNA reagent kit after de-complexion from MNP using heparin (20 µg/ml). The experimental loading efficiency of miR was computed using the following equation:

$$\text{Loading efficiency} = \frac{\text{Total amount of NEG-miR extracted}}{\text{Original theoretical loading of NEG-miR}} \times 100\% \quad (\text{Equation 2})$$

The release kinetics of miR from the scaffold was evaluated under static conditions. NEG-miR-incorporated scaffolds (1.55 ± 0.1 mg) were incubated in 1.2 ml 1x TE buffer at 37 °C. Three samples were used for each time point during this study. At each time point, 600 µl of TE buffer was withdrawn and replaced by an equal volume of fresh buffer. The supernatant was then used to measure the amount of miR that was released over time using Quant-iT™ RiboGreen® RNA reagent kit after treating with heparin (20 µg/ml). The cumulative release of miR (%), after taking into account the loading efficiency, is presented as mean ± standard deviation (S.D.).

4.2.4 Spinal cord injury and scaffold implantation

Adult female Sprague Dawley rats (6-8 weeks, 200-250 g) were obtained from In Vivos Pte Ltd (Singapore). All experimental procedures were approved by the Institutional Animal Care and Use Committee, Nanyang Technological University (IACUC, NTU). All methods were performed in accordance with the relevant guidelines and regulations.

Rats were anesthetized using a combination of ketamine (73 mg/kg) and xylazine (7.3 mg/kg) right before surgery. The surgical field was shaved and cleaned with 70% ethanol and then Betadine. Thereafter, the dorsal skin

was incised 3-4 cm caudally from the base of the head. Muscle layers were opened along the midline to gain access to the cervical spine. Following that, the C5 spine was located using T2 process as an anatomic landmark and its right side was opened to expose the C5 spinal cord. Afterward, the dura was cut open and a 1/3 incision was made using a pair of fine micro-scissors. Thereafter, scaffolds (1.0 mm in length) were implanted into the incision site such that the nanofibers were aligned to the longitudinal axis of the spinal cord. Subsequently, the dura was sutured back and a layer of fat tissue was used to cover the injury area. The muscle was then sutured and the skin was closed with auto clips. Animals were randomly divided into five treatment groups as presented in Table 4.1.

	Group	Description	Number of animals
1	Control	Animals received plain scaffolds	N = 3
2	Cy5-ODN	Animals received cy5-ODN encapsulated scaffolds	N = 4
3	NEG-miR	Animals received NEG-miR encapsulated scaffolds	N = 3
4	miR-222	Animals received miR-222 encapsulated scaffolds	N = 3
5	NT-3	Animals received NT-3 encapsulated scaffolds	N = 11

Table 4.1. Experimental groups

All animals were injected with buprenorphine subcutaneously (0.05 mg/kg) twice a day for 3 days post-operation. Subsequently, they were fed

with meloxicam (5.0 mg/ml, 1.0 ml in 500 ml drinking water) continuously for the following 7 days.

4.2.5 Immunohistochemistry

At 7, 10, 14, and 28 days post-injury, animals were perfused with 0.9% saline followed by 4% ice-cold PFA. After perfusion, 5 cm of spine containing the spinal cord with the lesion site was harvested from each animal and post-fixed for 16 - 18 h at 4 °C before transferring to 15% sucrose for 24 h. Thereafter, 1.5 cm of the spinal cords containing the lesion sites were removed from the spines and stored in 30% sucrose at 4 °C until cryosectioned. Spinal cord samples were sectioned coronally on a cryostat set at 25 µm thickness. Subsequently, the spinal cord sections were blocked in 5 % NGS and 0.1% Triton X-100 for 1 h at room temperature. The sections were then incubated with primary antibodies diluted in the above block solution at 4 °C overnight followed by secondary antibodies diluted in PBS for 2.5 h at room temperature. The following primary antibodies were used respectively: rabbit anti-neurofilament 200 (NF, 1:700 dilution), chicken anti-NF (1:1000 dilution), rabbit anti-glial fibrillary acidic protein (GFAP, clone GA5, 1:1000 dilution), mouse anti-myelin associated glycoprotein (MAG, clone 513, 1:200 dilution), and mouse anti-CD11b/c (anti-Ox42, 1:200 dilution). Secondary antibodies were Alexa Fluor 488-conjugated Goat Anti-Mouse/Rabbit IgG (1:700 dilution) and Alexa Fluor 555-conjugated Goat Anti-Mouse/Rabbit IgG (1:1000 dilution). Nuclear staining was performed by incubating the sections with DAPI (1:1000 dilution) at room temperature for 10 minutes after the secondary antibodies.

Thereafter, all samples were examined using an Olympus IX71 inverted microscope and a Zeiss LSM710 META confocal microscope.

4.2.6 Statistical analysis

All data are presented as mean $\pm$ standard deviation (S.D.). Statistical comparison for fiber diameter, after verifying to have equal variances, was analyzed using one-way ANOVA followed by Turkey post-hoc test.

4.3 Results

4.3.1 Scaffold characterization

4.3.1.1 *Fiber topography*

MiR incorporation did not alter the morphology and alignment of the electrospun fibers. Specifically, bead-free and uniformly aligned PCLEEP nanofibers were fabricated with and without miR encapsulation (Fig. 4.2A and B). Their average fiber diameters were 747 ± 97 nm and 818 ± 11 nm respectively and were not significantly different.

Figure 4.2. Scaffold characterization.

(A-D) SEM images of scaffolds. (A,B) Aligned PCLEEP nanofibers (A) with and (B) without encapsulation of miR (NEG-miR). (C, D) Cross-sectional view of nanofibers-hydrogel scaffold. (D) High magnification image of inset in (C). (E) *In vitro* degradation rate of the nanofibers-hydrogel scaffold.

4.3.1.2 Scaffold degradation

As shown in Fig. 4.2E, the scaffolds were gradually degraded overtime. In particular, total mass losses of ~ 24.8 , 33.4 , and 51.6% were respectively reported after 1, 2, and 3 months *in vitro*.

4.3.1.3 NT-3 release kinetic

A total loading of 54.6 ng NT-3/mg scaffold was achieved with a loading efficiency of 1.17 %. As indicated in Fig. 4.3A, ~ 90.1 % of NT-3 was released within the first week. Thereafter, the release rate of NT-3 was decreased and steady for up to 3 months (99.7 ± 0.07 %).

Figure 4.3. *In vivo* regeneration of aligned neurofilaments (NF⁺, green) within NT-3-incorporated nanofibers-hydrogel scaffolds after spinal cord injury.

(A) *In vitro* NT-3 release kinetics of NT-3-incorporated nanofibers-hydrogel scaffolds. (B) Schematic illustration and corresponding light micrograph depicting nanofibers-hydrogel scaffolds that were implanted into spinal cord tissues. Dotted line: tissue-implant interface. SC:

spinal cord tissue. (C) *In vivo* regeneration of aligned neurofilaments within injury site at (i-iii) 1 week, (iv-vi) 2 weeks and (vii-ix) 4 weeks post-implantation. (ii, v, and viii) High magnification images of insets in i, iv, and vii respectively. (iii, vi, and ix) Corresponding bright-field images of nanofibers implanted within spinal cord tissues for ii, v, and viii respectively.

4.3.1.4 miR release kinetic

By incorporating miRs into both nanofibers and collagen hydrogel, a total loading of 0.5 μg miR/mg scaffold was achieved with an experimental loading efficiency of $\sim 16\%$. As shown in Fig. 4.4A, a total of $27.1 \pm 3.38\%$ miR was rapidly released within the first month. Thereafter, the miR release rate was decreased and steady for at least another 2 months.

Figure 4.4. Enhanced regeneration of aligned neurofilaments (NF⁺, red) within nanofibers-hydrogel scaffolds that incorporated miR-222 at 10 days post-implantation. (A) Distribution of 488-ODN that was encapsulated within PCLEEP electrospun fibers and miR release profile of the nanofibers-hydrogel scaffold. (B) Neurofilament regeneration within nanofibers-hydrogel scaffolds at 10 days post-implantation. (C) Evaluation of microglia (Ox42⁺, green) and astrocyte (GFAP⁺, magenta) reactions within nanofibers-hydrogel scaffolds depicted no adverse side effects in the presence of miR-222.

4.3.2 miR delivery *in vivo*

Fig. 4.5 illustrates the distribution of encapsulated cy5-ODN within the spinal cords. Specifically, after 14 days post-implantation, Cy5-ODN was detected in the surrounding tissues up to $\sim 300 \mu\text{m}$ from the edge of the scaffolds (Fig. 4.5).

Figure 4.5. miR distribution within spinal cord tissues after implanting Cy5-ODN incorporated nanofibers-hydrogel scaffolds for 7 days.

Left: Cy5-ODN uptake (red, circled) in surrounding spinal cord tissues. Right: Merged image of Cy5-ODN and DAPI staining depicting the proximity between the released cy5-ODN and scaffold. Dotted line: tissue-implant interface. *: scaffold.

4.3.3 Axon regeneration

As shown in Fig. 4.3Ci and ii, aligned regenerated axons were found extensively throughout the implant as early as one week post-implantation. The extent of regeneration was sustained even at later time points of 2 (Fig. 4.3Civ and v) and 4 weeks post-implantation (Fig. 4.3Cvii and viii). Importantly, the newly regenerated axons were found to penetrate the scaffolds, following nanofiber alignment (Figure 4.3C).

On the other hand, after 10 days post-implantation, robust neurite ingrowth was observed in PCLEEP-collagen hybrid scaffolds that incorporated

miR-222 (Fig. 4.4B). This axon regeneration was more extensive as compared to the plain scaffolds (Control) and scaffolds that encapsulated NEG-miR.

4.3.4 Axon remyelination

Immunostaining for myelin-associated glycoprotein (MAG, a marker of oligodendrocyte myelination) revealed that MAG^+ structures were extensively observed throughout the scaffolds at 4 weeks post-implantation (Fig. 4.6B) and co-localized with NF^+ axons (Fig. 4.6C, D, and F). In addition, these MAG^+ structures were extended along the aligned PCLEEP nanofibers (Fig. 4.6D – F).

