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**BIOMIMICKING SCAFFOLDS FOR NEURAL TISSUE
REGENERATION AND INNATE IMMUNOMODULATION**

By

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University

in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarized materials and has not been submitted for a higher degree to any other University or Institution.

23rd August 2019

Date

Lin Junquan

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the writing and research are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

23rd August 2019

Date

Assoc. Prof Chew Sing Yian

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal where I was an author. It also contains material that was partially contributed by other fellow lab members as indicated below.

Chapter 3 has been submitted to the journal *ACS Applied Materials and Interfaces* (In Revision) as Junquan Lin, William Ong, Wai Hon Chooi, Na Zhang, Marie E. Bechler, Charles ffrench-Constant, Sing Yian Chew. Molecularly oriented and sustained protein expression on electrospun fibers for evaluating functionality of cells.

The contributions of the co-authors are as follows:

- Assoc. Prof Chew Sing Yian and Marie E. Bechler generated the hypothesis
- William Ong, Wai Hon Chooi, Na Zhang and I performed the experiments and analyzed the data.
- Assoc. Prof Chew Sing Yian and I wrote the manuscript.
- Marie E. Bechler and Charles ffrench-Constant edited and proofread the manuscript.
- Assoc. Prof Chew Sing Yian guided the project and provided the funding.

Chapter 4 was published as U Milbreta, J Q Lin, C Pinese, W Ong, J S Chin, H Shirahama, R Mi, A Williams, M E Bechler, J Wang, C french-Constant, A Hoke, S Y Chew, 'Scaffold-mediated sustained, non-viral delivery of miR-219/miR-338 promotes CNS remyelination', *Molecular Therapy*, <https://doi.org/10.1016/j.ymthe.2018.11.016>.

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- Assoc. Prof Chew Sing Yian guided the project and provided the funding.

Chapter 5 has been submitted to the journal *Biomaterials Science* (In revision) as Junquan Lin, Dollaporn Anopas, Ulla Milbreta, Jiah Shin Chin, Na Zhang, Wutian Wu, Seng Kwee Wee, Adela Tow, Wei Tech Ang, Sing Yian Chew. Regenerative rehabilitation: Exploring the synergistic effects of rehabilitation and implantation of a bio-functional scaffold in enhancing nerve regeneration.

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- Assoc. Prof Chew Sing Yian provided the funding.

Non-viral delivery of microRNAs to modulate host-implant integration by regulating foreign body reaction in Chapter 6 has been contributed by various co-authors and will be submitted to the journal *Advanced Healthcare Materials* shortly.

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- Prof David Becker and his lab provided advice and resources for sample preparations
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ABSTRACT

The application of tissue engineering for replacing damaged, injured or diseased tissues has come a long way since conceived in the late 1980s. Currently, scaffold-based treatments, as compared to cell-based treatments, pose minimal risks following implantation and enable versatile fabrication procedures. Electrospun synthetic fibers are one of the many avenues for attaining a scaffold and be easily modified to affect neural cell behaviour and function. For example, polycaprolactone fibers coated with poly-DOPA, a mussel-inspired bioadhesive, for affixing Protein A and subsequently recombinant L1 Cell Adhesion Molecule (L1CAM) through specific interactions led to enhanced axonal growth. Additionally, when L1CAM was replaced with Neuregulin-1 type III (Nrg1 type III), a known enhancer of myelination, oligodendrocytes (OLs) began to display better myelination in terms of myelin sheath length. The reverse, where OLs myelinated poorly, occurred when Nrg1 type III is further replaced with Junctional Adhesion Molecule 2 (JAM2), a known inhibitor of myelination. Besides surface modification, these electrospun synthetic fibers can also be assembled into a 3 dimensional (3D) scaffold which can delivery therapeutics in a sustained fashion when implanted into spinally injured rats. Therapeutics delivered, such as microRNA-219 (miR-219) and microRNA-338 (miR-338), which have been shown to promote the differentiation of oligodendrocyte progenitor cells (OPCs) during development, were further demonstrated to be able to increase the differentiation and myelination of OPCs in an injury. When the scaffold was loaded with a pro-regenerative factor (Neurotrophin-3 (NT-3)) instead and coupled with rehabilitation, animals showed increased motor recovery and force exertion. Lastly, these fibers can also be encapsulated with miRs for directing macrophage polarization, which may possibly be associative with the formation of fibrous capsule. With the delivery of miR-124, it was

observed that fibrous encapsulation and blood vessels formation can be limited and enhanced respectively, which are indicative of host-implant integration. This thesis describes the endeavour towards treating spinal cord injury. It is hoped that through this series of experiments, readers will be able to gain some insight into not only the difficulties present but also most importantly, a possible way forward. In this work, I have chosen biocompatible, synthetic electrospun fibers as the scaffolding material for supporting all the biological components (cells and tissues). I show that these fibers can be loaded with small nucleic acids such as microRNAs and, depending on their orientation (aligned or randomly oriented), can be used for directing cellular behaviour. Taken together, tissue engineering has limitless potential for tackling neural-related deficits.

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CHAPTER 1

INTRODUCTION

1.1. STATEMENT OF PROBLEM

Central nervous system (CNS) deficits, especially those resulting from severe traumatic injuries, remains untreatable and highly debilitating. As axons are crucial for signal conduction which eventually innervates and dictate motor function, much focus were commonly placed on their extension and restoration of synaptic connections. However, this approach is similar to viewing the shadow of a 3D object and often misrepresents the complexity of the problem. This is evident from studies that despite robust anatomical recovery of mature axons and in some cases, descending neural tracts which control locomotion such as the rubrospinal and corticospinal tracts, functional recovery is never complete. In truth, traumatic injuries in the CNS are multifaceted and have to be tackled from all possible aspects. For example, one of these facets may involve target cell types. If we assume that all cells within the body have a certain function for maintaining homeostasis, then we can be sure that restoration of the various glial cells damaged in the injured CNS tissue is necessary to achieve normalcy. On a similar note, other considerations may entail the re-training of lost motor functions as well as the delivery of growth and survival factors that were present previously but reduced following injury. To this end, the employment of tissue engineering techniques and rehabilitation for augmenting conventional therapeutics administration following CNS nerve injury has the potential to enhance functional outcomes.

1.2. HYPOTHESIS

In this thesis, the various factors that are potentially useful for CNS nerve regeneration both in form and function were investigated using tissue engineering strategies. We hypothesize that traumatic CNS nerve injury requires a multipronged treatment approach and that rehabilitation coupled with scaffolds functionalized with the appropriate therapeutics can enhance the behaviour of both neurons and neuroglial cells for achieving anatomical and functional regeneration of the compromised CNS tissue.

1.3. OBJECTIVES

This thesis aims to explore the various potential treatment options (protein enhancers, RNA interference, rehabilitation) for traumatic CNS nerve injury and evaluate the fundamental science behind these approaches using a rodent spinal cord injury (SCI) as a model via the:

1. Investigation of functionalized fibrous scaffold in effecting neural cells
 - a. Fabrication of electrospun scaffold for surface functionalization
 - b. Detailed characterization of functionalized scaffold
 - c. Testing of platform using primary neural cells
2. Evaluation of the potential of neural glial cells for enhancing spinal cord regeneration
 - a. Fabrication of scaffold for therapeutic delivery and implantation
 - b. Assessing the effects of RNA interference on oligodendroglial lineage cells
3. Exploration of the effect of rehabilitation coupled with regenerative measures for improving functional outcomes
 - a. Rehabilitating spinally injured rats
 - b. Evaluating the effects of rehabilitation on anatomical and locomotor recovery
 - c. Conducting and analyzing behavioural tests

4. Examination of immunomodulation as an avenue for boosting tissue recovery
 - a. Fabrication of fibrous scaffold for therapeutic delivery and implantation
 - b. Evaluating the effects of RNA interference on fibrous encapsulation and blood vessel formation
 - c. Assessing the effects of RNA interference on macrophages

1.4. SCOPE OF THESIS

Chapter 1 provides a succinct description of the statement of problem. Subsequently, the hypothesis and objectives of this thesis are presented.

Chapter 2 provides the relevant literature review of this thesis. These include the history of tissue engineering and the fundamental properties a biomaterial scaffold should possess before application. Besides that, the review also includes the common biomaterials used for tissue engineering as well as the various methods of scaffold fabrication. Following the description of the concepts of tissue engineering, the review proceeds to introduce their application for the regeneration of neural tissues and the additional therapies that should be incorporated in order to achieve better functional outcomes.

Chapter 3 investigates the effect of protein enhancers on the functions of neural cells, specifically on cortical neurons' axonal outgrowth and oligodendrocytes' extent of myelination. These were all conducted on an aligned, biomimicking electrospun fiber platform for better correlations with the *in vivo* setting. Additionally, a molecular orientor, Protein A, is added for accessing protein configuration-based effects on neural cells. Overall, I show that for instances requiring receptor and protein binding, the configuration of the

protein presented to the receptor is important in determining if the outcome can be enhanced. This effect is dependent on both the protein of interest and the cell of interest.

Chapter 4 explores the potential of RNA interference for enhancing myelination in an injury setting. Specifically, miR-219/miR-338 is loaded into a 3D hybrid electrospun fiber collagenous scaffold, implanted into spinally injured rats and evaluated for their effects on oligodendroglial lineage cells through immunohistochemical staining and electron micrography. I demonstrate that during a spinal cord injury, miR-219/miR-338 is able to preserve the viability, enhance the rate and extent of differentiation and promote myelination of oligodendroglial lineage cells. Furthermore, these miRs do not affect glial scar formation and neurite outgrowth.

Chapter 5 examines the combinatorial regenerative and rehabilitative approach for enhancing functional recovery following spinal cord injury. This work involves the implantation of a pro-regenerative NT-3 loaded 3D hybrid electrospun fiber collagenous scaffold into spinally injured rats followed by 12 weeks long of intense rehabilitation. Our results show that rats that underwent rehabilitation had better axonal ingrowth into the scaffold, increased percentage of anti-inflammatory macrophages, reduced percentage of pro-inflammatory macrophages, enhanced ankle flexion strength and improved functional recovery.

Chapter 6 investigates the use of miRs to modulate macrophage phenotype, specifically, Let-7c and miR-124 for inducing anti-inflammatory macrophages and Anti-Let-7c for inducing pro-inflammatory macrophages to regulate foreign body reaction. Here,

electrospun fibrous scaffolds containing each of the miRs were implanted subcutaneously into the back of mice and assessed for extent of fibrous encapsulation (thickness) after 2 and 4 weeks. Immunostaining were also conducted to verify the effect of the miRs on macrophage polarization. In all, results show that miR-124 was the best candidate for reducing fibrous capsule thickness and improving blood vessel formation in the within the scaffold. Furthermore, there was an associative relationship between the anti-inflammatory macrophage phenotype and thickness of fibrous capsule at early time points.

Chapter 7 ends off with concluding remarks on all the work shown in this thesis and discuss possible future directions.

CHAPTER 2

TISSUE ENGINEERING AND ITS APPLICATION IN NEURAL REGENERATION

Summary

Tissue engineering has been around for close to three decades since it was first conceptualized in the late 1980s to fulfil an unmet need of organs by patients. Since then, the collective ingenuity of biomedical engineers have laid down the general foundations required for fabricating an ideal scaffold fit for replacing the damaged, injured or diseased tissue. While it seems that tissue engineering has reached the pinnacle, we are apparently way behind when it comes to applying it in the central nervous system (CNS). Complete recovery remains elusive and is currently limited by our knowledge of the diverse cell responses following tissue engineering treatments. Moreover, it is also becoming clear that the restoration of anatomical features will not suffice for attaining complete functional recovery and that rehabilitation must be coupled with regenerative approaches for significantly enhanced outcomes.

2.1. INTRODUCTION

Tissue regeneration, which is synonymous with tissue engineering, is the Holy Grail and pervading concept for treating diseases, injuries and traumas sustained by the human body. This paradigm of restoring the malfunctioning tissue, which often requires the development of biological substitutes using biocompatible supporting scaffold architectures^{1, 2}, is a complete shift from conventional treatments. Usually, the diseased or damaged tissues are replaced with healthy ones either from other parts of the body in the same patient (autograft) or from one individual to another (allograft)³. These techniques revolutionized clinical treatments when it was first demonstrated by Joseph Murray in 1954⁴ and have since found great success in offering a second chance for patients faced with life-threatening events such as serious burns⁵, failing kidneys⁶, broken skull⁷, etc. However, there remains several notable problems. Firstly, harvesting autografts is costly, painful, constrained by anatomical limitations and may lead to donor-site morbidity due to infections and/or hematoma³. Additionally, patients receiving allografts and transplants are subjected to the risks of rejection by their own immune system as well as to the risks of infection or disease from the donor. To overcome tissue rejection, patients may need to be administered immunosuppressive medications but doing so may lead to other immune-related complications. Lastly, the availability of tissues or organs for transplantation is limited and is worsening in this rapidly aging world population¹. For certain hard-to-obtain organs such as kidney, heart or liver, patients may spend up to decades on the waiting list before having the opportunity to proceed with the transplantation. Some die waiting for it⁸. Therefore, the solutions to these downsides are to either engineer artificial but functional, biocompatible tissue for replacement or to completely forgo tissue replacement and encourage endogenous tissue regeneration instead. Basically, tissue engineering.

Pioneered by Robert Langer and Joseph Vacanti in the 1990s, the field of tissue engineering is highly multidisciplinary and requires, not limited to, expertise from materials science, life science, clinical medicine and mechanical engineering^{3, 9}. In the most initial approach, tissue engineering involves the utilization of a 3D porous biodegradable scaffold as a supporting structure for cells (*in vitro* cultured) to infiltrate, attach, proliferate and interact, eventually forming a living piece of functional construct after long term *in vitro* culturing for implantation into the diseased or injured region^{3, 8}. Not long after, tissue engineering went on to encompass the implantation of just the cells alone as well as the implantation of scaffolds alone for stimulating *in situ* tissue regeneration^{10, 11}. Naturally, this stratification consists of key differences and presents various strategies one can undertake depending on the pros and cons as summarized in Table 2.1.

Tissue engineering approach	Advantages	Drawbacks
Transplantation of isolated cells or cell substitutes	<ul style="list-style-type: none"> • Can interact easily with host cells • Easy to administer using injectables, minimally invasive 	<ul style="list-style-type: none"> • Low cell survivability • Cells that do not manage to attach will get displaced to other regions of the body • Compatible cells may not be easily obtainable • Not easy to scale up • Not easily approved by regulatory bodies • Low chance to enhance recovery significantly

Implantation of matrices (tissue/organ-like) with cells seeded within.	<ul style="list-style-type: none"> • High cell survivability • Cells on the scaffold do not get displaced • High chance to enhance recovery significantly 	<ul style="list-style-type: none"> • Requires time and specialized equipment to 'grow' <i>in vitro</i>, highly complex process • Requires surgery for implantation • Compatible cells may not be easily obtainable • Not easy to scale up • Not easily approved by regulatory bodies
Implantation of matrices (non-tissue/organ-like) with cells seeded within.	<ul style="list-style-type: none"> • High cell survivability • Cells on the scaffold do not get displaced • Decent chance to enhance recovery significantly 	<ul style="list-style-type: none"> • Requires surgery for implantation • Compatible cells may not be easily obtainable • Not easy to scale up • Not easily approved by regulatory bodies
Implantation of non-cellular matrices derived either naturally or synthetically	<ul style="list-style-type: none"> • No cell issues, relatively easy to scale up, relatively easy to get approval from regulatory bodies 	<ul style="list-style-type: none"> • Requires surgery for implantation • Chance to significantly enhance recovery dependent on tissue of interest

Table 2.1. Comparison between different tissue engineering strategies

2.2. SCAFFOLDS IN TISSUE ENGINEERING

2.2.1. Design criteria

Given the immense advantage offered by the use of a scaffold for either cellular or acellular implantation to enhance recovery, it is imperative to take into consideration the design criteria of the scaffold in order to attain optimal outcomes.

2.2.1.1. *Biocompatibility*

For any scaffold to be applicable in tissue engineering, it must absolutely be biocompatible. This means that the scaffold must allow cells to survive well, whatever the means, as if they are residing in a part of a tissue in the body. Whether the scaffold can promote proliferation, differentiation or mount an immune response is secondary and depends on its intended use. Most importantly, cells must be able to survive in and outside the scaffold.

2.2.1.2. *Biodegradability*

The original intention of the scaffold is for it to act as a temporary supporting structure³ for cells to reside and secrete their own extracellular matrix (ECM) so that it can replace the scaffold eventually¹². The by-products of this degradation process should also be non-toxic and clearable by the body without causing interference to the other body organs.

2.2.1.3. *Biointerfacibility*

Scaffolds do not only act as a physical structure and passively support the residing or infiltrating cells. They can be designed to regulate cellular activity actively through topographical¹³, mechanical^{14, 15} or biochemical means¹⁶. For example, mature cells such as

epithelial cells, myocytes and neurons show dissimilar morphologies and characteristics when cultured in substrates possessing non-native stiffness¹⁷. As such, scaffolds should be designed to be as physically and mechanically biomimicking as possible depending on the architecture of the ECM in the region of application. These considerations commonly include, but not limited to, scaffold pore size, scaffold pore alignment, scaffold stiffness, scaffold geometry, scaffold permeability and scaffold surface chemistry. Besides that, scaffolds can also be modified to contain biological cues such as cell-adhesive ligands or encapsulated with growth factors that can enhance tissue re-growth and regeneration¹⁸.

2.2.2. Biomaterials

Currently, the materials used in tissue engineering, also known as biomaterials, are classified into three individual groups; ceramics, synthetic polymers and natural polymers. Commonly utilized ceramics are hydroxyapatite (HA), β -tricalcium phosphate (TCP), and biphasic calcium phosphate (BCP) – mixture of HA and TCP¹⁹. Generally, bioceramics are characterized with having high mechanical stiffness, low elasticity and hard brittle surface^{3, 19}. Therefore, they are mostly used as a scaffolding material for hard tissue regeneration such as bone since these properties have been shown to enhance osteoblast differentiation and proliferation^{20, 21}. Additionally, other less well-known ceramics such as aluminium oxide (alumina) and zirconium dioxide (zirconia)²² have been used as dental implants in place of titanium, which has been a clinical gold standard for more than three decades²³. However, this class of materials have poor biocompatibility and biodegradability as compared to synthetic and natural polymers, which limits their use clinically²⁴.

Synthetic polymers are widely favoured in tissue engineering as the polymer chains are highly tunable, allowing their properties such as degradation rate and stiffness to be controllable. Materials used extensively for fabricating scaffolds include polycaprolactone (PCL), poly-L-lactic acid (PLLA), polyglycolic acid (PGA) and poly-DL-lactic-co-glycolic acid (PLGA). Besides being biocompatible, the degradation by-product is non-toxic and bioresorbable^{25, 26}. For example, lactic and glycolic acid, produced by PLGA upon hydrolytic degradation, are substances that exist naturally in metabolic pathways of the body and can be resorbed to participate in the cycle²⁷. Another advantage of synthetic polymers is the ease of functionalization with drugs²⁸, proteins²⁹, nucleic acids³⁰ and even vitamins³¹ through either encapsulation or chemical conjugation. Furthermore, the release of these molecules is usually sustained since it is associated with the rate of degradation of the polymers. A limitation of using polymers such as PLLA, PGA or PLGA though, is the dip in local pH, which may be detrimental to surrounding cells³². Besides that, although PCL is a material approved by FDA, it takes years to completely degrade³³, which limits its efficacy as a sustained drug delivery platform for certain relatively short-term applications.

Natural polymers are the first type of biodegradable biomaterials to be used clinically³⁴. They can be further classified into proteins (silk, collagen, gelatin), polysaccharides (cellulose, dextran, chitosan, hyaluronic acid) or polynucleotides (DNA, RNA)³⁵, although polynucleotides are not suitable for scaffold fabrication. Unlike synthetic or ceramic polymers, natural polymers are bioactive and usually promote excellent cell adhesion and proliferation since some of them, such as collagen and hyaluronic acid, are components of the ECM³. Additionally, they are biodegradable, biocompatible and similar to synthetic polymers, can be easily loaded with therapeutics for delivery. Some drawbacks of using natural polymer as a

scaffolding material include immunogenicity, source variability, processing difficulty and poor mechanical properties for hard tissue engineering^{3, 19}.

Of note, while there exist intrinsic limitations in each group of biomaterials, it is becoming increasingly common to fabricate composite scaffolds using materials from at least two of these groups^{36, 37}. This could effectively help to draw out more advantages and compensate drawbacks which would not have been possible if a single material was used.

2.2.3. Fabrication/assembly techniques

Since the 1980s till now, there are multitudes of literature describing many different ways of fabricating/assembling 3D scaffolds for tissue engineering applications. Depending on the intent (to replace whole organs, to replace part of a tissue, etc.) and tissue/organ of interest (bone, bladder, cartilage, spinal cord), it is crucial to select the relevant technique(s) so that the scaffold produced will conform to the design criteria stated previously. Currently, there are three major scaffolding methods that have been described.

2.2.3.1. *Pre-made porous scaffold*

Conventionally, 3D porous scaffolds are produced using the solvent casting/particle leaching technique. This method requires porogens to first be dispersed into either natural or synthetic polymeric materials and the resulting mixture is then dispensed into a mold to allow solidification. Subsequently, the porogens are removed from the scaffold and the void left behind will form the pores of the scaffold³⁸. Of note, the porogens used for this method can be gaseous (carbon dioxide), liquid (water), or solid (ice crystals, salt particles)¹⁸. As a result, this strategy has been widely used to fabricate 3D porous scaffolds since it is extremely

amenable for use in a diversified choice of biomaterials. Electrospinning is another technique to obtain 3D porous fibrous scaffolds. Using a high DC voltage, synthetic or natural polymers dissolved optimally in solvent systems can be made to electrostatically repel internally to form fibers of controllable diameters. These fibers can then be collected, piled together and bonded using thermal energy or adhesives to provide a porous meshwork³⁹. Moreover, an advantage of using these electrospun fibers lie in the ease of fabricating scaffolds with aligned pores, which is preferable for applications in tissues that are relatively aligned such as smooth muscles and spinal cord. Besides solvent casting/particle leaching and electrospinning, solid free-form fabrication or rapid prototyping technologies have recently also been gaining recognition as a potent tool to obtain 3D porous scaffolds⁴⁰. These technologies, which include selective laser sintering, stereolithography and 3D printing, rely on precise delivery of light or heat energy to defined points within photopolymerizable materials for crosslinking so that solid 3D structures can be formed^{40, 41}.

2.2.3.2. Decellularized ECM

Naturally derived ECM obtained from allogenic or xenogenic sources are the most biomimicking scaffolds as they possess the original structural, biochemical and biomechanical cues^{18, 42}. However, in order to prevent an immunogenic response from the host tissues while at the same time remain viable for utilization, the ECM has to be fully cleared of cellular and genetic components without altering its protein content and mechanical properties⁴². This is usually achieved through a combination of physical, chemical and enzymatic treatments^{43, 44}. Once the ECM is completely decellularized, it can then be implanted into the body region of interest for the host cells to repopulate. Till date, this technique has been used to recreate

various types of tissues and organs including blood vessel^{45, 46}, heart valve^{47, 48}, kidney^{49, 50}, heart^{51, 52} and many more.

2.2.3.3. Hydrogel

Hydrogels are 3D porous structures that consist of hydrophilic polymers crosslinked through either covalent bonds or physical intramolecular and intermolecular attractions⁵³. Due to their ability to absorb huge amounts of fluid while maintaining its porous structure, hydrogels are ideal for acellular implantation since the host cells can easily infiltrate and adhere within. Alternatively, cultured cells can also be easily encapsulated within the hydrogels before implantation. Commonly used natural polymers for fabricating hydrogels include sodium alginate⁵⁴, agarose⁵⁵, chitosan⁵⁶ and type 1 collagen⁵⁷ while synthetic polymers include polyethylene glycol (PEG)⁵⁸ and polyvinyl alcohol (PVA)⁵⁹.

2.3. TISSUE ENGINEERING FOR NEURAL TISSUE REGENERATION

2.3.1. Factors limiting central nervous system (CNS) regeneration

The capacity for regeneration differs between species as well as between the tissues/organs of a specific species⁶⁰. Generally in mammals, scar-free healing and self-regeneration is only restricted to the early stages of life and lost progressively during development⁶¹. On the other hand, many adult non-mammalian vertebrates retain the ability to regenerate tissues, organs and even limbs after injury⁶². A detailed summary of the regenerative capacity of tissues in different species by Xia et al.⁶⁰ is shown in Figure 1.

Figure 2.1. Tissue regeneration capacity in different species.

As seen in Figure 1, salamanders, being lower vertebrates, are able to regenerate their spinal cord, if injured, throughout their life. Conversely, this ability is only applicable to mouse pups that are younger than P7 given that they are higher vertebrates as compared to salamanders. From this trend, we can thus infer that in humans (highly evolved central nervous system (CNS)), complete regeneration of the injury spinal cord is even less possible. This lack of regeneration is closely associated with the complexity of the CNS where the more

complex it is, the less likely it can fully regenerate or return to its initial state. For clarification, the complexity in this context refers to the interactions between the various cells present in the CNS, especially between neurons where networks of synapses are formed.

Besides being unable to regenerate at a tissue level, not all cell types in the CNS can repopulate themselves as well. The nervous system consists of two cell types: neurons and neuroglia⁶³. Neurons are the functional elements of the nervous system, which is to transmit information from the brain to the nerves that are present throughout the body for innervating the muscles. However, once matured and differentiated, they possess no ability to undergo cell division⁶³. This means that in the event of a disease, trauma or injury, neurons that are lost will not be replenished which as a result, will impair the functionality of the nervous system. On the other hand, neuroglia, glial cells that support neurons metabolically and functionally, have the capacity for mitosis. Yet even so, due to the extremely close interactions between neurons and glial cells, as well as between glial cells themselves, dysregulation of any cell type would almost certainly impair the coherence and functionality of the nervous system as a whole. Not to mention the dysregulation of the entire nervous tissue itself, which occurs in a neurotraumatic injury.