Figure 4.6. *In vivo* regeneration of aligned neurofilaments (NF^+ , green) and remyelination (MAG^+ , red), which colocalized within the nanofibers-hydrogel scaffolds at 4 weeks post-implantation.

(A, B) Overview of the representative longitudinal spinal cord section. (C, D) High magnification images of the insets in A and B respectively. (E) Corresponding bright-field image of nanofibers in C and D. (F) Merged images of C, D, and E. Arrow heads indicate colocalization of NF-MAG signals.

4.3.5 Host-implant integration

As shown in Fig. 4.3C and 4.7, our PCLEEP-collagen hybrid scaffold integrated well with the host tissues and showed no sign of cyst formation throughout this study. In addition, extensive cell penetration into the scaffolds was observed as early as one week post-implantation (Fig. 4.7). Moreover, the incorporation of miRs did not alter the integration of the nanofibers-hydrogel implants with surrounding host tissues (Fig. 4.4C). In particular, no significant difference was observed in microglia and astrocyte reactions among the three experimental groups.

Figure 4.7. Extensive cellular infiltration into nanofibers hydrogel scaffolds at 1 week post implantation.

Left: DAPI staining for cell nuclei. Right: Merged image of DAPI staining and bright field showing scaffold with aligned nanofibers. Dotted line: implant-tissue interface.

4.4 Discussion

Many growth inhibitory factors exist after SCI. Among these, the formation of a cyst, which is a fluid filled cavity, has been a long withstanding problem. In the absence of an underlying supporting substrate, nerve/tissue regeneration is poor. As such, tissue scaffolds have been explored to provide bridges for nerve regrowth across injuries. Among the scaffolds analyzed, hydrogels⁹⁻¹⁷ and self-assembled peptide nanofibers^{18,19} are most popular.

However, the directions in which nerve regenerates within these scaffolds are always haphazard, often resulting in very few axons exiting the implant and reconnecting with host tissues. Here, we hypothesize that biomimicking aligned nanofiber topography is beneficial in guiding axonal regeneration. In addition, nerve regeneration may be further assisted by the synergistic supplementation of biomolecules such as neurotrophic factors (e.g. NT-3) and miRs (e.g. miR-222).

While the role of neurotrophic factors in nerve regeneration is well recognized, the involvement of miRs and RNA interference (RNAi) are only beginning to be elucidated. RNAi is widely adopted in treatments related to cancer therapy and genetic diseases. However, RNAi is also useful in regenerative medicine. Unfortunately, one of the major stumbling blocks to the translational use of RNAi is the development of proper non-viral delivery systems for effective and efficient delivery with minimal off-target effects in the body. Traditional approaches of systemic delivery for cancer and genetic disease therapy does not benefit traumatic tissue injuries since the latter often involves confined injuries that require the reestablishment of tissue architecture. MiR delivery using scaffolds, as demonstrated here, represents a more appropriate approach since scaffolds provide the necessary structural support and directional cues for tissue regrowth and restructuring. Scaffolds also provide localized delivery of drugs, hence minimizing off-target systemic side effects. In terms of neuronal regrowth, controlling the intrinsic growth ability of neurons by miRs will provide more robust treatment targets and outcomes. This is because although growth factor therapy is commonly used,

most factors offer selective support for only the subclass of neurons that express the appropriate receptors to allow neurons to take up these factors. In cases where the expression levels of the receptors are low and nerve damage involves a wide spectrum of neurons (such as in diabetes), growth factor-based approaches are ineffective¹⁸⁵.

Besides serving as a drug/gene delivery scaffold, our PCLEEP-collagen hybrid substrate provides aligned topographical signals for synergistic contact guidance effect over neuronal regeneration. As compared to hydrogels and micron-sized structures, nanofibers more closely imitate the topographical features of the natural extracellular matrix. By combining aligned electrospun nanofibers with collagen hydrogel, the orientation and alignment of PCLEEP nanofibers were retained even after implantation into the injured spinal cord (Fig. 4.3). Close examination of the nanofibers-hydrogel scaffold revealed the presence of loosely packed, three-dimensionally distributed aligned nanofibers within the collagen gel (Fig. 4.2C and D). This loose arrangement of aligned fibers in turn facilitated robust *in vivo* cell penetration (Fig. 4.7) and neurite infiltration (Fig. 4.3, 4.5, and 4.6).

Aside from providing a biomimicking 3D architecture, collagen possesses inherent cell adhesivity that supports cell attachment as compared to other biomaterials that have been widely explored for SCI treatment, such as agarose, chitosan, and fibrin²⁹. To evaluate the stability and degradability of the scaffold, we examined its *in vitro* degradation rate under physiologically-relevant conditions. As shown in Fig. 4.2E, the scaffolds were gradually

degraded overtime and only 48.4 % total mass was remained at 3 months *in vitro*. In addition, our previous *in vivo* study showed that PCLEEP fibers retained their morphology and alignment after 3 months post-implantation into the rat's spinal cords ⁷⁵. Thus, degradation occurred mostly within the collagen hydrogel throughout the duration of our studies.

NT-3 is a neurotrophic factor commonly used for SCI treatment to promote neuronal survival and axonal regrowth ^{58,73-75}. Here, we incorporated NT-3 into the collagen hydrogel matrix for localized and controlled delivery. We could not encapsulate NT-3 into the nanofibers while keeping the nanofiber architecture (fiber diameter and alignment) unchanged. As such, we did not include this part into this study. In order to regulate the release rate of NT-3, heparin was added in combination with NT-3 ¹⁷⁸. The release profile of NT-3, shown in Fig. 4.3A, was likely resulted from the non-specific binding of NT-3/heparin to the collagen hydrogel. Nonetheless, the initial supplementation of NT-3 after SCI is essential to minimize neuronal apoptosis as well as support neurite regrowth ⁶⁶. Correspondingly, we observed robust axonal regeneration at 1, 2, and 4 weeks post-implantation (Fig. 4.3C). Furthermore, when compared to currently available scaffold systems, our PCLEEP-collagen hybrid scaffold prolonged the availability of NT-3. For example, using the same platform of NT-3/heparin nonspecific binding with collagen, our previous electrospun nanofiber scaffold almost completely released NT-3 (99.2 ± 3.91 %) after only 2 months versus 3 months as seen in our current platform ¹⁷⁸. Furthermore, as compared to our current scaffold, a notably lower amount of NT-3 could be loaded into the nanofiber construct (~

21 ng NT-3/mg scaffold)¹⁷⁸. In another example, biodegradable poly(ethylene glycol) hydrogels fully released NT-3 over a 2-week period albeit possessing a lower burst release after 24 h (40 % vs 87 % in our system)¹⁸⁶. Similarly, the fibrin scaffold by Taylor *et al.* could only deliver NT-3 for 2 weeks post-implantation due to the rapid scaffold degradation rate¹⁸⁷. This rapid scaffold degradation further resulted in the formation of cystic cavities at 12 weeks after injury. Meanwhile, our scaffolds integrated well with the host tissues since no sign of cyst formation was detected throughout this study (Fig. 4.3C and 4.7) and even after 12 weeks post-implantation⁷⁵.

The effectiveness of our NT-3 incorporated scaffolds in supporting axonal ingrowth is depicted in Fig. 4.3C. In particular, besides providing good host-implant integration, NT-3 incorporated scaffolds greatly promoted axon regeneration (Fig. 4.3C). Notably, this supportive effect was sustained until 4 weeks post-implantation (Fig. 4.3Cvii and viii). As compared to our previous work⁷⁵, in this study, we deliberately maximized the loading capacity of our scaffold with twice the amount of NT-3. Correspondingly, the degree of axonal regrowth was also significantly enhanced. Furthermore, the newly regenerated axons were found aligned with the nanofibers (Fig. 4.3C). These results clearly indicate the contact guidance role of our scaffold in directing *in vivo* axon regeneration.

We next extended our analysis to evaluate the efficacy of the nanofibers-hydrogel scaffolds in supporting *in vivo* remyelination. As shown in Fig. 4.6, MAG⁺ structures were extensively observed throughout the scaffolds at 4 weeks post-implantation (Fig. 4.6B). Interestingly, these MAG⁺ structures

were co-localized with NF⁺ axons (Fig. 4.6C, D, and F). This result suggests the ability of our PCLEEP-collagen hybrid scaffold in facilitating axonal remyelination *in vivo*. In addition, these MAG⁺ structures were extended along the aligned PCLEEP nanofibers (Fig. 4.6D – F), further highlighting the contact guidance effect of our substrates. Although our scaffold implantation resulted in the extensive axonal ingrowth and remyelination, this 1/3 incision SCI model would not be sufficiently severe to result in significant differences in rat locomotion⁷⁵. We believe that the spontaneous recovery process following incomplete SCIs contributed to this outcome^{75,165-168}. Thus, in this study, behavior recovery was not assessed. Future works would focus on using the complete transection model to evaluate functional outcomes.

Apart from protein encapsulation, our scaffolds also allowed the easy incorporation of nucleic acids for non-viral gene transfection. MiRs are labile molecules. As such, the incorporation of miRs into PCLEEP-collagen scaffolds was an attempt to protect these drugs from biodegradation. Here, we demonstrate the capacity to encapsulate miRs either into nanofibers during the electrospinning process and/or into the collagen hydrogel (Fig. 4.1) to achieve non-viral delivery of these nucleic acid therapeutics. Notably, the encapsulated oligonucleotides were distributed uniformly along the electrospun nanofibers and did not alter nanofiber architecture (Fig. 4.2A, 4.2B, and 4.4A). The loading efficiency of miRs into the scaffold is found to be much higher as compared to NT-3. We speculate this greater retention of miR inside the scaffold to be due to charge interaction and molecular size. Since miR/MNP complexes are slightly positive and small in size, they can be

homogenously entrapped inside the neutralized collagen hydrogel with a net charge of ~ 0 ¹⁸⁸. In contrast, heparin is highly negatively charged and large in size, making it hard to be retained inside the collagen hydrogel. The release kinetic of small, non-coding RNAs from our PCLEEP-collagen hybrid scaffold (Fig. 4.4A) was greatly improved as compared to other existing delivery systems. In particular, Krebs *et al.* demonstrated the release of short-interfering RNA (siRNA) for only ~ 2 weeks from collagen hydrogels¹⁸⁹. The mechanism of siRNA/miR release over time is likely a combination of polymer charge, hydrogel porosity and biodegradation. It is possible that our high collagen concentration hydrogel (6.0 mg/ml) might have favoured RNA retention as compared to Krebs' 3.0 mg/ml collagen hydrogel.

To evaluate the capacity of our scaffold in providing localized gene delivery *in vivo*, scaffolds incorporated with Cy5-ODN, which were of similar size as miRs) were implanted into the same SCI model. As depicted in Fig. 4.5, Cy5-ODN was detected in the surrounding tissues up to ~ 300 μm from the edge of the scaffolds after 14 days post-implantation. This finding suggests the ability of our PCLEEP-collagen hydrogel scaffold in providing localized and sustained delivery of miRs.

Within the body, axons can extend for long distances. For efficient cell function, protein synthesis occurs locally at the terminal and growth ends of axons so that the transport of biomolecules from the cell soma is not critical. Such controlled local protein synthesis at distal axons has allowed severed axons to undergo regeneration within hours after injuries^{35,45}. This local

protein synthesis is finely controlled by miRs, such as miR-222. Indeed, miR-222 enhanced axonal regrowth in injured adult neurons *in vitro*^{82,83,89,90}. However, there is no study on miR-222's effects on SCI treatment to date. Given this knowledge gap, we next closely examined the efficacy of scaffold-mediated miR-222 delivery in supporting *in vivo* axon regeneration. As shown in Fig. 4.4B, axon regeneration was more robust in the miR-222 incorporated scaffold group as compared to the plain scaffolds (Control) and scaffolds that encapsulated NEG-miR after 10 days post-implantation. Moreover, the incorporation of miRs did not alter microglia and astrocyte reactions (Fig. 4.4C). Taken together, these results demonstrate the efficacy of our scaffolds in providing effective non-viral delivery of bioactive miR-222 to promote nerve regeneration after SCI.

The process of tissue repair is contributed by a complex cascade of signalling pathways involving various biological factors¹⁹⁰⁻¹⁹². Hence, spatially controlled and sequential release of drugs could enhance the tissue regeneration process^{190,193}. Accordingly, the flexibility of our scaffold design allows multiple drugs to be incorporated in different locations of the scaffold, either in the nanofibers or the hydrogel. The different nature of nanofibers and hydrogel would allow drugs to be released with distinct release kinetics, possibly mimicking the natural healing process. Such combinational delivery of drugs, such as miRs with growth factors, in the scaffold may be exploited for SCI treatment. For example, after SCI, a dramatic loss of OLs occurs, which frequently results in axonal demyelination¹⁹⁴. To replenish this OL pool, OPCs proliferate and are recruited to the lesion site for differentiation and

remyelination^{194,195}. Many growth factors have been known to promote OPC proliferation, such as PDGF-A, FGF-2, and IGF¹⁹⁴. On the other hand, miR-219/miR-338 enhance OPC differentiation and maturation¹⁹⁵. Thus, a possible strategy may be to combine these growth factors and miR-219/-338 in our scaffold platform for future SCI treatments.

4.5 Conclusions

In this study, we introduced an aligned nanofibers-hydrogel scaffold as a promising bio-functional platform for nerve injury treatment. Our scaffold provided localized and sustained release of drugs and nucleic acid molecules for non-viral transfection to enhance axon regeneration and myelination *in vivo*. Since non-viral gene delivery was employed, the application of our scaffolds may be translated to the clinical setting without raising biosafety concerns associated with viral-mediated methods. Additionally, the aligned nanofibers provided topographical signals that effectively directed neurite extensions and supported myelination within the lesion sites. These scaffolds also demonstrated good host-implant integration. Further works are currently ongoing to evaluate in detail, the ability of our nanofibers-hydrogel scaffold in supporting functional neuronal reconnections, myelination, and functional recovery after SCI.

Chapter 5. The effects of miR-219/miR-338 on microglia and astrocyte gene expression, activation, migration, and interaction with oligodendrocyte precursor cells

5.1 Introduction

Spinal cord injuries (SCI) result in a complex series of events that eventually lead to persistent neurological dysfunction. Unlike the peripheral nervous system, where damaged axons can regenerate depending on the extent of the injuries, damaged spinal axons often do not regenerate in their native inhibitory environment ^{1,2}. In addition, OLs, the myelin-forming cells of the central nervous system (CNS), are highly susceptible to cell death after SCI ^{194,196}. Correspondingly, the loss of OLs causes axonal demyelination and further impedes the survival and functions of spared intact neurons. Axon remyelination in the CNS has been documented in many studies ¹⁹⁴. However, this remyelination is not performed by preexisting mature OLs but is instead regulated by OL progenitor cells, OPCs ^{31,33,194}. Following SCI, the proliferation rate of OPCs increases markedly, especially at the lesion borders, to compensate for OL loss ^{194,197}. However, despite such spontaneous response and activation of OPCs, remyelination after SCI remains suboptimal possibly because these OPCs cannot successfully differentiate and mature into myelinating OLs ^{196,198}.

The oligodendroglial development is regulated by miRs, a class of small non-coding RNAs^{195,199}. Among them, miR-219/miR-338 are critical regulators of OL differentiation and maturation^{195,199}. Zhao *et al.* showed that the transfection of miR-219/miR-338 mimics into mouse OPCs resulted in a 5.1-fold increase in the percentage of MBP⁺ OLs as compared to the treatment with scrambled miR at 4 days post-transfection⁹³. Correspondingly, we demonstrated that nanofiber-mediated reverse transfection of miR-219/miR-338 significantly improved OL maturation by 5 folds with respect to scrambled miR on day 7¹⁹⁷. Therefore, treatment with these miRs, especially when coupled with nanofiber topography, would be a promising strategy to enhance OPC differentiation and maturation for axonal remyelination after SCI.

Recently, we have developed a biodegradable, three-dimensional aligned nanofibers-hydrogel scaffold to provide contact guidance and sustained therapeutic drug and non-viral gene delivery for SCI treatment²⁰⁰. We envision that the incorporation of miR-219/miR-338 into our scaffold platform can promote OL differentiation and maturation, leading to enhanced remyelination for SCI treatment. However, the biological responses following SCI are very complicated and involve a vast number of cell types beside OPCs, such as microglia and astrocytes. Upon injuries, microglia and astrocytes are activated and undergo dramatic alteration in morphology, expression of cell surface molecules, and release of cytokines²⁰¹⁻²⁰³. The activation of these cells possesses both beneficial and detrimental effects on nerve repair^{204,205}. Furthermore, many reported evidences suggest that astrocyte reactivity influences the development of OL lineage and remyelination

²⁰⁶⁻²⁰⁹. Thus, these complex cellular interactions need to be carefully considered when developing treatments for SCI.

This study was carried out to identify any potential effects that miR-219/miR-338 may have on microglia and astrocytes. In particular, we investigated the effects of miR-219/miR-338 on gene silencing and activation of microglia and astrocytes both *in vitro* and *in vivo*. For the *in vivo* study, a hemi-incision model at C5 level in the rat spinal cord was chosen. Our aligned nanofibers/hydrogel scaffold was employed to provide localized and sustained released of miR-219/miR-338 following SCI. Additionally, we also examined the effects of miR-219/miR-338 on OL maturation in the context of astrocyte/OPC scratch assay, an injury mimicking condition *in vitro*. In this setup, aligned electrospun fluorescent poly (ϵ -caprolactone)-based (PCL-DPP-PCL) fibers were used for OPC culture.

5.2 Materials and Methods

5.2.1 Materials

TFE ($\geq 99.0\%$), poly-D-lysine (PDL), poly-L-lysine (PLL), ITS, L-glutamine (G8540), sodium pyruvate (P2258), BSA (A9649), apo-transferrin (T2252), insulin (I6634), sodium selenite (S5261), D-biotin (B4501), hydrocortisone (H0888), Triton X-100, NaOH, NGS, poly (ϵ -caprolactone) (PCL), 3,4-Dihydroxy-L-phenylalanine (DOPA), Bicine, NaCl, and fluoromount were purchased from Sigma–Aldrich. Cell strainer (70 μm) was purchased from BD Biosciences, USA. Sterile screening pouch (20 μm) was purchased from Sefar American, USA. Rat-tail Collagen type I (10.08 mg/ml) and T75

flasks were purchased from Corning. DMEM (11960-044), DMEM high glucose (11995065), DMEM GlutaMAX (10569-010), 0.25% Trypsin (25200-072), 1x PBS (10010-023), Penicillin-Streptomycin (P/S, 15140-122), and FBS (11995-065) were purchased from Gibco. PDGF-AA (100-13A) and bFGF (100-18B) were purchased from Peprotech. TransIT-TKO was purchased from MirusBio. 10x PBS was purchased from 1st Base, Singapore. LIVE/DEAD® cell viability Kit, DAPI (1:1000 dilution), Dulbecco's phosphate-buffered saline (DPBS), HBSS, 10x PBS, scrambled negative miR (NEG-miR), miR-219, miR-338-3p, and miR-338-5p mimics were purchased from Life Technologies. RNeasy Mini Kit was purchased from Qiagen. M-MLV Reverse transcriptase was purchased from Promega. IQ SYBR Green Supermix was purchased from Bio-Rad. Primers were purchased from Integrated DNA Technologies. Paraformaldehyde (PFA), mouse anti-integrin α M (CD11b) (clone Ox-42, CBL1512Z, 1:200 dilution), rabbit anti-oligodendrocyte transcription factor 2 (Olig-2, AB9610, 1:500 dilution), and rat anti-myelin basic protein (MBP, MAB386, 1:100 dilution) were purchased from Millipore. Rabbit anti-glial fibrillary acidic protein (GFAP, Z033401, 1:2000 dilution) was purchased from Dako. Alexa Fluor 488-conjugated Goat Anti-Rabbit/Rat IgG (1:500 dilution), Alexa Fluor 555-conjugated Goat Anti-Mouse IgG (1:1000 dilution), and Alexa Fluor 633-conjugated Goat Anti-Rabbit IgG (1:500 dilution) and Phalloidin 488 (1:500 dilution) were purchased from Invitrogen.