Essentially, the condition of the CNS environment is another factor that dictates regeneration. This is especially true in the sacrosanct CNS where there even exist external controls such as physical barriers (blood-brain/spinal cord barrier⁶⁴ and meninges) to regulate materials traversing between the nervous system tissue and the circulatory system.

Figure 2.2. Pathophysiology of traumatic spinal cord injury (SCI). Damage progression in the spinal cord over time is caused by the initial necrotic debris, the presence of blood components and the immune response. With the help of phagocytic cells to remove the inhibitive debris and astrocytes and fibroblasts to form new extracellular matrix, the damage eventually becomes confined. However, this confinement resulted in the formation of an inhibitive chondroitin sulfate proteoglycan (CSPG)-rich scar which restricts both axonal regeneration and OL remyelination. More detailed description of the pathophysiology can be referred from Ahuja *et al.*⁶⁵.

Naturally, the environment is also regulated internally by the different glial cell types present^{66, 67}. However, the tighter the regulations, the harder it is to restore this balance when it gets disrupted. Over a certain threshold, the initial balance is lost forever and never gets re-established. For instance, in a severe traumatic SCI, the disruption is maximal, with well-established sequence of events following injury. At the initial traumatic impact, the spinal cord may undergo contusion, compression or even transection, which immediately destroys the neurons, glial cells, vasculature system and compromise the blood-spinal cord barrier⁶⁵. Locally, with both the external (physical barriers) and internal (cells) regulators dysregulated, the spinal cord starts to enter a secondary injury cascade, which leads to further protracted damage lasting for weeks to months⁶⁵.

2.3.2. Response to injury

During the secondary injury cascade, biochemical signals of death and destruction continue to ripple outwards radially towards the other surrounding cells in the region of injury. Upon receiving this information, the spinal cord neurons and axons and all the various glial

cells (astrocytes, microglial, oligodendrocytes) begin to respond, albeit incoherently, with the aim of preserving the integrity of the remaining spared tissues. Importantly, knowing how these cells/structures will react in an injury can facilitate treatment strategies that can specifically target certain responses in order to attain a more favourable outcome.

2.3.2.1. *Neurons and axons*

Neurons are the functional asset of the nervous system for transmitting signals and coordinating physical processes and movements for survival. Anatomically, it consists of a cell soma, dendrites for propagating signals received from other neurons and axons for propagating signals to other neurons. Depending on the site of the injury, neurons do not die immediately when their axons are severed. If the trauma occurs far away from the neuronal cell soma, then the neuron will have a high chance of survival. Conversely, too near to the cell soma and the neuron will undergo necrosis or apoptosis rapidly. Attempts to reseal the axolemma by the severed axons occur immediately following the primary trauma so as to restore homeostasis and limit exposure to toxic components in the injured area⁶⁸. For smaller diameter axons that seal faster, their survival rate will be relatively higher as compared to larger diameter axons since during the sealing process, the organelles present in their cytoplasm will not be exposed to as much cytotoxic materials. Despite that, in an extremely severe injury, it is very likely that even if the neurons are spared, they may undergo atrophy which would lead to their retraction over time⁶⁵. Even though neurons are unable to proliferate, they may regenerate or sprout new processes⁶³ once the condition has stabilized and form new synaptic connections⁶⁹ which can be used to initiate locomotion below the level of injury.

2.3.2.1.1. In vitro/In vivo evaluation of axonal regeneration

Axon regeneration is strictly defined by the ability of a severed axon to regrow into or across the lesion site⁷⁰. Naturally, the best method to probe for axonal regeneration is the complete transection of the entire spinal cord as it will clear any doubts regarding the possibility of axonal sprouting instead of regeneration. For both in vitro and in vivo, the most commonly used method for determining axonal regrowth is immunolabeling⁷⁰. This approach is preferred when the axons of interest display unique proteins. For example, matured axons can be immunostained by NF200. On the other hand, Tuj1 is a pan-axonal marker that labels all axons, both immature and matured. There are more markers specific for axons in the CNS in vivo such as 5-hydroxytryptamine (5HT, identifies serotonergic axons), dopamine beta hydroxylase (DBH, stains for cerulospinal projections); as well as Substance P, IB4 and CGRP which labels different subclasses of primary afferents from dorsal root ganglion cells. While these markers are able to depict the exact location of the axons of interest, it is unable to definitively reveal if they originate from severed axons (therefore regeneration). This would not be a problem if a full transection model is utilized. For other spinal cord injury models such as hemisection, contusion or compression, other more robust methods for evaluating axonal regeneration should be conducted. Tract tracing is one such approach and it involves labelling specific axonal tracts originating from different regions of the brain/peripherals with biotinylated dextran amines (BDAs). Descending tracts are mostly derived from the brain and control motor functions while ascending tracts start from the limbs and involve sensory functions. There also exist intraspinal tracts where the axons are neither derived from the brain or the peripherals but within the spinal cord itself. Some of the most commonly studied motor tracts are the corticospinal tract (CST), rubrospinal tract, raphespinal tract, reticulospinal tract and serotonergic projections. Cerulospinal and propriospinal axons

modulate the activity of intraspinal circuitry. The ascending tracts are less studied and are defined collectively as dorsal column sensory axons⁷⁰.

2.3.2.2. Astrocytes

Astrocytes constitute the largest proportion of all cells in the mammalian CNS (around 20 to 40%)⁷¹. They function to control neuronal activity and well-being through neurometabolic coupling⁷² and helps to remove excess neurotransmitters, potassium⁷³ and glutamate⁷⁴ from the extracellular space. Following a traumatic insult, astrocytes become activated or reactive, displaying functional and morphological changes that constitute astrogliosis⁷⁵. Reactive astrocytes begin to upregulate key astrocyte intermediate filaments such as glial fibrillary acidic protein (GFAP) and various secreted factors⁷⁶. Interestingly, their level of reactivity lies on a spectrum and those astrocytes which are 'mildly' reactive only show some signs of cellular processes and nuclear hypertrophy as well as minimal proliferation and domains overlap⁷⁷. However, those astrocytes that are 'highly' reactive exhibit robust upregulation of GFAP, exaggerated hypertrophy, upregulated secretion of cytokines (TGF- β , IL-1 β , IL-6, etc.) and other factors (COX-2, iNOS, S100 β , etc.)⁷⁸. The most intense reaction usually occur right beside lesion area where the astrocytes will proliferate, migrate and align to form a glial scar that surrounds the region⁷⁹. The gradual formation of such a structure around the trauma site leads to both beneficial and detrimental outcomes. Without the glial scar as a physical barrier to contain the cytotoxic components and the peripheral inflammatory macrophages present in the lesion, the surrounding cells may be harmed as well which can lead to much greater damage to the spinal cord tissue⁸⁰. This has been shown previously by Sofroniew *et al.* where genetically ablating astrocytes led to severe consequences post injury^{81, 82}. Unfortunately, this approach of protecting the tissue integrity

will result in the impediment of tissue recovery since axons situated rostrally will not be able to pass through the barrier of astrocytes to form synaptic connections with the axons situated on the caudal side, vice versa. Additionally, the glial scar also contains CSPGs and tenascins which acts to inhibit axonal growth locally⁸³. Currently, there is no good solution to bypass the glial scar 'necessary evil' conundrum. However, reducing the amount of CSPGs present on the glial scar using chondroitinase ABC (chABC) has been shown to improve axonal regeneration and functional axon reconnection⁸⁴, suggesting that strategies for circumvention can be executed even after the formation of the glial scar.

2.3.2.3. Microglial

Being the main immune cell residing in the CNS, microglial are strongly activated during a traumatic injury where excess extracellular damage-associated molecular patterns (DAMPs) such as haemoglobins, purine metabolites and inflammatory factors are present⁸⁰. Similar to astrocytes, microglial exhibit varying levels of activation phenotypes following an injury. Quiescent microglial are ramified, with many long and fine processes; moderately activated microglial are hypertrophic with shorter processes and are mildly inflammatory; fully activated microglial becomes morphologically spherical (amoeboid-like) and exhibit robust inflammatory expressions⁸⁰. The functions of microglial in an injured and dysregulated environment are relatively comparable to peripheral blood derived macrophages, which is to initially facilitate debris clearance followed by tissue healing and wound resolution in the long-term. Within a week post injury, microglial and recruited macrophages make up most of the cells in the epicentre of the lesion site, maintaining their duties to sterilize the area and phagocytose cellular debris⁸⁵. However, unlike the immune response in the non-CNS tissue where excess immune cells will be cleared eventually through apoptosis or circulation⁸⁶, these

cells will persist into the chronic time phase with sustained inflammation⁸⁷, hindering tissue repair and functional recovery. As these immune cells reflect the immunological state of the microenvironment, the persistence of chronic inflammation suggests that the injured CNS is pro-inflammatory. Therapeutics which can alter the immune landscape towards pro-reparative may therefore enhance tissue preservation.

2.3.2.4. *Oligodendrocytes*

Oligodendrocytes (OLs) exist to myelinate axons (enables saltatory conduction) and provide metabolic support for neurons⁸⁸. They are extremely sensitive to a dysregulated environment and die easily if exposed to oxidative materials⁸⁹, excess glutamate and ATP^{90, 91}, pathologic lipids⁹² and inflammatory cytokines⁹³. In just hours following a traumatic injury, spared oligodendrocytes and its myelin components around the lesion start to undergo apoptosis and degeneration respectively⁹⁴. In rats, this phenomenon will persist for at least 3 weeks^{93, 95}. Fortunately in the CNS, there exist endogenous pools of oligodendroglial progenitor cells (OPCs) which have the ability to proliferate, differentiate into OLs and remyelinate spared axons after injury⁹⁶. However, the newly formed myelin sheaths are usually thinner and shorter and easily inhibited by components of previously fragmented myelin sheath (myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG)) as well as proteoglycans (CSPGs, aggrecans and neurocans). Given that dysfunctional myelin formation will affect the rate of signal conduction and subsequently, recovery, therapies to promote remyelination post injury may potentially result in enhanced motor functions.

2.3.3. Treatment using tissue engineering approaches

Nerve regeneration following by functional recovery in the diseased or injured CNS is limited and attempts to enhance this process have proved to be arduous and seemingly impossible. Yet this has not deterred researchers from constantly finding innovative methods to achieve that and using tissue engineering strategies is one of them. Conventional non-tissue engineering treatments usually include administration of neuroprotective drugs^{97, 98} during the onset of the disease or injury and subsequently rehabilitation or drugs that improve lost functions^{84, 99}. While these measures are widely utilized, their effects are usually suboptimal and do not lead to complete functional recovery for severe cases. Given that neural cells and tissue structure are lost in both the diseased and injured state, it makes sense that restoring the entire tissue using tissue engineering concepts will provide the highest chance for attaining full recovery.

2.3.3.1. *Transplantation of stem cells*

Since the successful attempts to reliably culture pluripotent mouse and human embryonic stem cells (ESCs) *in vitro* in 1981 by Evans and Kaufman¹⁰⁰ and 1998 by Thomson et al.¹⁰¹ respectively, many believe that stem cell therapy is the 'silver bullet' which can treat or even cure any diseases and injuries. Rightfully so, pluripotent stem cells can divide indefinitely without senescence and have the potential to, when exposed to defined factors, differentiate into any cells (except placenta cells) found in the body¹⁰². As such, they boast the capacity to replace lost or damaged cells (especially neurons) when transplanted into the defective CNS region, making this method the earliest form of clinical treatment for neurological defects. Specifically, ESCs derived from different species have been used for generating dopaminergic¹⁰³ and tyrosine hydroxylase¹⁰⁴ neurons as well as oligodendrocyte precursors¹⁰⁵ when injected into the CNS neural tissues in animal models. While ESCs hold

great promise in restoring defective tissues, its use has also sparked ethical debates pertaining to the source of derivation, the allowable length of culture *in vitro* and the issue of tumors and teratomas formation *in vivo*. These limitations were circumvented in 2006 when Yamanaka et al. demonstrated the possibility of deriving induced pluripotent stem cells (iPSCs) from adult mouse somatic fibroblast cells¹⁰⁶. This groundbreaking discovery was then later applied in human cells to obtain human iPSCs. Similar to ESCs in its differentiation potential, human iPSCs can be used for potentially treating Parkinson disease¹⁰⁷, for improving motor function following traumatic SCI¹⁰⁸ and for conferring neuroprotective functions in an ischemic stroke model¹⁰⁹. Besides ESCs and iPSCs, neural stem cells (NSCs)¹¹⁰ and mesenchymal stem cells (MSCs) have been used for implantation as treatment strategies as well, although MSCs were meant for playing an immunomodulatory instead of a substitutional role^{111, 112}. An issue with transplanting cells though, is the numerous regulatory barriers present towards clinical relevance. Despite that, various clinical trials using this stem cell transplantation technique is already in motion for treating Parkinson disease^{113, 114}, amyotrophic lateral sclerosis (ALS)^{115, 116} and SCI^{117, 118}.

2.3.3.2. *Implantation of matrices seeded with cells*

Besides regulatory concerns, another major downside of transplanting cells directly into the diseased or injured region of the CNS through injection is the loss of cell viability and failed integration caused by the ailing environment they were supposed to replace^{119, 120}. Therefore, an improved strategy to transplant cells into the body reliably is to encapsulate them within 3D hydrogels, or other forms of matrices which can either be synthetic or natural, before implantation. Correspondingly, the inclusion of such matrices will be able to protect the transplanted cells' survival following implantation by reducing their direct exposure to the

toxic components present in the region, providing a conducive environment for them to thrive and eventually form tissue-like structures. Additionally, these scaffolds will also hold the transplanted cells in place, preventing them from being relocated/washed away by the constant ebb and flow of the cerebrospinal fluid (CSF) as well as can act as a depot for the sustained release of therapeutics for synergistically enhancing the treatment of neurological defects. As a result of the advantages mentioned, this method is currently most promising in achieving anatomical replenishment. Specifically, researchers managed to bridge the entire transected portion of the rat spinal cord by implanting human neural stem cells (hNSCs) encapsulated within growth factors-laden fibrin matrices¹²¹. These hNSCs mostly differentiated into neurons and were even able to extend extremely long distances into the surrounding intact spinal cord tissues¹²¹. As cells are involved, this cell-scaffold implantation method will also be subjected to regulatory scrutiny and there is currently only one reported clinical study underway¹²².

2.3.3.3. *Implantation of matrices alone*

The implantation of porous acellular matrices is another common approach used for tissue regeneration and localized, sustained therapeutics delivery in the CNS. Without the inclusion of stem cells, this method has to rely on the proliferation and migration of the intact nervous tissue, specifically the various types of glial cells, to populate the scaffold. As neurons are unable to divide after development, the implanted scaffold will usually be loaded with growth or survival factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3) to spur surrounding spared neurons to sprout and extend their axons. Alternative, the matrices can also be modified to contain cell binding sites (RGD, IKVAV, etc.) to enhance attachment and growth. Such matrices, when

implanted into the diseased/damaged tissue in the brain, will encourage axonal ingrowth¹²³ and when implanted into the injured spinal cord, can act as a bridge for axons to cross the defect gap^{124, 125}. Additionally, to further augment this concept's clinical relevance, magnetic resonance imaging (MRI) and 3D printing can be used in combination to probe the geometric properties of the lesion site non-invasively before 'printing' the scaffold out precisely for implantation¹²⁶. Till date, biomaterials used for fabricating such scaffold includes agarose¹²⁷, fibrin matrices¹²¹, chitosan^{128, 129}, collagen¹³⁰, gelatin¹³¹, etc. As it is preferential to incorporate topographical cues into the scaffold for enhancing axonal ingrowth, most of them were designed to include aligned channels or pores.

2.4. RNA INTERFERENCE AND THEIR POTENTIAL IN NERVE INJURY TREATMENT

Elucidated by Fire, Mello *et al.* in 1998¹³² and hailed as the 'Breakthrough of the year' in 2002¹³³, RNA interference (RNAi) can be employed as a potent mechanism for overwriting DNA-based regulation in cells, thereby enabling manipulation of cellular functions and behaviour. This was a huge revelation at that time because RNAs have always been thought to be inferior to DNAs and only function to convert genetic information into proteins¹³³. It turns out that RNAs are more than that and are capable of executing RNAi, which is a natural phenomenon that exists even across various kingdoms (in plants, fungi and animals)¹³⁴ for regulating gene transcription and protein translation. Additionally, the mechanism of RNAi is well-established and more information regarding this process can be found in these excellent reviews¹³⁴⁻¹³⁷. Although its role has not been fully explored till date, it is clear that RNAi is rightfully an evolutionary conserved defence mechanism against molecular invaders such as

viruses and transposable elements (TE) where foreign double stranded RNA (dsRNA) were often introduced^{134, 138}. More recently, it has also been recognized that RNAi plays an important role in development¹³⁹ such as neurogenesis^{140, 141}, axonal outgrowth¹⁴²⁻¹⁴⁴ and myelination¹⁴⁵ in the central nervous system (CNS). Given these huge responsibilities for ensuring the smooth progress of such crucial processes, it is almost certain that RNAi is involved in their maintenance as well. Indeed, using RNAi microarray analysis, studies are revealing that microRNAs (miRNAs), naturally occurring components for regulating RNAi, are commonly dysregulated following nerve injuries^{137, 146, 147} and during disease progression¹⁴⁵.

2.4.1. Drivers of RNAi

RNAi is fundamentally triggered by the presence of short sequences of RNA strands, which may be single or double stranded and contains around 19 to 29 nucleotides, within the cell. Currently, the known drivers of RNAi are small interfering RNA (siRNA), microRNA (miRNA), short hairpin RNA (shRNA) and antisense oligonucleotides (ASO). These RNA sequences in their mature form can be easily obtained through commercial means and despite being so similar in their size, each of them have their own uniqueness and efficiency for achieving the intended outcome.

2.4.1.1. *SiRNA*

SiRNAs form the main arm of RNAi and are, in design, the most potent molecule to elicit a transient single gene target knockdown once it interacts with the cell's machinery responsible for eliciting RNAi¹³⁴⁻¹³⁷. Functional siRNAs were observed to be within 21 to 23 base pairs in length and can easily be synthesized *in vitro* by cleaving long dsRNAs into a pool of siRNAs using Dicer¹³⁴. The main mechanism of siRNA in leading to RNAi is gene regulation

where it will bind to perfectly complementary segments of messenger RNA (mRNA) to initiate degradation and prevent transcription¹⁴⁸.

2.4.1.2. *MiRNA*

MiRNAs, unlike siRNAs, exist endogenously. They are transcribed by RNA polymerase II and usually consist of 19 to 25 nucleotides. Contrary to siRNAs, miRNAs induce RNAi by mainly repressing translation¹³⁵, although occasional degradation of mRNA to prevent transcription can occur as well¹⁴⁹. The modulation of protein at the translational level in animals usually stems from their imperfect base-pairing with target mRNAs^{150, 151}. However in plants, their miRNAs generally bind to mRNAs with near-perfect complementarity which leads to target mRNA degradation instead^{152, 153}, which is similar to the mechanism displayed by siRNAs. In a sense, the difference in RNAi regulatory mechanisms between siRNAs and miRNAs is determined by the degree of complementarity during the binding to mRNAs. Yet mind-blowingly, due to the slight mismatch of miRNAs towards mRNAs, miRNAs have no true target mRNAs and can therefore bind to hundreds or even thousands of free mRNAs, resulting in massive amounts of translational regulation. Since their discovery¹⁵⁴, we now know that miRNAs govern physiology at an organism level, especially since they are involved in the entire range of cellular processes such as cell proliferation^{155, 156}, differentiation^{141, 155, 157, 158}, senescence¹⁵⁹⁻¹⁶¹ and even apoptosis^{162, 163}.

2.5. REGENERATIVE REHABILITATION

Generally, neural tissue engineering approaches to regenerate or replace diseased or injured CNS tissues have, in recent years, undergone tremendous progress in terms of the basic knowledge obtained and its applicability in the clinical settings. While this growing field

constitutes a potent tool for regenerative medicine, it is increasingly clear that the focus on anatomical recovery do not fully translate to functional recovery. This is especially true in the CNS where since the neural connections are extremely complex, the growth of new axonal circuits following treatment cannot be expected to attain function immediately, but require their integration into functional networks through use-dependent plasticity^{164, 165}. Correspondingly, in order to allow the newly sprouted axons to form synaptic connections with intact ascending and descending tracts, rehabilitation is necessary.

From this, it becomes apparent that tissue regeneration has to be applied in combination with rehabilitation to achieve maximal beneficial effects since the latter alone remains suboptimal in promoting motor function recovery despite being the gold standard clinical treatment for neural diseases or injuries. Likewise, the various US federal funding agencies have also recently endorsed the assimilation of rehabilitation into tissue engineering, termed regenerative rehabilitation¹⁶⁶, for promoting functional outcomes. Indeed, based on studies from other body regions, the implantation of a 3D scaffold alone for tissue regeneration yielded poorer functional outcomes as compared to a combination of scaffold implantation and rehabilitation^{167, 168}. Currently, there are almost no neural regenerative studies that included rehabilitative regimes and almost no neural rehabilitative studies that included regenerative medicine, which represents a knowledge gap for the combinatorial treatment. Future works on neural tissue regeneration should include both of these disciplines to provide better insights on the applicability of this strategy in the clinics.

2.6. FOREIGN BODY RESPONSE AND HOST IMPLANT INTEGRATION

In tissue engineering where treatments revolve around scaffold implantation as a tissue/organ replacement, a major concern that always exist is the extent of foreign body response induced, which is a process that occurs purely through the interaction between the contents in blood and the implanted material following surgical procedures¹⁶⁹. In the worst case scenario, this event can lead to implant rejection, as indicated by the dense fibrous encapsulation of the implanted material by ECM-producing cells such as fibroblasts as well as a chronically inflamed environment within and/or around the implant¹⁷⁰. Being a vital limitation to the field of tissue engineering, there have been a plethora of studies dedicated to establishing it mechanistically as well as preventing it. Moreover, the formation of fibrosis on top of glial scarring in the engineered CNS tissue is a situation that has to be avoided at all costs. Although it is certain that prevention is not possible, there are a wide array of strategies to attenuate foreign body responses so as to attain better host implant integration.

2.6.1. Modulation through cells

In the sequence of events spanning the entire process of the foreign body response starting from blood protein adsorption on biomaterial, immune cells attachment on biomaterial, initiation of acute and chronic inflammation, formation of foreign body giant cells (FBGCs) to lastly fibrous capsule development¹⁶⁹, macrophages and fibroblasts are the reputed keystone cells that are involved. However, macrophages are the most commonly targeted cell type for modulating the foreign body reaction due to its ability to rapidly respond and transform the immune status of the microenvironment, making them master regulators of this process. Accordingly, one can easily tune their influences by targeting their activation states, either towards classical M1 activation (induced by LPS and IFN- γ) which is pro-inflammatory, or towards alternative M2 activation (induced by IL-4 and IL-13) which is anti-

inflammatory¹⁷¹. Depending on their polarization states, macrophages will secrete cytokines that will not only dictate inflammation, but also wound healing and fibrosis¹⁷². Of note, attempting to utilize macrophages for enhancing host implant integration and wound healing is incredibly complex and cannot be achieved by simply polarizing them towards M1 or M2. Even till now, there have been conflicting results by different groups on which activation state can really prevent fibrosis. Taking into consideration the myriad of cytokines produced by macrophages for orchestrating a single outcome, factors that can lead to a large amount of genotypic changes in them may potentially solve the problem. This concept is based on the assumption that if there exist a specific set of cytokines for promoting wound healing and subsequently prevent fibrosis, then by extensively modulating their gene expression, there will be a greater chance that more of the cytokines within that specific set can be produced to elicit the desired effect. An alternate but less viable option is to include the entire combination of cytokines known to prevent fibrous encapsulation along with the scaffold during implantation. Furthermore, given that the scaffold can be used as a delivery vehicle, these factors can be loaded within for long-term sustained release.

2.6.2. Modulation through intrinsic properties of biomaterials

Since the foreign body response is caused by the interaction between the implanted biomaterial and blood components, it turns out that different types of biomaterials will elicit different responses when implanted¹⁷³. This suggests that the intrinsic surface chemistry and physical properties of implants can be tuned for reducing fibrous encapsulation and enhancing host integration. For example, biologically derived biomaterials prepared by decellularization techniques that largely preserve the native architecture and composition have been shown to, as compared to synthetic biomaterials, promote better tissue

remodelling¹⁷³. Besides possessing unique surface topologies and ligand landscapes¹⁷⁴, these decellularized scaffolds may also attract vastly different molecules to adsorb on its surface, therefore inducing beneficial cellular responses and leading to more favourable responses than synthetic materials¹⁷³. However, as biologically derived biomaterials are not easily obtained, synthetic and also natural biomaterials (collagen, chitosan, cellulose, etc.) may at times be more practical. As scaffolds made from these materials can be fabricated reproducibly, their physical properties can therefore be exploited to ameliorate fibrous encapsulation. Specifically, it has been shown that porous scaffolds with 30-40 μ m pores led to minimal fibrosis and highest vascularity, suggesting great host implant integration^{175, 176}. Additionally, for fibrous scaffolds, randomly aligned fibrous scaffolds are able to elicit better responses as compared to aligned fibrous scaffolds¹⁷⁷.

2.7. CONCLUSION

Tissue engineering has become an inevitable approach for treating diseased or injured tissue/organs in the clinic given how it can seamlessly integrate with other disciplines such as life science, mechanical engineering and materials science to enhance therapeutic outcomes. There are however, certain recommendations to comply with (biocompatibility, biodegradability and biointerfacibility) during scaffold fabrication in order for it to be safe and effective when applied. Given the plethora of materials that fulfil these criteria, the possibilities are nearly limitless. Such potential is especially helpful for CNS tissue regeneration which, till date, remains unsolved over a millennium. Despite that, one major advantage we possess now is the relatively vast amount of knowledge on the individual cell types within the CNS that can be tapped upon, which will definitely be useful for devising more novel and effective treatment strategies. Importantly, a holistic approach, where both

‘form’ and ‘function’ recovery presided by regenerative and rehabilitative concepts respectively, should be considered.