5.2.2 Scaffold fabrication

5.2.1.1 Aligned PCLEEP nanofibers/hydrogel scaffold

Aligned PCLEEP nanofibers: The PCLEEP copolymer ($M_w = 40,509$, $M_n = 17,288$) was synthesized according to previously reported method^{133,134}. PCLEEP (25 % wt.%) was dissolved in TFE and electrospun using the two-pole air gap electrospinning technique¹³⁵. In brief, the spinning solution was loaded into a 1 ml syringe that was capped with a 21-gauge blunt-tipped needle. This solution was then charged at + 10 kV and released at a flow rate of 1.9 ml/h. The PCLEEP fibers were deposited within a 5.0 cm air gap area that was between two stationary collector poles (- 5.0 kV). Each set of fibers was obtained after 1 min of spinning. These fiber bunches were then sterilized under UV light for 30 min.

Aligned PCLEEP nanofibers/hydrogel scaffold: The aligned PCLEEP nanofibers/collagen hydrogel scaffolds were fabricated according to our reported method²⁰⁰. Briefly, 10x PBS, collagen type I, 1.0 N NaOH, and DI water were added into a sterile microtube and mixed properly to get a final collagen concentration of 6.0 mg/ml. For miR incorporation, miRs and TKO were complexed in DMEM for 15 min at room temperature. Following that, the complex (29 vol%) was added into the collagen mixture in place of DI water. The detailed components for each scaffold are listed in Table 5.1. 7.5 μ l of miRs (100 μ M) was the maximum amount of miRs that could be added into 100 μ l of the collagen mixture without causing the precipitation of collagen. To obtain scaffolds with aligned nanofibers, a sterilized cylinder mold (5.0 mm in length and 4.5 mm in inner diameter) was pre-set with 4 sets of fibers in the

core region prior to the addition of the collagen mixture. The scaffolds were then incubated at 37 °C for 45 minutes for hydrogel formation. Thereafter, the scaffolds were frozen at - 80 °C then lyophilized and kept at - 20 °C until used.

	Name of experimental groups	Component volume (μl)/scaffold				Number of animals
		miR (100 μM)		TKO	DMEM	
		NEG-miR	miR-219: miR-338-3p: miR-338-5p (1:1:1 volume ratio)			
1	NEG-miR	3.75	-	7.5	17.86	N = 3
2	miR-219/miR-338	-	7.5	7.5	14.11	N = 3

Table 5.1. Experimental groups *in vivo*

5.2.1.2 Aligned PCL-DPP-PCL nanofibers

The polycaprolactone co-di(thiophene-2-yl)-diketopyrrolopyrrole (PCL-DPP-PCL) polymer (average Mw = 38,300) was synthesized according to previously reported method²¹⁰. To fabricate aligned nanofibers, PCL (12 wt.%) and PCL-DPP-PCL polymer (2 wt.%) was first mixed and dissolved in TFE. The solution was then loaded into a 3 ml syringe that was capped with a 21-gauge blunt-tipped needle. This solution was then charged at + 10 kV and released at a flow rate of 1.5 ml/h. Fibers were collected on a negatively charged rotating plate (- 4 kV, 2500 rpm) at a distance of 14 cm away from the needle tip. Thereafter, the scaffolds were punched into circular shape with a

diameter of 16 mm to fit 24-well tissue culture plates for downstream cellular studies.

5.2.3 Poly-DOPA and PDL coating of the PCL-DPP-PCL nanofibers

Poly-DOPA coating of the PCL-DPP-PCL nanofibers was performed following our published protocol ²¹¹. Briefly, the scaffolds were pre-wet with 70 % ethanol for 15 min followed by DI water for another 15 min at 150 rpm on an orbital shaker. Thereafter, they were incubated in 0.5 mg/ml DOPA that was dissolved in poly-DOPA coating buffer (10 mM Bicine and 250 mM NaCl, pH 8.5) at 150 rpm, room temperature, for 4 h. The scaffolds were then washed with DI water 3 times, 30 min/time, and lyophilized overnight. Subsequently, the poly-DOPA coated scaffolds were further coated with PDL ²¹². In particular, the scaffolds were incubated in 5.0 µg/ml of PDL diluted in DPBS at 37 °C and 100 rpm overnight. On the next day, the scaffolds were washed with sterile 1x PBS twice for 15 min each time, and then immersed in DMEM until cell seeding.

5.2.4 Scaffold characterization

The aligned PCL-DPP-PCL nanofibers and aligned PCLEEP nanofibers/collagen hydrogel scaffolds were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 120 s and 140 s, respectively. Thereafter, the samples were observed under the scanning electron microscope (SEM; JEOL, JSM-6390 LA) at an accelerating voltage of 10 kV. The fiber diameters were measured using the ImageJ software (<http://imagej.nih.gov/ij/>) on the SEM images. At least 100 fibers were measured.

5.2.5 Preparation of PLL coated coverslips for microglia culture

Coverslips for microglia culture were pre-coated with PLL according to previously reported method ²¹³. The coating solution was prepared by dissolving PLL in DPBS (40 µg/ml). Coverslips were then incubated in the coating solution for 1.5 h at 37 °C. Thereafter, the coverslips were washed with sterile DI water 3 times, air-dried in the culture hood, and kept at room temperature until used.

5.2.6 Animals

Adult female (6-8 weeks, 200-250 g) and time-pregnant Sprague Dawley rats were obtained from In Vivos Pte Ltd (Singapore). All experimental procedures were approved by the Institutional Animal Care and Use Committee, Nanyang Technological University (IACUC, NTU). All methods were performed in accordance with the relevant guidelines and regulations.

5.2.7 Spinal cord injuries and scaffold implantation

Spinal cord injury in rats was performed according to our reported protocol ²⁰⁰. A 1/3 incision was made on the right side of the spinal cord at C5 level (Fig. 5.1A). Thereafter, a 1.0-mm long scaffold was implanted into the lesion site such that the fibers were parallel to the long axis of the spinal cord. Animals were randomly divided into two treatment groups as presented in Table 5.1. At day 4 post-implantation, rats were anesthetized and 4 mm-portion of the spinal cord at the injury site was taken out and kept in ice-cold HBSS until used for real time-PCR (Fig. 5.1A).

Figure 5.1. Experimental set-up.

(A) Schematic illustration of scaffold-implanted spinal cord samples that were collected for PCR. (B) The set-up of astrocyte scratch assay-OPC co-culture.

5.2.8 Primary cell isolation and culture

Cells from P1-2 neonatal rat cortices were isolated according to our reported protocol¹⁹⁷. Thereafter, these cells were seeded in T75 flasks at a density of 10^7 cells/flask. The DMEM20S medium, which comprised of DMEM, 20 % FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 µg/ml streptomycin, was used and replaced every 3 days. After 10 days of culture, microglial was purified by shaking the cell culture flasks on an orbital shaker for 30 min at 37 °C, 250 rpm. The purified microglia were then seeded onto 13 mm glass coverslips at a density of 20,000 cells per coverslip and

cultured in the glial medium that comprised of DMEM GlutaMAX, 10 % FBS, and 1 % P/S.

After harvesting microglia, fresh DMEM20S medium was added into the flasks. OPCs were then collected by shaking the flasks for 18–20 h at 37 °C, 200 rpm. Following that, the cell suspension was passed through a 20 µm sterile screening pouch. The purity of OPC by using this approach was ~ 96%¹⁹⁷. Thereafter, the OPCs were seeded onto the DOPA-PDL coated PCL-DPP-PCL scaffolds at a density of 50,000 cells per scaffold and cultured in DMEM high glucose supplemented with modified SATO²¹⁴, 1 % P/S, ITS, 0.5 % FBS, and freshly prepared 10 ng/ml of PDGF-AA and 10 ng/ml of bFGF. On the next day, scaffolds were flipped over and placed on top of the astrocyte culture as shown in Fig. 5.1B for astrocyte scratch assay-OPC co-cultures.

The remaining flasks, after replaced with the glial medium, were further shaken for 6-8 h at 37 °C, 250 rpm. Following that, the medium was removed, the flasks were rinsed with PBS and trypsinized for 5 min to isolate astrocytes. Astrocytes were then seeded onto 13 mm glass coverslips at a density of 20,000 or 50,000 cells per coverslip for astrocyte and scratch assay cultures, respectively, and cultured in the glial medium.

5.2.9 miR transfection

MiRs and TKO were complexed in DMEM for 15 min at room temperature. At 24 h after cell seeding, 100 µl of the miR/TKO complex was added into the cell cultures. As listed in Table 5.2, different miR:TKO ratios

(1:1, 1:2, and 1:4) were used. With respect to the *in vivo* study, the miR-219/miR-338 (1:4) group was added here to serve as a group of extremely high dosage of transfection reagent TKO, in order to provide us more insights into any possible effects of TKO on glial cells.

	Name of experimental groups	miR:TKO ratio	Component volume (μl)/coverslip			
			miR (100 μM)		TKO	DMEM
			NEG-miR	miR-219: miR-338-3p: miR-338-5p (1:1:1 volume ratio)		
1	Untreated	-	-	-	0	100
2	NEG-miR	1:1	0.25	-	0.25	99.5
3	miR-219/miR-338 (1:1)	1:1	-	0.25	0.25	99.5
4	miR-219/miR-338 (1:2)	1:2	-	0.25	0.5	99.25
5	miR-219/miR-338 (1:4)	1:4	-	0.25	1.0	98.75

Table 5.2. miR/TKO complex components

5.2.10 Cell viability assay

At day 2 post-transfection, three coverslips from three independent cultures for each experimental group were collected for live-dead assay using

LIVE/DEAD® cell viability kit following the manufacturer's protocol. All samples were examined and captured using a Leica DMI8 inverted microscope and ImageJ was used to quantify the images. At least 500 cells were counted for each group.