CHAPTER 3

MOLECULARLY ORIENTED AND SUSTAINED PROTEIN EXPRESSION ON ELECTROSPUN FIBERS FOR EVALUATING FUNCTIONALITY OF CELLS

ABSTRACT

A proper protein orientation is often required in order to achieve specific protein-receptor interaction to elicit a desired biological response. As such, having a platform that allows control over the specific display of proteins is crucial in allowing one to understand such protein-receptor interactions. Here, I present a Protein A-based biomimicking platform that is capable of efficiently orienting proteins for evaluating cellular behaviour. By absorbing Protein A onto aligned bio-mimicking polycaprolactone (PCL) fibers, I demonstrate that protein binding could be retained on these fibers for at least 7 days under physiologically relevant conditions. I further show that Protein A served as a molecular orientor to arrange the recombinant proteins in similar orientations. Such protein-orienting scaffolds were further verified to be cyto-compatible and biologically functional by using sensitive primary rat cortical neurons (CNs) and oligodendrocyte progenitor cells (OPCs), as model neural cells for a stringent proof of concept. Specifically, CNs that were seeded on fibers coated with Protein A and a known enhancer of neurite growth (L1 Cell Adhesion Molecular L1CAM) displayed the longest total neurite length ($462.77 \pm 100.79 \mu\text{m}$, $p < 0.001$) and longest neurite length ($352.99 \pm 88.21 \mu\text{m}$, $p < 0.001$) as compared to the controls. Besides that, OPCs seeded on fibers coated with Protein A and Neuregulin-1 Type III (Nrg1 type III)(myelin enhancer) produced the longest myelin ensheathment length ($19.8 \pm 11.69 \mu\text{m}$). In contrast, when Nrg1 type III was replaced with Junctional Adhesion Molecule 2 (JAM2)(myelin inhibitor), the

shortest myelin ensheathment length ($13.57 \pm 6.37 \mu\text{m}$) was obtained. These results demonstrate the efficacy of this platform for protein screening applications.

3.1. INTRODUCTION

Signaling molecules, either in the form of soluble factors or biomolecules that are found on cell surfaces and in the extracellular matrix (ECM), modulate almost every aspect of cellular function.¹⁷⁸ The recognition of a protein by its receptor depends on the availability of binding sites. Additionally, this specific binding can only be fulfilled when there is strong binding affinity and proper orientation. Furthermore, a proper protein orientation is often required in order to achieve specific protein-receptor interaction for eliciting a desired biological response. As such, having a platform that allows control over the specific display of proteins is critical in allowing one to understand such protein-receptor interactions.

Electrospun fibers have been widely used for central nervous system (CNS) tissue engineering to investigate the effects of topographical and biochemical cues on neurite outgrowth as well as myelination^{179, 180}. The choice of using electrospun fibers stems from their bio-mimicking architecture, which resembles the extracellular matrix in neurite outgrowth assays, and artificial axons in the case of myelination. Correspondingly, good correlation could be obtained between *in vitro* drug screening results and *in vivo* delivery outcomes using such electrospun scaffolds¹⁸¹. However, the establishment of a facile yet robust electrospun fiber platform with sustained and controllable presentation of proteins to study protein effects has not been achieved. Conventionally, approaches to display proteins on electrospun fibers include simple methods like direct encapsulation¹⁸² and non-specific surface adhesion¹⁸³ or the more complicated chemical conjugation approach which might not be feasible for most labs without chemistry knowledge. Building on these works, I took a step further to control the orientation of proteins on electrospun fibers by using Protein A.

Protein A is a highly stable surface receptor found in *Staphylococcus aureus* and is widely used for the purification of immunoglobulin Gs (IgGs) from serum¹⁸⁴. Its value lies in the strong binding affinity to the Fc region on IgGs that are produced from certain hosts (e.g. human, rabbit, etc.). As recombinant proteins can be modified to include a Fc region, I hypothesized that Protein A can be utilized as a molecular orientor for orienting the configuration of proteins of interest to better enhance cellular functions. To test the efficacy of this scaffold (based on the criterion that the cells' responses to the proteins used were as reported), I used primary rat cortical neurons (CNS) and oligodendrocyte progenitor cells (OPCs) as a proof of concept.

Within the developing CNS, axons have to traverse long distances to reach their targets in the spinal cord. To achieve that, growth cones formed from the tip of axons have to interact constantly with the environment via cell adhesion molecules (CAMs)¹⁸⁵(the most commonly studied being L1CAM) for guidance. Through these specific protein-receptor interactions, the axon-tract can develop normally; while a lack of these interactions results in severe neural diseases.¹⁸⁶ Neurons, in turn, share a mutualistic relationship with oligodendrocytes (OLs), the glia cells which are responsible for forming myelin sheaths and facilitating saltatory signal conduction in axons¹⁸⁷. Interestingly, despite the presence of many other cell types within the CNS, myelination only occurs exclusively on axons under normal circumstances. Studies have shown that much of this regulation lies in the myelinating OL-neuron interaction through axonal topography¹⁸⁸, neuronal activity¹⁸⁹ and surface protein signalling¹⁹⁰. Specifically for cell surface proteins, Neuregulin 1 type III (Nrg1 type III) was demonstrated to lead to hypermyelination of the axons during development when

abundantly expressed in genetically modified mice¹⁹⁰. On the other hand, Junctional Adhesion Molecule 2 (JAM2) found on the neuronal soma was able to inhibit myelination¹⁹¹. As the fibers used here mimic both the ECM and axons, it could ideally be utilized as a L1CAM-rich matrix to model axonal outgrowth and Nrg1 type III-, JAM2-rich axon to model OL myelination.

In this work, 3,4-dihydroxy-L-phenylalanine (DOPA) was utilized as a versatile bioadhesive¹⁹² for the long term adsorption of proteins onto electrospun fibers. The distribution, availability and configuration of these bound proteins were evaluated using fluorescently tagged antibodies as model proteins. Furthermore, I demonstrated the functionality of these protein-decorated fibers using rat primary neural cells such as CNs and OLs as a proof of concept. These cells were chosen as they are generally more sensitive to the environment and relatively tougher to maintain in culture, hence providing a stringent test criteria for the robustness of this platform.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Polycaprolactone (PCL, MW: 70,000-90,000), 2,2,2-trifluoroethanol (TFE), 3,4-dihydroxy-L-phenylalanine (DOPA), Triton X-100, DNase 1 type IV (DNase), Poly-D-Lysine (PDL), N-Acetyl-L-Cysteine (NAC), D-biotin, ITS, Putrescine, L-Thyroxine (T4), Tri-iodothyronine (T3), progesterone and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was acquired from Hyclone. B-27 supplement (B-27), Neurobasal medium, DMEM high glucose medium (DMEM), natural mouse laminin protein (23017015), penicillin-streptomycin (pen/strep), GlutaMax supplement (GlutaMax), 6x-His Tag antibody (His Tag antibody, R930-25), goat anti-rabbit IgG (H+L) Alexa Fluor 488 antibody

(anti-rabbit AF488 IgG, A27034), rabbit anti-mouse IgG (H+L) Alexa Fluor 555 antibody (rabbit AF555 IgG, A21427), goat anti-mouse IgG (H+L) Alexa Fluor 555 antibody (goat anti-mouse AF555, A21422), goat anti-rabbit IgG (H+L) Alexa Fluor 555 antibody (goat anti-rabbit AF555, A27039) and goat anti-rat IgG (H+L) Alexa Fluor 488 antibody (goat anti-rat AF488, A11006) were obtained from Life Technologies. NS21 supplement was procured from Miltenyi Biotec. Papain suspension was obtained from Worthington. Mouse anti- β III Tubulin (Tuj1, PRB-435P) was purchased from Covance. Rat anti-Myelin Basic Protein (MBP, aa82-87) was obtained from Bio-Rad. Rabbit Oligodendrocyte Transcription Factor 2 (Olig2, AB9610) was purchased from Merck. Protein A was acquired from Peprotech. Recombinant human L1CAM protein (Fc-tagged, 777-NC), recombinant mouse JAM2 protein (Fc-tagged, 988-VJ) and recombinant human Nrg1 type III isoform SMDF protein (378-SM) were procured from R&D Systems.

3.2.2. Preparation of protein functionalized PCL fiber scaffold

The fabrication of PCL fiber scaffolds with an area of approximately 0.78 cm² was carried out as previously described¹⁸⁰. Briefly, fibers were electrospun onto glass cover slips and silicone glue was applied at the edge. Following that, DOPA was used to coat the fibers at a concentration of 0.5 mg/ml for 2 h at room temperature with slight shaking. Thereafter, the poly-DOPA coated fibers were washed thrice with distilled water (DI water) before coating with Protein A (25 μ g/scaffold) for 2 h at 37 °C. For all experiments that required IgG (rabbit AF555 IgG, anti-rabbit AF488 IgG) or protein (L1CAM (1 μ g/scaffold), Nrg1 type III (2 μ g/scaffold), JAM2 (2 μ g/scaffold)) binding, the fibers were first blocked with 5% BSA for 1 h at 37 °C before the addition of the IgG (1 μ g/scaffold) or protein at 37 °C for 1 h. Additionally for Nrg1 type III binding, an adaptor His Tag antibody (1 μ g/scaffold) was adsorbed onto Protein A (37 °C, 1 h) first before Nrg1 type III was added as this specific recombinant protein

lacked an Fc fragment but contained a polyhistidine-tag at its C-terminal. For the control group in the CNs culture, mouse laminin (1 $\mu\text{g}/\text{scaffold}$) was adsorbed directly onto the poly-DOPA coated fibers (37 $^{\circ}\text{C}$, 1 h). The same goes for the control group in oligodendrocyte progenitor cells (OPCs) culture, where Nrg1 type III (2 $\mu\text{g}/\text{scaffold}$) was coated directly on poly-DOPA fibers. PDL (5 $\mu\text{g}/\text{scaffold}$) was then coated last (37 $^{\circ}\text{C}$, 1 h) in all groups before cell seeding. These adsorption procedures are summarized in Table 3.1. Additionally, the coating protocol for evaluating protein binding orientation is compiled in Table 3.2.

Naming convention	Scaffold processing steps					
	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
Poly-DOPA/Protein A/IgG fibers	Coat fibers with DOPA	Coat Protein A	Block with 5% BSA	Coat Rabbit AF555 IgG	—	—
Poly-DOPA/Protein A fibers	Coat fibers with DOPA	Coat Protein A	—	—	—	—
Poly-DOPA/IgG fibers	Coat fibers with DOPA	Block with 5% BSA	Coat Rabbit AF555 IgG	—	—	—
Poly-DOPA fibers	Coat fibers with DOPA	—	—	—	—	—
Protein A/IgG fibers	Coat Protein A	Block with 5% BSA	Coat Rabbit AF555 IgG	—	—	—
Protein A fibers	Coat Protein A	—	—	—	—	—
IgG fibers	Coat Rabbit AF555 IgG	—	—	—	—	—
Plain fibers	—	—	—	—	—	—
Poly-DOPA/Protein A/L1CAM fibers (For CNs)	Coat fibers with DOPA	Coat Protein A	Block with 5% BSA	Coat L1CAM	—	—
Poly-DOPA/L1CAM fibers (For CNs)	Coat fibers with DOPA	Coat L1CAM	—	—	—	—
Poly-DOPA/Laminin fibers (For CNs)	Coat fibers with DOPA	Coat Laminin	—	—	—	—
Poly-DOPA/Protein A fibers (For CNs)	Coat fibers with DOPA	Coat Protein A	—	—	—	—
Poly-DOPA/Protein A/Nrg1 fibers (For OPCs)	Coat fibers with DOPA	Coat Protein A	Block with 5% BSA	Coat His Tag antibody	Coat Nrg1	Coat PDL
Poly-DOPA/Protein A/JAM2 fibers (For OPCs)	Coat fibers with DOPA	Coat Protein A	Block with 5% BSA	Coat JAM2	Coat PDL	—
Poly-DOPA/Nrg1 fibers (For OPCs)	Coat fibers with DOPA	Coat Nrg1	Coat PDL	—	—	—
Poly-DOPA/Protein A fibers (For OPCs)	Coat fibers with DOPA	Coat PDL	—	—	—	—