5.2.11 Immunohistochemistry

At indicated time points, coverslips and scaffolds were fixed with 4% ice-cold PFA for 20 and 30 min, respectively. Thereafter, the samples were permeabilized with 0.3% Triton X-100 in 1x PBS for 15 min and then blocked in 10 % NGS for 1 h at room temperature. Subsequently, the samples were incubated with primary antibodies that were diluted in 5 % NGS at 4 °C overnight followed by secondary antibodies, which were diluted in PBS for 1.5 h at room temperature. The following primary antibodies were used: rabbit anti-GFAP, mouse anti-Ox-42, rabbit anti-Olig-2, and rat anti-MBP. Secondary antibodies were Alexa Fluor 488-conjugated Goat Anti-Rabbit/Rat IgG, Alexa Fluor 555-conjugated Goat Anti-Mouse IgG, and Alexa Fluor 633-conjugated Goat Anti-Rabbit IgG. DAPI was used for nuclear staining. Thereafter, all samples were mounted and examined using a Leica DMI8 inverted microscope and a Zeiss LSM800 inverted confocal microscope.

5.2.12 Evaluation of cell morphology

Cell morphology was assessed at day 2 and 4 post-transfection on GFAP⁺ astrocytes and Ox-42⁺ microglia. Specifically, a total of 150 cells per sample group (50 cells/coverslips, 3 coverslips/sample group) were outlined to

measure cell perimeter and area using ImageJ. Cell shape index (CSI) was defined as following:

$$CSI = \frac{P^2}{4\pi A}$$

where P is the cell perimeter and A is the cell area²¹⁵. A CSI value of 1 indicates a perfect circular cell shape while a larger value suggests a more ramified morphology.

5.2.13 Evaluation of gene uptake efficiency

Cy5-labeled oligonucleotides (Cy5-ODN) were used to visualize miR uptake by astrocytes and microglia. Cy5-ODN were synthesized with scrambled sequences: 5'-Cy5-CCTACGCCACCAATTTCTGTTT-3' (sense); 5'-ACGAAATTGGTGGCGTAGGTT-3' (antisense). At 24 h after cell seeding, Cy5-ODN/TKO complexes were added into the culture as listed in Table 3. At day 2 post-transfection, cell-seeded coverslips were washed with 1x PBS, fixed with 4% ice-cold PFA for 20 min, and then permeabilized with 0.3% Triton X-100 in 1x PBS for 15 min at room temperature. Thereafter, the samples were incubated with Phalloidin 488 and DAPI at 37 °C for 30 min. All samples were then mounted and examined using a Zeiss LSM800 inverted confocal microscope. The proportion of Cy5-ODN⁺ cells was expressed as a percentage of DAPI⁺ cells. At least 100 cells were counted for each group.

	Name of experimental groups	Component volume (μl)/coverslip		
		Cy5-ODN (100 μM)	TKO	DMEM
1	Untreated	0	0	100

2	Cy5-ODN only	0.25	0	99.75
3	Cy5-ODN (1:1)	0.25	0.25	99.5
4	Cy5-ODN (1:2)	0.25	0.5	99.25
5	Cy5-ODN (1:4)	0.25	1.0	98.75

Table 5.3. Cy5-ODN/TKO complex components

5.2.14 Scratch assay

5.2.13.1 *Astrocyte scratch assay*

Astrocytes were seeded onto 24-well sized coverslips at a density of 50,000 cells per coverslip and cultured in the glial medium until confluent. Thereafter, a scratch with a cross shape was made per coverslips using 2 glass pipettes held together at a distance of 0.5 cm apart. The cells were then transfected with the same condition as stated above. The cells were harvested at day 2 and 4 post transfection for immunostaining and PCR analysis.

5.2.13.2 *Astrocyte/OPC scratch assay*

OPC-seeded scaffolds were placed onto the astrocyte culture plates right after astrocytes were scratched and transfected as shown in Fig. 5.1B. The OPC-seeded scaffolds were fixed at day 4 and 7 post OPC seeding for immunostaining.

5.2.15 Real-time PCR

Spinal cords at day 4 post-implantation or cells at day 2 and 4 post-transfection were lysed by Trizol[®] reagent and RNA was extracted using RNeasy Mini Kit (Qiagen). Thereafter, 500 ng of RNA was used for reverse

transcription using M-MLV transcriptase according to the manufacturer's protocol. Real-time PCR was carried out using iQ SYBR Green Supermix in a StepOnePlus™ system (Applied Biosystems, USA) with the following program: 10 min at 95 °C, 15 s at 95 °C followed by 1 min at 59 °C for 40 cycles. The sequences of the primers are shown in Table 5.4. All our primers showed similar amplification efficiency under the parameters used. Hence the $\Delta\Delta C_t$ method was used for fold change analysis. All the results were normalized by the NEG-miR group, in which cells were transfected with NEG-miRs.

	Gene		Sequence (5'-3')
1	GAPDH	Forward	TCTCTGCTCCTCCCTGTTCT
		Reverse	TACGGCCAAATCCGTTTACA
2	18S	Forward	GCAATTATTCCCCATGAACG
		Reverse	GGCCTCACTAAACCATCCAA
3	RPL13A	Forward	GTGGTGGTTGTACGCTGTGA
		Reverse	CGAGACGGGTTGGTGTTTCAT
4	Sox6	Forward	AATGGCGAGGATGAAATGGAAG
		Reverse	GCAAACAAAAGCTCCTCAGTTG
5	Hes5	Forward	ATGCGTCGGGACCGCATCAAC
		Reverse	GGCGAAGGCTTTGCTGTGCTTC
6	PDGFRα	Forward	GTTGGAGCTTGAGGGAGTGAA
		Reverse	AAAATCCGATAGCCCGGAGG
7	FoxJ3	Forward	CAACCTTCCAGAACGAGATAAA
		Reverse	TGCTAGGAGAGTGCTGATAG

8	ZFP238	Forward	TGAAGACGAAGGCCGAAGATGAC
		Reverse	AGGGGCTGGCTACTGTTTTTCC
9	TNFα	Forward	CCACACCGTCAGCCGATT
		Reverse	TCCTTAGGGCAAGGGCTCTT
10	IL-1α	Forward	ACATCCGTGGAGCTCTCTTTACA
		Reverse	TTAAATGAACGAAGTGAACAGTACAGATT
11	IL-1β	Forward	GCTGTGGCAGCTACCTATGTCTTG
		Reverse	AGGTCGTCATCATCCCACGAG
12	GFAP	Forward	TGGCCACCAGTAACATGCAA
		Reverse	CAGTTGGCGGCGATAGTCAT
13	Vimentin	Forward	GCGAGAGAAATTGCAGGAGGA
		Reverse	CGTTCAAGGTCAAGACGTGC
14	Serpina3	Forward	CTCTCAGGTGGTCCACAAGG
		Reverse	GAGGGTACAGTTTCGCAGACA
15	Olig2	Forward	TAACTCTTTGGCTGAACGAACA
		Reverse	CAGTGCTCTGCGTCTCTTCTAA
16	NG2	Forward	GTTGCTCTGGGGTTGGTTAATT
		Reverse	CCATATACTCCAGGGCTTCCAT
17	CNP	Forward	ATGCCCAACAGGATGTGGTG
		Reverse	GATGAGGGCTTGTCCAGGTC
18	MBP	Forward	TTCTTTAGCGGTGACAGGGGTG
		Reverse	GACTACTGGGTTTTTCATCTTGGGTC
19	MAG	Forward	ACAGCGTCCTGGACATCATCAACA
		Reverse	ATGCAGCTGACCTCTACTTCCGTT

Table 5.4. Sequences of primers for realtime-PCR.

5.2.16 Statistical analysis

All data are presented as mean $\pm$ standard deviation (S.D.). Student t-test was used to compare the results of a group at two different time points. When samples had equal variances, differences between groups were examined using one-way ANOVA followed by the Turkey post-hoc test. Otherwise, the Kruskal-Wallis and Mann-Whitney *U*-tests were used.

5.3 Results

5.3.1 Evaluation of gene expression *in vivo*

5.3.1.1 Effect of miR-219/miR-338 on downstream targets

Sox6, Hes5, PDGFR α , ZFP238, and FoxJ3 are known downstream targets of miR-219/miR-338^{93,94}. Thus, we examined their expression at 4 days post-implantation of the miR-219/miR-338-encapsulated scaffolds. Day 4 post-implantation was chosen as time point to evaluate gene expression according to previous related studies^{93,195,197}. In general, as compared to the scrambled NEG-miR group, miR-219/miR-338 treatment resulted in successful knockdown of their downstream targets. Specifically, when treated with miR-219/miR-338, 3 out of 5 downstream targets, namely Sox6, Hes5, and ZFP238, were significantly down-regulated at 4 days post-implantation (Fig. 5.2A). A general decrease in FoxJ3 expression was seen, while the expression of PDGFR α was increased (Fig. 5.2A).

Figure 5.2. Gene expression at day 4 post-implantation.

(A) miR-219/miR-338 downstream targets (B) Oligodendrocyte lineage markers. (C) Microglia activation markers. (D) Astrocyte activation markers. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (N = 3).

5.3.1.2 Effect of miR-219/miR-338 on OL lineage

As shown in Fig. 5.2B, there was no change in the expression level of Olig2 among the experimental groups. Meanwhile, NG2, an OPC marker, was markedly up-regulated in the miR-219/miR-338 group as compared to the NEG-miR group (1.6 vs. 1.0, $p \leq 0.01$, Fig. 5.2B). However, the expression levels of the middle and late OL markers (CNP, MBP, and MAG) were all significantly decreased with miR-219/miR-338 treatment (Fig. 5.2B).

5.3.1.3 Effect of miR-219/miR-338 on microglia activation

As illustrated in Fig. 5.2C, miR-219/miR-338 suppressed the expression of pro-inflammatory cytokines *in vivo*. In particular, when treated with miR-

219/miR-338, $\text{TNF}\alpha$ was significantly down-regulated as compared to the NEG-miR group at day 4 post-implantation (0.1 vs. 1.0, $p \leq 0.05$, Fig. 5.2C). In addition, a general decreased in $\text{IL-1}\beta$ expression was seen (Fig. 5.2C). On the other hand, the expression of $\text{IL-1}\alpha$ appeared unaltered in both the experimental groups (Fig. 5.2C).

5.3.1.4 Effect of miR-219/miR-338 on astrocyte activation

MiR-219/miR-338 treatment did not enhance the expression of markers of astrocyte activation (Fig. 5.2D). In fact, GFAP was significantly knocked-down when treated with miR-219/miR-338 as compared to the NEG-miR group (0.5 vs. 1.0, $p \leq 0.05$, Fig. 5.2D). The results suggested that miR-219/miR-338 diminished astrocyte activation *in vivo*.

5.3.2 *In vitro* evaluation of cell viability after miR transfection

As depicted in Fig. 5.3, non-viral miR transfection significantly decreased the survival rate of microglia *in vitro* after 2 days. However, miR-219/miR-338 transfection showed no additional effect on the viability of microglia since there was no observed difference between treatments with NEG-miR vs. miR-219/miR-338 (1:1) (Fig. 5.3). In addition, increasing the amount of transfection reagent, TKO, did not further affect microglia viability (Fig. 5.3).

Figure 5.3. Microglia live-dead assay results.

(A) The Untreated group. (B) The NEG-miR group. (C) The miR-219/miR-338 (1:1) group. (D) The miR-219/miR-338 (1:2) group. (E) The miR-219/miR-338 (1:4) group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (N = 3).

Similarly, miR-219/miR-338 transfection showed no effect on the viability of astrocytes since there was no observed difference between the NEG-miR and the miR-219/miR-338 (1:1) groups (Fig. 5.4). However, unlike microglia, increasing the amount of TKO decreased astrocyte viability significantly (87.8 % of the miR-219/miR-338 (1:1) group vs. 70.9 % of the miR-219/miR-338 (1:4) group, $p \leq 0.01$, Fig. 5.4).

Figure 5.4. Astrocyte live-dead assay results.

(A) The Untreated group. (B) The NEG-miR group. (C) The miR-219/miR-338 (1:1) group. (D) The miR-219/miR-338 (1:2) group. (E) The miR-219/miR-338 (1:4) group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (N = 3).

5.3.3 Evaluation of *in vitro* gene uptake

As illustrated in Fig. 5.5B and F, 58.3% of microglia took up Cy5-ODN in the absence of the transfection reagent, TKO. Furthermore, there was no significant difference in the percentage of microglia that took up Cy5-ODN between treatments with Cy5-ODN alone vs. Cy5-ODN (1:1) (Fig. 5.5B, C and F). However, the percentage of microglia that took up Cy5-ODN significantly increased to 98.3 % and 98.2 % in the Cy5-ODN (1:2) and Cy5-ODN (1:4) groups, respectively ($p \leq 0.05$, Fig. 5.5C-F). Contrary to the microglia, without TKO, astrocytes did not take up Cy5-ODN (Fig. 5.6B and F). In addition, higher amounts of TKO resulted in significantly higher percentages of astrocytes that took up Cy5-ODN (Fig. 5.6C-F).

Figure 5.5. Cy5-ODN uptake by microglia.

(A) The Untreated group. (B) The NEG-miR group. (C) The miR-219/miR-338 (1:1) group. (D) The miR-219/miR-338 (1:2) group. (E) The miR-219/miR-338 (1:4) group. (F) Quantification of microglia that took up Cy5-ODN at day 2 post transfection. * $p \leq 0.05$ (N = 3).

Figure 5.6. Cy5-ODN uptake by astrocytes.

(A) The Untreated group. (B) The NEG-miR group. (C) The miR-219/miR-338 (1:1) group. (D) The miR-219/miR-338 (1:2) group. (E) The miR-219/miR-338 (1:4) group. (F) Quantification of astrocytes that took up Cy5-ODN at day 2 post transfection. * $p \leq 0.05$ (N = 3).

5.3.4 Evaluation of *in vitro* gene expression of miR-219/miR-338's

downstream targets

In this study, *in vitro* gene expression was examined at 2 days post-transfection because it was the time point generally used in literature for target gene knockdown evaluation. While the timing of 4 days post-transfection was chosen to correspond with the timing of the *in vivo* study. We demonstrated here the capacity of miR-219-338 to knock down their downstream targets in both microglia and astrocytes (Fig. 5.7).

The downstream targets of miR-219/miR-338, namely Sox6, Hes5, PDGFRA α , ZFP238, and FoxJ3, were expressed at low levels in microglia. As shown in Fig. 5.7A, only ZFP238 gene expression of the miR-219/miR-338 (1:4) group was significantly down-regulated at 2 days post-transfection as compared to the NEG-miR group (0.7 vs. 1.0, $p \leq 0.05$). Meanwhile, the expression of PDGFR α was elevated when treated with miR-219/miR-338

(1:2) (1.5 vs. 1.0, $p \leq 0.05$, Fig. 5.7A). Furthermore, no significant difference in the expression of these downstream targets was observed among the miR-219/miR-338 treated groups (Fig. 5.7A). On the other hand, Sox6, Hes5, and FoxJ3 expression were significantly decreased at 4 days post-transfection when treated with miR-219/miR-338 (1:4) (Fig. 5.7B). Moreover, Sox6 is the only target that was down-regulated in the miR-219/miR-338 (1:1) group while only Hes5 was down-regulated in the miR-219/miR-338 (1:2) group (Fig. 5.7B). The expression of PDGFR α and ZFP238 appeared unaltered regardless of the treatments (Fig. 5.7B).

Similarly to microglia, the miR-219/miR-338 downstream targets were also expressed at low levels in astrocytes. As shown in Fig. 5.7C, the miR-219/miR-338 (1:2) treatment successfully knocked down 4 out of 5 downstream targets, except for Hes5. Meanwhile, three of them, namely Sox6, ZFP238, and FoxJ3, were down-regulated when treated with miR-219/miR-338 (1:4) (Fig. 5.7C). ZFP238 was the only target that possessed a significant decrease in gene expression when treated with miR-219/miR-338 (1:1) as compared to the NEG-miR group (0.6 vs. 1.0, $p \leq 0.05$, Fig. 5.7C). On the contrary, only Hes5 expression was markedly suppressed at day 4 post-transfection in the miR-219/miR-338 groups (Fig. 5.7D). Moreover, the silencing efficiency of the miR-219/miR-338 (1:4) group was higher than that of the miR-219/miR-338 (1:2) group (0.2 vs. 0.6, $p \leq 0.05$, Fig. 5.7D).

According to our *in vitro* results so far, there was not much difference on gene uptake and downstream targets' expression among the miR-219/miR-

338 treated groups. Furthermore, miR-219/miR-338 (1:1) treatment gave the best cell viability as compared to other miR-219/miR-338 groups. Thus, miR-219/miR-338 (1:1) treatment was chosen for the subsequent *in vitro* experiments.

Figure 5.7. Gene expression of miR-219/miR-338 downstream targets at 2 and 4 days post-transfection. (A, B) Microglia culture. (C, D) Astrocyte culture. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (N = 3).

5.3.5 Effect of miR-219/miR-338 on glial cell morphology and gene

expression of activation markers *in vitro*

5.3.5.1 Microglia

It has been known that the change in morphology of microglia²¹⁶ and astrocytes²¹⁵ is indicative of their activation status. Thus, to have a better understanding of the effect of miR-219/miR-338 on the activation of glial cells, cell morphology analysis were performed. As shown in Fig. 5.8, there was no significant difference in microglial morphology, in terms of CSI value and cell

surface area, among the experimental groups at both day 2 and 4 post-transfection.

Figure 5.8. Microglia morphology at day 2 and 4 post transfection. (A, D) The Untreated group. (B, E) The NEG-miR group. (C, F) The miR-219/miR-338 (1:1) group. (G, H) Quantification of microglia CSI and cell area at day 2 and 4 post transfection. [#] $p \leq 0.05$ versus the same group at day 2 (N = 3).

As depicted in Fig. 5.9A, there was no difference on microglial expression of the pro-inflammatory cytokines, $\text{TNF}\alpha$, $\text{IL-1}\alpha$, and $\text{IL-1}\beta$, among the experimental groups at 2 days post-transfection. However, they were all down-regulated significantly at day 4 when treated with miR-219/miR-338 as compared to the NEG-miR group ($p \leq 0.05$, Fig. 5.9B). Taken together, the

results suggested that miR-219/miR-338 inhibited microglia activation and thus calming down their pro-inflammatory responses *in vitro*.

Figure 5.9. Gene expression of microglia and astrocyte activation markers at 2 and 4 days post-transfection.

(A, B) Microglia culture. (C, D) Astrocyte culture. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (N = 3).

5.3.5.2 Astrocytes

On the other hand, miR-transfected astrocytes possessed higher CSI values at day 2 with respect to the Untreated group (Fig. 5.10A-C, and G). Moreover, the CSI distributions of these groups clearly skewed toward higher values (Fig. 5.10I). At day 4, these morphological changes became significant

($p \leq 0.05$, Fig. 5.10D-G and J). This indicated that miR-transfected astrocytes adopted more ramified morphologies that are often associated with astrocyte activation. In addition, the CSI value of the miR-219/miR-338 (1:1) group was significantly higher than that of the NEG-miR group (6.2 vs. 5.1, $p \leq 0.05$, Fig. 5.10E-G). Meanwhile, no significant difference was observed in cell area of astrocytes among the experimental groups at day 2 (Fig. 5.10A-C and H). However, cell areas of astrocytes were markedly decreased when treated with miR-219/miR-338 (1:1) as compared to the Untreated group at day 4 (1061.7 vs. 2217.9 μm^2 , $p \leq 0.05$, Fig. 5.10E, F, and H).