Table 3.1. Coating protocol for the various types of fibers

Conditions	Scaffold processing steps					Expected outcomes
	Step 1	Step 2	Step 3	Step 4	Step 5	
Protein A included, blocking after rabbit AF555 IgG addition	Coat fibers with DOPA	Coat Protein A	Coat rabbit AF555 IgG	Block with 5% BSA	Add anti-rabbit AF488 IgG	AF555 fluorescence will be present but AF488 fluorescence will be absent
No Protein A, blocking after rabbit AF555 IgG addition	Coat fibers with DOPA	Coat rabbit AF555 IgG	Block with 5% BSA	Add anti-rabbit AF488 IgG	—	Both AF555 and AF488 fluorescence will be present
Protein A included, blocking before rabbit AF555 IgG addition	Coat fibers with DOPA	Coat Protein A	Block with 5% BSA	Coat rabbit AF555 IgG	Add anti-rabbit AF488 IgG	AF555 fluorescence will be present but AF488 fluorescence will be absent
No Protein A, blocking before rabbit AF555 IgG addition	Coat fibers with DOPA	Block with 5% BSA	Coat rabbit AF555 IgG	Add anti-rabbit AF488 IgG	—	Both AF555 and AF488 fluorescence will be absent

Table 3.2. Coating protocol used to evaluate antibody binding orientation

3.2.3. Characterization of protein functionalized PCL fibers

In order to evaluate the effectiveness of IgG adsorption, the loss of signals from the fiber surface over time and the availability of Protein A on the fiber surface over time, three different formulations of culture medium were compared. The medium formulations were as follows: DMEM only (no serum medium), DMEM with 0.5% FBS (low serum medium) and DMEM with 10% FBS (high serum medium). Fibers were characterized through the detection of fluorescent signals from fluorophore-conjugated IgGs using a fluorescence microscope (Leica Dmi8). Images were obtained either following the completion of functionalization, or every two days, starting from Day 1 to Day 7. In the experiment to determine the cause of the loss of fluorescence, poly-DOPA fibers coated with Protein A (poly-DOPA/Protein A fibers) were retrieved after incubation at 37 °C for 5 min, 3 days, 5 days and 7 days. They were then washed with PBS thrice, blocked with 5% BSA (37 °C, 1 h), incubated with rabbit AF555 IgG (1 µg/scaffold, 37 °C, 1 h) and washed thrice with PBS before imaging. The total number of

fluorescent pixels per region of interest (0.15 mm², ROI) after thresholding was defined as the amount of fluorescence present and subsequently used for comparison and statistical analysis. Three ROIs were imaged per scaffold and experiments were repeated thrice. The camera settings used were identical for all the images obtained.

3.2.4. Isolation and culture of primary rat CNs and OPCs

The isolation and culture of rat primary cells¹⁸⁰ were approved by the Institutional Animal Care and Use Committee (IACUC), Nanyang Technological University, Singapore. Both CNs and OPCs were derived from the cortices of postnatal Day 0 to 2 (P0 – P2) pups. The cortices were first dissociated through enzymatic digestion with 1.2 U Papain and 40 µg/ml DNase at 37 °C for 1 h. Following that, the partially dissociated tissues were triturated with a 21G needle to obtain a suspension of single cells, which were then divided for different cultures.

CN culture: Cells were seeded directly onto the poly-DOPA/Protein A/L1CAM fibers and its various control groups at a seeding density of ~265 cells/mm². The cells were then maintained for 3 days in Neurobasal medium containing 10% FBS, 2% B-27, 1% Pen/Strep and 1% GlutaMax.

OPC culture & differentiation: Cells were seeded onto PDL-coated T75 flasks and maintained in DMEM containing 10% FBS and 1% Pen/Strep. After 10 days, the OPCs were separated from the other glial cells within the flask using an orbital shaker set at 37 °C, 200 rpm. The flasks were first shaken for 1 h to remove the loosely attached microglial, followed by an additional 16 h to remove the OPCs. Thereafter, the OPCs were either seeded onto the

poly-DOPA/Protein A/Nrg1 type III fibers or the poly-DOPA/Protein A/JAM2 fibers and their various control groups at a seeding density of ~ 265 cells/mm². The OPCs were cultured for 14 days in myelin medium (medium renewed every 2 to 3 days) to direct their differentiation into oligodendrocytes. The myelin medium comprised of DMEM:Neurobasal (50:50), NS21, 10 ng/ml biotin, 5 μ g/ml NAC, SATO (100 μ g/ml BSA, 60 ng/ml Progesterone, 16 μ g/ml Putrescine, 400 ng/ml T3 and T4) and 1% Pen/Strep, 1% ITS and 1% GlutaMax.

3.2.5. Immunofluorescent staining

CNs and differentiated oligodendrocytes were fixed with 4% paraformaldehyde at Day 3 and Day 14 respectively. Both samples were then permeabilized in 0.1% Triton X-100 for 10 min and further blocked in 10% goat serum for 1 h. After blocking, the samples were incubated with primary antibodies overnight at 4 °C followed by incubation with secondary antibodies for 1 h at room temperature. The primary antibody used for evaluating CNs was Tuj1 (1:500) and the secondary antibody was goat anti-mouse AF555 (1:1000). For evaluating oligodendrocyte differentiation, the primary antibodies used were Olig2 (1:1000) and MBP (1:200) and the secondary antibodies were goat anti-rabbit AF555 (1:700) and goat anti-rat AF488 (1:700). The cell nuclei were counterstained with DAPI. Following the staining procedures, samples were mounted onto a 60 mm glass-coverslip using Fluoromount-G, dried and used for subsequent image analyses.

CNs were imaged under the epi-fluorescent microscope (Leica DMI8) at 20x magnification and the total neurite length and longest neurite length were quantified using Simple Neurite Tracer from Fiji¹⁹³. To calculate the myelin ensheathment length formed by oligodendrocytes as well as the number of ensheathments per Olig2⁺ cell, the samples were

imaged as a z-stack (0.3 μm thickness per slice, 40x objective magnification, 10x camera magnification) under the confocal microscope (Zeiss LSM 800) and quantified using ImageJ (NIH, USA). For both of these cultures, each group consisted of 2 technical replicates and were repeated using cells from 3 different animals.

3.2.6. Statistical analysis

Kruskal-Wallis test followed by the Mann-Whitney post-hoc test was utilized to evaluate the differences between groups. All data are shown as mean $\pm$ standard deviation (S.D.).

3.3. RESULTS

3.3.1. Poly-DOPA coating enhances functionalization of IgG on electrospun PCL fibers

Poly-DOPA coating on electrospun PCL fibers (poly-DOPA fibers) enables prolonged display of biomolecules to cells^{181, 194-196}. As such, I explored the feasibility of using this coating to functionalize electrospun fibers with Protein A. From initial optimization experiments, coating 50 $\mu\text{g}/\text{ml}$ of Protein A for 2 h on poly-DOPA fibers at 37°C followed by the addition of 2 $\mu\text{g}/\text{ml}$ of rabbit AF555 IgG provided the most uniform fluorescence on individual fibers (Figure 3.1 to 3.3). These homogenous and distinct signals observed on poly-DOPA/Protein A/IgG fibers incubated in various concoction of culture medium, as opposed to the patchy and indistinct fluorescence on Protein A/IgG fibers (Figure 3.4), indicated that Protein A was well-distributed on the fiber surface (Figure 3.5A to 3.5C). Correspondingly, it also demonstrated that DOPA was required for the coating to be optimal.

Figure 3.1. Representative images comparing PCL fibers coated with various concentrations of protein A.

Figure 3.2. Representative images comparing different duration of protein A coating on PCL fibers.

Figure 3.3. Representative images comparing different concentrations of IgG added to protein A fibers.

Figure 3.4. Representative high magnification images showing distinct signals in poly-DOPA coated fibers as compared to non-poly-DOPA coated fibers.

Figure 3.5. Poly-DOPA coating enhances functionalization of IgG on fibers. Representative images showing **(A)** poly-DOPA coated and **(B)** non-poly-DOPA coated fibers in various medium with the respective controls at Day 0. Number of fluorescent pixels quantified for all groups is depicted in (C). Data are represented as mean $\pm$ SD; n = 3 experimental repeats; *: p-value < 0.05; D/A/I = Poly-DOPA/Protein A/IgG fibers; D/A = Poly-DOPA/Protein A fibers; D/I = Poly-DOPA/IgG fibers; D = Poly-DOPA fibers; A/I = Protein A/IgG fibers, A = Protein A fibers; I = IgG fibers; Plain = plain fibers.

3.3.2. Poly-DOPA coating minimizes loss of IgG signals over time

Next, I evaluated the binding stability of the Protein A/IgG complexes on the poly-DOPA coated and non-poly-DOPA coated fibers by measuring the changes in fluorescent

intensity over 7 days in culture medium (DMEM) with varying amounts of serum. For both of these groups incubated in DMEM only, I was able to observe an eventual decrease of the fluorescence over time (Figure 3.6). However, this decrease was more rapid in non-poly-DOPA coated fibers where the signals were almost indistinguishable by Day 3 (Figure 3.6B and 3.6C). On the other hand, the fluorescence on poly-DOPA coated fibers decreased more gradually, suggesting a sustained binding of IgGs over the course of 7 days (Figure 3.6A and 3.6C). In order to further investigate the effects of protein displacement on the fiber surface,¹⁹⁷ the scaffolds were incubated in DMEM that consisted of different amounts of serum (low (0.5%) and high serum (10%)). Consistent with the results obtained in DMEM only, poly-DOPA coated fibers retained higher fluorescent signals over time as compared to non-poly-DOPA coated fibers regardless of the amount of serum added into DMEM (Figure 3.6A to 3.6C). More importantly, there were no notable differences in the number of fluorescent pixels at every time point between poly-DOPA coated fibers incubated in DMEM only and in low and high serum media (Figure 3.6A and 3.6C). This suggests that serum proteins within the medium did not play a significant role in the displacement, if any, of Protein A from the poly-DOPA coated surface or IgG from Protein A.

Figure 3.6. Poly-DOPA coating minimizes loss of IgG signals over time. Representative images showing the decrease in fluorescent signals in **(A)** poly-DOPA coated and **(B)** non-poly-DOPA coated fibers in various medium over 7 days. The number of fluorescent pixels were quantified for all groups and depicted in **(C)**. Data are represented as mean $\pm$ SD; *: p-value < 0.05 between poly-DOPA coated and non-poly-DOPA coated fibers for each of the different time points; n = 3 experimental repeats; D/A/I = Poly-DOPA/Protein A/IgG fibers; A/I = Protein A/IgG fibers.

3.3.3. Poly-DOPA coating promotes sustained presentation of Protein A on fibers

To determine the cause of the loss of fluorescence as observed in Figure 3.6, we adsorbed Protein A only onto both poly-DOPA coated and non-poly-DOPA coated fibers and incubated them in DMEM containing no, low and high serum. Thereafter, at predetermined time points, the fibers were exposed to rabbit AF555 IgG to assay the presence of Protein A. In the case where Protein A is still present on the fibers, the IgG will be immobilized and provide fluorescence. As observed from Figure 3.7A and 3.7B, the fluorescent signals from poly-DOPA coated fibers were more prominent and well maintained at all time points screened as compared to non-poly-DOPA fibers. Besides that, no significant difference was detected between the no, low and high serum groups for both poly-DOPA and non-poly-DOPA coated fibers. (Figure 3.7A to 3.7C). As fluorophores from the IgGs were observed to be very stable in culture medium (data not shown), these results suggested that the signal losses detected in Figure 3.6 were likely due to the displacement of IgGs from Protein A and were lost during medium change. Based on these, I can conclude that the specific binding ability of the poly-DOPA/Protein A fibers were retained for at least 7 days. This duration was reduced considerably in Protein A only fibers.

Figure 3.7. Poly-DOPA coating promotes sustained availability of functional Protein A. Representative images showing the amount of fluorescent signals in **(A)** poly-DOPA coated and **(B)** non-poly-DOPA coated fibers. IgG was added only after fibers were incubated in

various medium for a pre-defined duration. The number of fluorescent pixels were quantified for all groups and depicted in **(C)**. Data are represented as mean $\pm$ SD; n = 3 experimental repeats; *: p-value < 0.05 between poly-DOPA coated and non-poly-DOPA coated fibers for each of the different time points, &: p-value < 0.05 between Day 0 and Day 5 as well as Day 0 and Day 7 of non-poly-DOPA coated fibers, #: p-value < 0.05 between Day 3 and Day 7 of non-poly-DOPA coated fibers; D/A/I = Poly-DOPA/Protein A/IgG fibers; A/I = Protein A/IgG fibers.

3.3.4. Protein A dictates IgG binding configuration

As Protein A binds specifically to the Fc region, I hypothesized that this interaction can be exploited to control the orientation of immobilized proteins. A schematic depicting the expected outcomes of this experiment is proposed (Figure 3.8A). Theoretically, in the presence of bound Protein A, IgGs can only assume a single configuration due to the specific binding between Protein A and its Fc region. On the other hand in the absence of Protein A, IgGs that are adsorbed onto the fiber surface can be oriented in any configuration. When a second IgG that recognizes the first IgG is added, the former will only bind specifically to the Fc region of the first IgG. The initial availability of Protein A will therefore physically prevent the second IgG from binding to the first IgG. Conversely, without Protein A, the second IgG is free to bind to the first IgG.