As illustrated in Fig. 5.9C and D, miR-219/miR-338 suppressed the expression of markers of astrocyte activation *in vitro*. In particular, the miR-219/miR-338 (1:1) treatment resulted in a general decrease in GFAP expression at both time points with respect to the NEG-miR group, although it was not statistically significant (Fig. 5.9C and D). Meanwhile, the decrease in gene expression of Vimentin was seen at day 2 (Fig. 5.9C) and became significant at day 4 ($p \leq 0.05$, Fig. 5.9D). In addition, Serpina3 was knocked-down at both time points as compared to the NEG-miR group ($p \leq 0.05$, Fig. 5.9C and D).

Figure 5.10. Scratch assay at day 2 and 4 post transfection.

(A, D) The Untreated group. (B, E) The NEG-miR group. (C, F) The miR-219/miR-388 (1:1) group. (G, H) Quantification of astrocyte CSI and cell area at day 2 and 4 post-transfection. (I, J) CSI distribution at day 2 and 4 post transfection. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ versus other groups at the same time point. # $p \leq 0.05$, ## $p \leq 0.01$, and ### $p \leq 0.001$ versus the same group at day 2 (N = 3).

5.3.6 Effect of miR-219/miR-388 on astrocyte migration and gene expression in scratch assay

In the scratch assay, a gap of 500 μm was created on each confluent layer of astrocytes. As depicted in Fig. 5.11A, D, and G, the gap sizes of the

Untreated group drastically decreased by 8.6-fold and 29.4-fold at 2 and 4 days post-transfection. Thus, these gaps were mostly closed at day 4 with the gap size of only $5.7 \pm 8.5 \mu\text{m}$. In addition, only the Untreated and NEG-miR groups possessed significant declines in the gap distance at day 4 with respect to day 2 (Fig. 5.11A, B, D, F, and G). Meanwhile, non-viral miR transfection reduced the extent of gap closure significantly at both time points as compared to the Untreated group (Fig. 5.11). However, there was no observed difference between the NEG-miR and the miR-219/miR-338 (1:1) groups (Fig. 5.11B, C, E, F, and G). On the other hand, miR-219/miR-338 treatment, in general, did not affect gene expression of their downstream targets as well as astrocyte activation markers with respect to the Untreated group in the scratch assay at both time points (Fig. 5.12).

Figure 5.11. Scratch assay at day 2 and 4 post-transfection.

(A, D) The Untreated group. (B, E) The NEG-miR group. (C, F) The miR-219/miR-338 (1:1) group. (G) Quantification of the gap distance at day 2 and 4 post-transfection. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (N = 3).

Figure 5.12. Gene expression of miR-219/miR-338's downstream targets and astrocyte activation markers in scratch assay. (A, B) 2 days post-transfection. (C, D) 4 days post transfection. * $p \leq 0.05$ (N = 3).

5.3.7 PCL-DPP-PCL scaffold characterization

Fig. 5.13 shows the morphology and appearance of electrospun PCL-DPP-PCL fibers. Bead-free and uniformly aligned nanofibers with an average diameter of $2.0 \pm 0.3 \mu\text{m}$ were obtained. This diameter was chosen based on our previous study¹⁹⁵. In that study, we showed that upon miR-219/miR-338 incorporation, $2.0 \mu\text{m}$ aligned fibers enhanced OL maturation significantly more than 300 and 700 nm aligned fibers¹⁹⁵. In addition, the PCL-DPP-PCL fibers were highly fluorescent in a uniform manner during the course of this study.

Figure 5.13. Morphology and appearance of electrospun PCL-DPP-PCL fibers.
 (A) Light microscope. (B) Confocal microscope at 568 nm wavelength.

5.3.8 Evaluation of OPC maturation and differentiation in astrocyte scratch assay-OPC co-culture

We next examined OPC reactions in response to astrocytes that have been transfected with miR-219/miR-338. Once OPCs and astrocytes were both transfected, higher portions of MBP⁺ cells were detected in the miR-219/miR-338-treated group as compared to the NEG-miR group at both day 4 and day 7 (Fig. 5.14B, C, E, F, and G). Meanwhile, no significant difference in the percentage of MBP⁺ cells was observed between the Untreated and the NEG-miR groups at day 4 (Fig. 5.14A, B, and G). Yet, at day 7, NEG-miR treatment significantly reduced MBP expression with respect to the Untreated

group (7.4 % vs. 14.4 %, $p \leq 0.05$, Fig. 5.14D, E, and G). Overall, *in vitro* OL maturation was markedly promoted when treated with miR-219/miR-338.

Figure 5.14. MBP immunostaining of mature OLs in scratch assay at day 4 and 7 post OPC seeding (OPCs and astrocytes were co-transfected). (A, D) The Untreated group. (B, E) The NEG-miR group. (C, F) The miR-219/miR-338 (1:1) group. (G) Quantification of the number of MBP⁺ cells at day 4 and 7 post OPC seeding. . * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ versus other groups at the same time point. # $p \leq 0.05$, ## $p \leq 0.01$, and ### $p \leq 0.001$ versus the same group at day 2 (N = 3).

On the other hand, we also performed the co-culture when only astrocytes were transfected as the control. The results showed that there was no significant difference in the percentage of MBP⁺ cells among all the miR-treated groups at both time points (Fig. 5.15B, C, and E-G). In addition, treatment of miRs, regardless of NEG-miR or miR-219-338, significantly decreased MBP expression with respect to the Untreated group at both time points ($p \leq 0.05$, Fig. 5.15). Thus, non-viral miR transfection in astrocytes inhibited OL maturation *in vitro*.

Figure 5.15. MBP immunostaining of mature OLs in scratch assay (only astrocytes were co-transfected).

(A, D) The Untreated group. (B, E) The NEG-miR group. (C, F) The miR-219/miR-338 (1:1) group. (G) Quantification of the number of MBP⁺ cells at day 4 and 7 post OPC seeding. . * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ versus other groups at the same time point. # $p \leq 0.05$, ## $p \leq 0.01$, and ### $p \leq 0.001$ versus the same group at day 2 (N = 3).

5.4 Discussion

Following SCI, a dramatic loss of OLs occurs, which frequently results in axon demyelination¹⁹⁴. Despite the increase in OPC proliferation in

response to the injuries, axon remyelination remains suboptimal due to unknown factors that inhibit oligodendrocyte differentiation and maturation^{194,196-198}. Our previous work showed that miR-219/miR-338 cocktail, coupled with fiber topography, was able to enhance OL differentiation and maturation¹⁹⁷. In addition, we developed a nanofiber platform that can provide localized and sustained release of drugs and nucleic acid molecules for non-viral transfection after SCI²⁰⁰. Thus, a potential strategy for SCI treatment is to combine miR-219/miR-338 with our scaffold design. However, besides OPCs, the biological responses following SCI involve many other cell types in the CNS, such as microglia and astrocytes. Yet, there is no report on miR-219/miR-338's effects on these cells to date. Given this knowledge gap, this study was done to identify any potential effects that miR-219/miR-338 may have on microglia and astrocytes.

Sox6, Hes5, PDGFR α , ZFP238, and FoxJ3 are proteins involved in promoting OPC proliferation and inhibiting OL differentiation^{93,94}. Thus, by inhibiting the production of these genes, miR-219/miR-338 promotes OL differentiation^{93,94}. As shown in Fig. 5.2A, the implantation of miR-219/miR-338-encapsulated scaffolds resulted in the significant knockdown of 3 out of 5 downstream targets, except PDGFR α and FoxJ3, as compared to the NEG-miR group at 4 days post-injury. Hence, in this study, we demonstrated the capacity of our scaffold in miR delivery and gene silencing *in vivo*.

NG2 is widely used as a reliable marker for OPCs²¹⁷. Here we showed that NG2 expression level significantly increased when treated with miR-

219/miR-338 as compared to the scrambled NEG-miR treatment (Fig. 5.2B). This observation is in accordance with a study by Fan *et al.* demonstrated that miR-219 promoted the differentiation of neural precursor cells into OPCs ²¹⁸.

With the success in knocking down the OPC-expressed proliferation-promoting genes, the miR-219/miR-338-incorporated scaffolds were expected to enhance OL differentiation. However, as depicted in Fig. 5.2B, the expression levels of the middle and late OL markers (CNP, MBP, and MAG) extensively decreased in the miR-219/miR-338 group with respect to the NEG-miR group. Unfortunately, the reason for this observation remains unknown.

Microglia and astrocytes are activated in response to nerve injuries ^{202,219}. Depending on the environmental stimuli, activated microglia would acquire the pro-inflammatory phenotype ^{202,220,221}. This phenotype is associated with the increase in expression and release of pro-inflammatory cytokines, such as TNF α , IL-1 α , and IL-1 β ^{202,220}. As illustrated in Fig. 5.2C, our miR-219/miR-338-incorporated scaffolds markedly decreased the expression of TNF α at day 4 post-implantation. Additionally, *in vitro* microglial expression levels of these three pro-inflammatory cytokines were all dropped significantly at 4 days post-transfection when treated with miR-219/miR-338 (Fig. 5.9B). Conclusively, these results revealed that miR-219/miR-338 hindered microglia activation and thus calming down their pro-inflammatory responses. Furthermore, a study by Yang *et al.* showed that treatment of IL-1 receptor antagonist for the first 3 days after SCI in rats markedly reduced

injury-induced apoptosis²²². Thus, miR-219/miR-338 treatment may be beneficial in reducing cell death following nerve injuries.

Similarly to microglia, astrocytes also undergo various alterations in gene expression upon activation²²³. In particular, GFAP, Vimentin, and Serpina3 expressions are usually up-regulated in reactive astrocytes^{223,224}. These reactive astrocytes seal off the lesion site by forming glial scar, which in turn acts as a physical barrier and thus inhibiting axonal regeneration^{219,225}. Our results showed that astrocytes were more quiescent when treated with miR-219/miR-338, since *in vivo* expression level of GFAP was significantly decreased at 4 days post-implantation (Fig. 5.2D). Moreover, treatment of miR-219/miR-338 *in vitro* also diminished the expression of Vimentin and Serpina3 as compared to the NEG-miR group at day 4 post-transfection (Fig. 5.9D). Taken together, these results implied that miR-219/miR-338 suppressed astrocyte activation despite of the observed morphological changes. As such, miR-219/miR-338 treatment may be helpful in suppressing/delaying the glial scar formation.