To test this, I first enabled non-specific IgG adsorption by exposing rabbit AF555 IgG to both of these fibers without a blocking procedure. Fibers that were non-specifically bound with rabbit AF555 IgG will fluoresce as well, as seen in Figure 3.8B. Following that, I implemented a blocking step before the addition of an AF488-conjugated IgG raised in goat (anti-rabbit AF488 IgG) that targets rabbit AF555 IgG. Even though both fibers were initially

bound with rabbit AF555 IgG, the addition of anti-rabbit AF488 IgG only resulted in poly-DOPA only fibers fluorescing in both wavelengths but not poly-DOPA/Protein A fibers (Figure 3.8A). Similar to immunohistochemistry, the anti-rabbit AF488 IgGs were only expected to bind specifically to the Fc region of the rabbit AF555 IgGs. However, this Fc region is also specific to Protein A. In other words, if rabbit AF555 IgGs managed to bind to Protein A first, its Fc region would not be accessible to anti-rabbit AF488 IgGs for binding.

To further confirm that the AF488 signals observed in Figure 3.8B were due to specific binding between anti-rabbit AF488 IgG and rabbit AF555 IgG, I next disabled non-specific IgG adsorption by blocking the fibers with 5% BSA before the addition of rabbit AF555 IgG. This led to fluorescence only in poly-DOPA/Protein A fibers and not in poly-DOPA only fibers (Figure 3.8B and 3.8C). Thereafter, without further blocking, I added anti-rabbit AF488 IgG to both fibers with the intention for it to bind to rabbit AF555 IgG. As expected, no signals from poly-DOPA only fibers were observed since it did not contain rabbit AF555 IgG on its surface. Similar as before, I did not observe fluorescence in the AF488 wavelength on poly-DOPA/Protein A fibers even though rabbit AF555 IgG was present (Figure 3.8B and 3.8C). Taken together, these results validated the use of Protein A as an alternative strategy for controlling the orientation of bound proteins.

Figure 3.8. Protein A dictates IgG binding configuration. (A) Schematic diagram illustrating the proposed binding mechanism in the presence and absence of Protein A. (B) Images of scaffolds depicting the outcome of multiple antibody binding when the blocking process was conducted after the addition of rabbit AF555 IgG. Quantification of the number of fluorescent pixels in those scaffolds are shown in (C). (D) Images of scaffolds depicting the outcome of multiple antibody binding when the blocking process was conducted before the addition of rabbit AF555 IgG. Quantification of the number of fluorescent pixels in those scaffolds are shown in (E). Data are represented as mean $\pm$ SD; n = 3 experimental repeats; *: p-value < 0.05.

3.3.5. Binding of L1CAM onto Protein A promotes neurite outgrowth

Having established the binding mechanisms between poly-DOPA, Protein A and IgG, I proceeded to test the efficacy of this platform using rat neural cells, specifically CNs, as a model primary cell. A commercial recombinant L1CAM protein, which contains an Fc fragment, was adsorbed onto the fibers (with its important functional domain outward facing/accessible) in place of the IgGs. As compared to other groups, CNs seeded on poly-DOPA/Protein A/L1CAM fibers had significantly greater longest total neurite length ($462.77 \pm 100.79 \mu\text{m}$). A similar trend was also observed when the longest neurite length was compared ($352.99 \pm 88.21 \mu\text{m}$) (Figure 3.9A to 3.9C). In the absence of Protein A, neurons that were cultured on poly-DOPA/L1CAM fibers also had encouraging neurite outgrowth ($355.97 \pm 92.11 \mu\text{m}$ for total neurite length and $268.19 \pm 74.69 \mu\text{m}$ for longest neurite length) relative to those in poly-DOPA/Laminin ($196.92 \pm 55.71 \mu\text{m}$ for total neurite length and $146.26 \pm 45.69 \mu\text{m}$ for longest neurite length) and poly-DOPA only fibers (Figure 3.9A to 3.9C). These results demonstrate that the orientation of bounded proteins led to observable differences in cellular behaviour. Most likely, the incorporation of Protein A effectively immobilized L1CAM in an optimal orientation for the neurons to bind to, hence allowing improved effects and efficient usage of the protein.

Figure 3.9. Binding of L1CAM onto Protein A promotes neurite outgrowth. (A) Representative images of P2 cortical neurons seeded on scaffolds coated with the various molecules. Quantification of the total neurite length and the longest neurite length for each coating conditions is shown in **(B)** and **(C)** respectively. Data are represented as mean $\pm$ SD; $n = 3$ experimental repeats; Each dot represents one measurement from a neuron; ***: p -value < 0.001 between all groups; D/A = Poly-DOPA/Protein A fibers, D/A/L1 = Poly-DOPA/Protein A/L1CAM fibers, D/L1 = Poly-DOPA/L1CAM fibers, D/Lam = Poly-DOPA/Laminin fibers.

3.3.6. Binding of Nrg1 type III onto Protein A moderately enhances myelin ensheathment length

Besides examining cortical neurons (CNs), we also tested this platform using rat oligodendrocyte progenitor cells (OPCs) as a model primary progenitor cell. Here, I used Nrg1 type III and JAM2, which support¹⁹⁸ and inhibit¹⁹¹ myelination respectively, to test the functionality of the poly-DOPA/ProteinA fibers. Results show that OPCs cultured on poly-DOPA/Protein A/Nrg1 type III fibers had significantly longer myelin ensheathment length as compared to cells on poly-DOPA/Nrg1 type III fibers ($19.8 \pm 11.69 \mu\text{m}$ vs. $16.26 \pm 8.24 \mu\text{m}$ respectively, $p < 0.01$, Figure 3.10A and 3.10B). These differences became more notable in contrast to poly-DOPA/Protein A/JAM2 ($13.57 \pm 6.37 \mu\text{m}$) and the poly-DOPA/Protein A control ($13.94 \pm 8.62 \mu\text{m}$)(Figure 3.10A and 3.10B). Although there were statistical significance for these comparisons ($p < 0.01$), I noticed that the effects were rather modest, especially between OPCs that were cultured on poly-DOPA/Protein A/Nrg1 type III and poly-DOPA/Nrg1 type III fibers. This suggests that the orientation of Nrg1 type III might not play a huge role in promoting myelination.

Figure 3.10. Binding of Nrg1 onto Protein A enhances myelin ensheathment length. (A)

Representative Day 14 images of OPCs seeded onto differently coated scaffolds. Quantification of the ensheathment length and the number of ensheathments per Olig2⁺ cell are shown in **(B)** and **(C)** respectively. Data are represented as mean $\pm$ SD; n = 3 experimental repeats; For ensheathment length, each dot represents one measurement; For number of ensheathment per Olig2⁺ cells, each dot represents the average measurement within an image; **: p-value < 0.01; N.S. = Not Significant; D/A = Poly-DOPA/Protein A fibers, D/A/Nrg1 = Poly-DOPA/Protein A/Nrg1 fibers, D/Nrg1 = Poly-DOPA/Nrg1 fibers, D/A/JAM2 = Poly-DOPA/Protein A/JAM2 fibers.

3.4. DISCUSSION

Signal transduction through the effects of signalling proteins is a fundamental mechanism for cells to “sense” their environment and respond appropriately. In the CNS where some of the most crucial events for ensuring survival occurs (myelination, development of axonal tracts, etc.), the signalling molecules involved are often known but not fully understood. While not the first, our work has added advantages and further demonstrates the feasibility of using electrospun fibers as artificial axons with customizable display of biomolecules to understand cell fate. Moreover, as such fibers greatly mimic the architecture of the extracellular matrix (ECM), they could also be expanded to understand the behaviour of other cell types. Notably, in a deeper sense, I realized that the orientation of a protein is as instrumental as specificity in achieving protein-receptor binding. Therefore in this study, I designed a protein screening platform that can optimally orientate immobilized proteins for efficient evaluation of its effects on cellular behaviour. More importantly, the

immobilized proteins could be expressed in a sustained fashion, allowing longer time point study to be carried out.

Protein A has long been used for IgG antibody extraction from various serum sources due to its exceptional affinity towards the Fc fragment¹⁸⁴. Besides that, previous studies have also utilized it to coat polystyrene beads¹⁹¹ as well as polymer thin films¹⁹⁹ and microfibrinous scaffolds¹⁸³ for protein binding. However, one major drawback of directly adsorbing Protein A onto the synthetic materials is that their binding is unstable. Based on our results, proteins that were coated directly onto our electrospun fibers were quickly lost within 3 days, as observed from the loss of fluorescence (therefore loss of rabbit AF555 IgGs, Fig 2) over time. This was also consistent in the studies referenced^{183, 191, 199} which used Protein A to bind Fc-tagged proteins, where the experiments were limited to 3 days.

In order to circumvent this problem, I included a bioadhesive, poly-DOPA, to secure Protein A onto the fibers. In fact, the binding of Protein A to poly-DOPA was so robust that even after a portion of IgGs were lost from Protein A after 7 days, most of the latter remained bounded to the fibers (Figure 3). To further clarify, rabbit AF555 IgGs were chosen as the model protein as it contains an Fc fragment (from rabbits) that has high affinity for Protein A.²⁰⁰ Once immobilized, the amount of fluorescence observed can then be a measure of how successful the coating is. One potential pitfall though, is that the IgGs were able to bind non-specifically to uncoated as well as poly-DOPA coated fibers. As a result, a blocking step using BSA, before the addition of IgGs, was adopted. Besides that, our platform was also tested in varying levels of serum in order to cater to the media formulations that are commonly used for cell culture.

A problem that may arise is that the binding of Protein A onto poly-DOPA is random. Theoretically, if most of the orientation of Protein A is random, we would expect the surface of the coated fibers to be patchy when the fluorescent antibodies were adsorbed after that. However, I did not observe that, suggesting that most of the Protein A were properly oriented for interaction. There are two reasons why Protein A can somehow achieve that. Firstly, Protein A (42 kDa) is relatively smaller than the other proteins (L1CAM is around 210 kDa, antibodies are around 150 kDa, Nrg1 is around 90 kDa) used in this paper. Being smaller means that its binding site has a higher chance of being exposed for specific interactions regardless of its orientation when it is adsorbed onto poly-DOPA. Secondly, also due to its small size, other proteins trying to bind to it may face steric hindrance. This would lead to quite a number of unbound Protein A. When bound, an antibody which is at least 3 times larger than a single Protein A will prevent other antibodies from binding to the immediate neighbouring Protein A, creating redundancies. These redundancies ensure that within a group of bound Protein A, as long as there is one that has an exposed binding site, subsequent bound proteins will be able to homogeneously line the fiber surface.

Using this platform, I first tested the effects of L1CAM, a well explored cell adhesion molecule that is implicated in axonal growth²⁰¹, on CNs. Our results were consistent with a similar study that was conducted by Cherry *et al.*¹⁹⁹ where neurite outgrowth was most prominent when both Protein A and L1CAM were present as compared to just Protein A alone. Moreover, despite differences in the source and age of the neurons used (E15 spinal cord neurons vs. P8 cerebellar neurons vs. hESC-derived neural progenitor cells vs. P0-P2 CNs), similar trends were observed, indicating that the effects of L1CAM is likely not discriminatory

towards neuron sub-types. Of note, we were able to observe significant differences in the length of neurite outgrowth between poly-DOPA/Protein A/L1CAM and poly-DOPA/L1CAM fibers ($p < 0.001$). This indicated that the orientation of L1CAM is a crucial factor for enhancing axonal elongation and also justify the addition of Protein A onto poly-DOPA fibers instead of just immobilizing L1CAM directly.

Besides that, while L1CAM is shown to promote axonal growth of most, if not all, neurons, the relative enhancement for different types of neurons have not been explored. Comparing the effects of 1 μ g L1CAM on the length of our P0 - P2 CNs to the E15 spinal cord neurons from Cherry *et al.*, I observed that the average total length of CNs were approximately 7 times longer than spinal cord neurons¹⁹⁹. This was reduced to 3 times when comparing P3 CNs to P6 cerebellar neurons from a study by Hoffman *et al.*²⁰², suggesting that L1CAM might not have identical effects on all neuronal cells and that different types of neuronal cells might have varying intrinsic responses towards L1CAM.

To further assess the reliability of our scaffold platform, I tested other proteins on rat-derived primary OPCs, a progenitor cell type found in the CNS. In our work, I chose Nrg1 type III, a trophic factor expressed by axons²⁰³ that was reported to support oligodendrocyte myelination *in vivo*^{190, 198}. I found that in our controls, the range of ensheathment lengths obtained from oligodendrocytes (derived from the cerebral cortex) were in accordance with those from Bechler *et al.*²⁰⁴. Besides that, the influence of JAM2, a myelin inhibitory protein¹⁹¹, was also observed to reduce myelination ensheathment length when compared to the controls. However, although I obtained significantly longer ensheathment length in poly-DOPA/Protein A/Nrg1 type III fibers as compared to poly-DOPA/Nrg1 type III fibers ($p < 0.01$),

the range of improvement was relatively modest. As compared to the ensheathment lengths obtained from our previous work using microRNAs, the miR-219/miR-338 treated groups yielded ensheathment lengths that are around 9 times longer¹⁸⁰. This indicated that Nrg1 type III might not be the only determining factor for promoting OPCs myelination *in vitro*, which is consistent with *in vivo* studies, which showed that myelination in the CNS did not improve in the abundance of Nrg1 type III^{190, 198}. Future works could explore other proteins that have the potential to enhance myelination *in vitro* as well as evaluate the underlying mechanisms. Taken together, this platform would be useful for protein screening studies that require a sustained and optimally oriented protein expression. A proper protein orientation refers to the orientation that enables the most efficient interaction with, in this case, a biological entity such as a cell. This suggests that for every protein bound, it will have a high chance to interact with the cell to elicit a desirable effect. As protein screening is usually comprehensive and exhaustive, having a platform that can enhance this chance of interaction means that less protein is required for an observable effect. Efficiency, therefore, refers to the interaction between the protein and the cell, which correspondingly translates to reduced cost.