The *in vitro* scratch assay has been employed to mimic cell migration during wound healing *in vivo*^{226,227}. Here, we used this assay to study the effect of miR-219/miR-338 on astrocyte migration in the injury-mimicking condition. Only the Untreated and NEG-miR groups possessed significant long distances of gap closure at day 4 as compared to day 2 post-transfection (Fig. 5.11A, B, F, G, and K). Meanwhile, when treated with miR-219/miR-338, the gaps remained widely opened and did not seal overtime (Fig. 5.11C-E, H-K).

These observations suggested that miR-219/miR-338 might be the factor that hindered astrocyte migration.

In addition, we also found that miR-219/miR-338 did not affect viability of both microglia and astrocytes at 2 days post-transfection. Specifically, there was no difference in their survival rates between treatments with miR-219/miR-338 vs. scrambled NEG-miR that were delivered with the same amount of transfection reagents (Fig. 5.3 and 5.4). However, transfection reagent TKO appeared to be toxic on astrocyte, but not microglia, within the range tested since higher amounts of TKO decreased astrocyte viability (Fig. 5.4). As such, the miR dosage and miR/TKO ratios used for nerve injury treatment must be monitored and adjusted in order to not affect other cell populations in the CNS.

OLs require close interactions with axons for precise myelination in the CNS. Thus, it is challenging to separately examine neuronal and oligodendroglial effects on myelination in their *in vitro* co-culture²²⁸. To resolve this, nanofibers mimicking axons can be employed to investigate only features of OLs that are related to myelination. A study by Lee *et al.* showed that 60% of OL lineage cells ensheathed large fibers of 2.0 - 4.0 μm diameter, while only ~ 5% ensheathed 0.2 - 0.4 μm fibers²²⁸. In addition, 2.0 μm aligned fibers promoted more MBP expression as compared to 300 nm or 700 nm aligned fibers¹⁹⁵. Taken together, in this study, we decided to use high fluorescence and photostable PCL-DPP-PCL aligned fibers with an average diameter of $2.0 \pm 0.3 \mu\text{m}$ for OPC culture and visualization²¹¹ (Fig. 5.13).

MBP is a known marker for mature OLs. Here, we evaluated the percentage of MBP⁺ cells as an indicator of the extent of OL maturation. Once OPCs and astrocytes were both transfected with miR-219/miR-338 (1:1) in the co-culture, OL maturation increased by 2.3 folds as compared to the NEG-miR treatment at day 7 (Fig. 5.14E-G). Meanwhile, a study by Diao *et al.* showed that when treated with miR-219/miR-338, MBP expression of OPC culture was increased by ~ 2.0 folds¹⁹⁵. Thus, miR-219/miR-338-transfected astrocytes seem to further support the maturation of miR-219/miR-338-transfected OPCs. On the other hand, when only astrocytes were transfected, there was no difference in the number of MBP⁺ cells between the NEG-miR and miR-219/miR-338 groups (Fig. 5.15). These observations implied that miR-219/miR-338 need to be acquired directly by OPCs in order to support OL maturation.

Additionally, the numbers of MBP⁺ cells of both miR-transfected groups were significantly lower than that of the Untreated group when only astrocytes were transfected (Fig. 5.15). Thus, TKO, via its effects on astrocytes, seems to be harmful for OL maturation. Moreover, most commercially available transfection reagents are latently cytotoxic. Therefore, for our future study, we would like to thoroughly search for reliable non-toxic transfection reagents and then using them to incorporate miR-219/miR-338 into our scaffold platform to minimize undesired effects.

5.5 Conclusions

The microglia and astrocyte cellular responses toward miR-219-338 were investigated in this study aiming to provide insights into the translational applications of these miRs treatment for nerve injuries. In particular, miR-219-338 reduced microglial expression of pro-inflammatory cytokines and suppressed astrocyte activation both *in vivo* and *in vitro*. Furthermore, these miRs also inhibited astrocyte migration in scratch assay. In addition, miR-219/miR-338 treatment promoted OPC maturation in the astrocyte/OPC scratch assay co-culture. However, the extent of this promoting effect varied with miRs/TKO ratios. Therefore, in order to exploit the therapeutic application of miR-219/miR-338 for nerve injury treatment, the dosage of miRs and miR/transfection reagent ratio must be wisely optimized.

Chapter 6. Conclusions & Future works

In this work, we introduced the three-dimensional hybrid scaffold as a potential platform for SCI treatment. Its aligned nanofibers provided topographical signals that effectively directed neurite extensions and neovascularization into the lesion sites. The scaffold also provided good host-implant integration, as it did not trigger additional inflammatory response and scar tissue formation. Importantly, our scaffold provided localized and sustained release of drugs and nucleic acid molecules for non-viral transfection to enhance axon regeneration and remyelination *in vivo*. Since non-viral gene delivery was employed, the application of our scaffolds may be translated to the clinical setting without raising biosafety concerns associated with viral-mediated methods.

The long-term goal of this study is to eventually develop a nanofiber construct that is capable of enhancing remyelination in the CNS following nerve injuries. Hence, miR-219/miR-338, the regulators of oligodendrocyte differentiation and maturation, were incorporated into our scaffold platform and implanted into rats. We successfully demonstrated the capacity of our scaffold in miR delivery and gene silencing *in vivo*. As a result, OPC maturation was significantly enhanced at the early stage after SCI under miR-219/miR-338 treatment. In future study, evaluation of cellular responses and behavior tests will be performed to investigate the ability of our miR-219/miR-338-incoproated scaffolds in supporting functional neuronal reconnections, remyelination, and functional recovery after SCI.

The C5 incision SCI model was chosen in this study as it is repeatable, thus allowing us to carry out preliminary morphometric evaluation and focus on critical factors affecting nerve regeneration, such as neurite extension, cell penetration, revascularization, host responses, and host–implant integration⁷⁵. However, it was not sufficiently severe to result in significant differences in rat locomotion among experimental groups. Therefore, future studies should involve a complete transection SCI model to examine the effectiveness of our scaffolds in promoting functional recovery.

The microglia and astrocyte responses to miR-219/miR-338 were investigated in this study aiming to provide insights into the translational applications of these miRs for nerve injury treatment. In particular, miR-219/miR-338 diminished microglial expression of pro-inflammatory cytokines and suppressed astrocyte activation both *in vivo* and *in vitro*. Furthermore, these miRs also inhibited astrocyte migration in scratch assay. Taken together, we demonstrated that the non-specific uptake of miR-219/miR-338 by microglia and astrocytes might cause undesired effects that potentially interfere with the regeneration process. As such, for future translational RNAi technology, reliable methods for targeted gene knockdown might be required to minimize undesired gene uptakes and effects.

Our current knowledge of many biological mechanisms is based mainly on studies of cells cultured on flat, 2D substrates²²⁹. However, *in vivo*, cells live in a complex environment that consists of different cell populations which constantly communicate with each other and an assortment of cell-secreted

factors. Thus, the simplicity of 2D culture might not closely mimic the cellular behaviours *in vivo*. Accordingly, miR-219/miR-338-encapsulated 3D scaffolds might be employed to provide insights into the process of axonal remyelination that would not have been observed in 2D systems.

Besides miR-219/miR-338, there are other miRs found to involve in the OL development. In particular, miR-7a maintains OPCs at the proliferation stage by inhibiting the expression of myelin genes ¹⁹⁹. Since ~ 50 % of local OPCs are lost after SCI ²³⁰, miR-7a treatment would be explored to replenish the OPC pool. On the other hand, miR-23a also supports OL differentiation of OPCs ¹⁹⁹. Thus, future studies can focus on finding the combination of miRs that possesses the best synergistic effects on promoting nerve repairs.

Figure 6.1. Schematic summary of the future works

List of Publications

1. Nguyen, L. H.; Gao, M.; Lin, J.; Wu, W.; Wang, J.; Chew, S. Y., Three-dimensional aligned nanofibers-hydrogel scaffold for controlled non-viral drug/gene delivery to direct axon regeneration in spinal cord injury treatment. *Scientific Reports* **2017**, *7*, 42212. DOI: 10.1038/srep42212, <https://www.nature.com/articles/srep42212#supplementary-information>.
2. Wu, X.; Tan, Y. J.; Toh, H. T.; Nguyen, L. H.; Kho, S. H.; Chew, S. Y.; Yoon, H. S.; Liu, X.-W., Stimuli-responsive multifunctional glyconanoparticle platforms for targeted drug delivery and cancer cell imaging. *Chemical Science* **2017**, *8* (5), 3980-3988. DOI: 10.1039/C6SC05251G.
3. Milbreta, U.; Nguyen, L. H.; Diao, H.; Lin, J.; Wu, W.; Sun, C.-Y.; Wang, J.; Chew, S. Y., Three-Dimensional Nanofiber Hybrid Scaffold Directs and Enhances Axonal Regeneration after Spinal Cord Injury. *ACS Biomaterials Science & Engineering* **2016**, *2* (8), 1319-1329. DOI: 10.1021/acsbiomaterials.6b00248.
4. Nguyen, L. H.; Diao, H. J.; Chew, S. Y., MicroRNAs and their potential therapeutic applications in neural tissue engineering. *Advanced Drug Delivery Reviews* **2015**, *88*, 53-66. DOI: <http://dx.doi.org/10.1016/j.addr.2015.05.007>.

List of Conferences

1. World Biomaterials Congress (WBC) **2016**, Montreal, Canada. Poster presentation: ***A three-dimensional hybrid scaffold as an in vivo-imitative platform to direct migration, maturation, and differentiation of oligodendrocyte precursor cells.***
2. Tissue Engineering and Regenerative Medicine International Society (Termis) **2015**, Boston, USA. Poster presentation: ***Scaffold mediated miR interference to direct and enhance axonal regeneration and remyelination after spinal cord injury.***

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