3.5. CONCLUSION

I have successfully developed a facile and robust electrospun fibrous platform for oriented and sustained presentation of proteins. I have also demonstrated the efficacy of such scaffolds for effecting primary cells derived from the CNS using cortical neurons and OPCs as model adult and progenitor cells respectively. Since fiber scaffolds mimic the architecture of the natural extracellular matrix, such scaffolds may also be extended to understand the effects of other proteins on cell types, beyond the CNS.

CHAPTER 4

SCAFFOLD-MEDIATED SUSTAINED, NON-VIRAL DELIVERY OF MIR-219/MIR-338 PROMOTES CNS REMYELINATION

ABSTRACT

The loss of oligodendrocytes (OLs) and subsequently myelin sheaths following injuries or pathologies in the central nervous system (CNS) leads to debilitating functional deficits. Unfortunately, effective methods of remyelination remains limited. Here, I present a scaffolding system that enables, sustained non-viral delivery of microRNAs (miRs) to direct OL differentiation, maturation and myelination. I show that miR-219/miR-338 promoted primary rat OL differentiation and myelination *in vitro*. Using spinal cord injury as a proof-of-concept, I further demonstrate that miR-219/miR-338 could also be delivered non-virally *in vivo* using an aligned fiber-hydrogel scaffold to enhance remyelination after a hemi-incision injury at C5 level of Sprague Dawley rats. Specifically, miR-219/miR-338 mimics were incorporated as complexes with the carrier, TransIT-TKO® (TKO), together with neurotrophin-3 (NT-3) within hybrid scaffolds that comprised of poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP) aligned fibers and collagen hydrogel. After 1, 2 and 4 weeks post treatment, animals that received NT-3 and miR-219/miR-338 treatment preserved higher number of Olig2⁺ oligodendroglial lineage cells as compared to those treated with NT-3 and scrambled miRs (Neg miR, $p < 0.001$). Additionally, miR-219/miR-338 increased the rate and extent of differentiation of OLs. At the host-implant interface, more compact myelin sheaths were observed when animals received miR-219/miR-338. Similarly within the scaffolds, miR-219/miR-338 samples contained significantly more myelin basic protein (MBP) signals ($p <$

0.01) and higher myelination index ($p < 0.05$) than Neg miR samples. These findings highlight the potential of this platform to promote remyelination within the CNS.

4.1. INTRODUCTION

Oligodendrocytes (OLs), glial cells that form the myelin sheaths within the central nervous system (CNS),²⁰⁵ are essential for facilitating saltatory signal conduction in axons as well as providing metabolic support for neuronal survival.^{187, 206} Without myelin ensheathment, energy-efficient conduction in axons cannot occur, which in turn results in perturbed axonal transport, degeneration and ultimately functional impairment.²⁰⁷ The importance of this structure was further displayed in patients with non-penetrating traumatic nerve injuries in the CNS, where even though a portion of axons was spared post-injury, functional recovery was still not observed due to demyelination.²⁰⁸ This suggests that although regeneration of axons is an end goal, they have to be myelinated in order to allow proper conduction of nerve impulses.

Within the spinal cord, oligodendrocyte progenitor cells (OPCs) exist endogenously and they continue to proliferate and differentiate into myelinating OLs in the adult CNS after injury. Much evidence suggests that OPCs residing in the white matter of the spinal cord represent the largest potential source of remyelinating OLs.²⁰⁹ However, although the proliferation rate of OPCs increases significantly post spinal cord injury (SCI), spontaneous remyelination remains limited, particularly in humans.²¹⁰ As pre-existing damaged OLs do not contribute to remyelination,^{211, 212} current strategies to encourage myelin regeneration include either transplantation of exogenous cells into the injury site²¹³ or activation of the endogenous pool of OPCs. While both approaches are feasible and have been demonstrated, the former method may present more issues since the availability of autologous OPCs is limited and heterologous transplantation requires long-term immunosuppression that may cause serious side effects.²¹³

As such, we focus on the latter. Early *in vitro* culture experiments involving primary OPCs from postnatal CNS indicated that in the absence of signals to maintain the cells as OPCs, they would rapidly differentiate into OLs.²¹⁴ This suggests that much of the regulation in CNS myelination might be at the level of inhibiting this otherwise default pathway of differentiation and the relief of inhibition might lead to OL differentiation and myelination.²¹⁴ Hence, strategies that aim to silence some of these inhibitory pathways may promote remyelination in the CNS.

MicroRNAs (miRs) are small non-coding RNAs (21 to 23 base pairs) that are involved in gene expression regulation via RNA interference.²¹⁵ They are intricately involved in directing the fate of OPCs and OLs. Specifically, microRNA-219 (miR-219) and microRNA-338 (miR-338) are effective in promoting OPC differentiation *in vitro* and *in vivo* by suppressing the expression of gene targets that promote OPC proliferation.^{194, 216-218} Furthermore, in a recent study, Lu *et al.* demonstrated that these miRs have a synergistic role in enhancing myelin repair in an experimental demyelinating model induced by lysolecithin (LPC).²¹⁹ Building on their well-substantiated findings, I sought to improve the delivery method of these miRs for long-term therapeutic purposes, using SCI as a proof-of-concept and an extended application. Importantly, while both of these models can induce demyelination, SCI presents a higher level of difficulty due to the plethora of factors that impede myelin formation around the injured region.⁹⁶

Therefore in this study, I fashioned a fiber-hydrogel scaffold for non-viral sustained delivery of both miR-219 and miR-338 (miR-219/miR-338) *in vivo*. Specifically, this hybrid

scaffold consisted of a core bundle of aligned poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP) fibers, which were structurally stabilized in a three-dimensional (3D) configuration by collagen hydrogel embedment. Within the collagen hydrogel, miRs and drugs can be loaded and delivered through diffusion to the microenvironment. Having established its efficacy in concurrently delivering miRs and neurotrophins,²²⁰ as well as providing topographical cues to enhance axonal regeneration,^{220, 221} I proceeded to assess the effects of this scaffold on OPC differentiation and myelin regeneration *in vivo* using a spinal cord C5 hemi-incision rodent model. Here, I show that rats implanted with miR-219/miR-338-loaded scaffolds retained a higher population of oligodendroglial lineage cells around the lesion site. These cells also displayed enhanced differentiation capabilities and myelinated more axons. Altogether, the results highlight the potential of these miR-incorporated fiber-hydrogel scaffolds in enhancing the survival of OPCs and promoting myelination within the injured CNS.

Figure 4.1. Graphical abstract. A electrospun fiber/collagen hybrid scaffold loaded with miR-219/miR-338 cocktail was implanted into a C5 1/3 hemisection injured rat.

4.2. MATERIALS AND METHODS

4.2.1. Materials

2,2,2-trifluoroethanol (TFE), heparin, bovine serum albumin (BSA), horse serum, poly-D-lysine, cytosine arabioside, 5-fluoro-2'-deoxyuridine, DNase, progesterone, putrescine and ITS liquid media supplement (ITS) were obtained from Sigma, USA. Rat tail collagen type

1 was purchased from Corning, USA. Neurotrophin-3 (NT-3) and nerve growth factor (NGF) was procured from PeproTech, USA. Negative scrambled microRNA (Neg miR), miR-219, miR-338-3p, miR-338-5p, Quant-iT™ RiboGreen, collagenase type 1, goat anti-mouse Alexa Fluor 555, goat anti-rat Alexa Fluor 555, goat anti-rat Alexa Fluor 633, goat anti-rabbit Alexa Fluor 488, goat anti-chicken Alexa Fluor 488, 4',6-diamidino-2-phenylindole (DAPI), N-2 supplement, laminin mouse protein (laminin), goat serum, neurobasal medium, DMEM (high glucose, L-glutamine, pyruvate), glutamax supplement and penicillin–streptomycin were acquired from Life Technologies, USA. TransiT-TKO (TKO) was obtained from Mirus Bio, USA. Tris-EDTA (TE) buffer (pH 8.0) and PBS (pH 7.4) were purchased from 1st Base, Singapore. NT-3 ELISA kit was bought from R&D Systems, USA. Mouse anti-CC-1 (ab16794) was obtained from Abcam, UK. Rabbit anti-Olig2 (AB9610) was acquired from Merck, USA. Rat anti-MBP (MCA409S) was purchased from Biorad, USA. Chicken anti-NF200 (822601) and mouse anti-βIII-Tubulin (801202) was procured from Biolegend, USA. Mouse anti-CNPase (AMAb91072) was bought from Atlas antibodies, Sweden. Rabbit anti-GFAP (Z033401) was obtained from DAKO, Denmark. DMEM/F12 medium was purchased from Lonza, Switzerland. Papain (LS003126) was bought from Worthington Biochemical Corporation, USA.

4.2.2. Cell culture

P0-P2 neonatal rat cortices, along with their meninges, were removed and enzymatically digested with 1.2 U Papain and 40 µg/ml DNase at 37 °C for 1 h. Thereafter, 8 ml of DMEM containing 10% Fetal Bovine Serum (FBS) were added and the dissociated tissues were triturated with 21G needle and syringe. Tissues from 6 digested cortices were seeded onto 4 PDL-coated T75 flasks and cultured in DMEM with 10% FBS and 1% Pen/Strep. After 9-11 days, the mixed glia culture was placed on an orbital shaker at 200 rpm for 1 h at 37 °C to

remove the loosely attached microglia. Following this, an additional 16-17 h of shaking was done to obtain the OPCs. To further purify the cells collected, a differential adhesion method (25 min) on untreated petri dishes was adopted. Purified OPCs were then seeded at a density of 45,000 and cultured for 14 days in myelin medium containing DMEM (high glucose, L-glutamine, pyruvate):Neurobasal (50:50), NS21, 1% Pen/Strep, glutamax supplement, ITS, 10 ng/ml biotin, 5 µg/ml NAC and SATO (100 µg/ml BSA, 60 ng/ml Progesterone, 16 µg/ml Putrescine, 400 ng/ml T3 and T4).

4.2.3. In vitro myelin sheath quantification

A myelin sheath is defined as a continuous, smooth tube of MBP staining surrounding a fiber. To measure the length of myelin sheaths on fibers, MBP signals were traced along individual fibers using 40x magnification confocal images (Zeiss LSM800). To compute the number of myelin sheaths per cell, MBP⁺ cells in miR-219/miR-338 and Neg miR group from 3 independent experiments were included in the analysis. All measurements and counting were done using Fiji.

4.2.4. Scaffold fabrication

The PCLEEP copolymer (M_w = 59,102, M_n = 25,542) was synthesized according to a procedure described previously¹⁹⁴ and a two-pole air gap electrospinning technique was used to obtain the aligned PCLEEP fibers.²²⁰ Briefly, the electrospinning solution was obtained by dissolving PCLEEP in TFE (35% w/v) and loaded into a 3 ml syringe that was capped with a 21 gauge blunt-tipped needle. The solution was charged at +12 kV and extruded at a flow rate of 1.1 ml/h. The PCLEEP fibers were deposited within a 5-cm air gap area between two stationary collector poles that were charged at -4 kV. Each set of fibers was obtained after 75 s of

electrospinning. Four sets of fibers were then sterilized under UV light for 45 min and set in place within a sterilized cylinder mold (5 mm in length and 4.5 mm in inner diameter). 200 μ l of rat tail collagen type 1 was then prepared to a concentration of 3 mg/ml following manufacturer's protocol and added into the mold. Hydrogel formation took place at room temperature for 30 min. The scaffolds were then frozen at - 20 °C for 3 h before being lyophilized overnight. Finally, the scaffolds were cut into 1 mm long pieces and sterilized under UV light for 45 min before *in vivo* implantation.

4.2.5. Drug loading

NT-3 was loaded into all the scaffolds and delivered together with the miRs. For calculations regarding neurotrophin-3 (NT-3) incorporation into the scaffolds, DI water within the collagen mixture was replaced with 1 μ l of NT-3 (Stock concentration is 2 μ g/ μ l, 2 μ g per scaffold for implantation), which was, in turn, reconstituted in 0.1% BSA and heparin (400 μ g/ μ l) (1:1 v/v). For calculations regarding miR incorporation, miR (2 μ g per scaffold containing equal parts of miR-219, miR-338-3p and miR-338-5p) and TKO (1:1 v/v) were complexed for 15 min at room temperature. After complexation, the solution was then added into the collagen mixture. For the control scaffold, Neg miR was used to complex with TKO instead.

4.2.6. Fiber diameter quantification

Scaffolds were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 120 s and observed under the scanning electron microscope (SEM; JEOL, JSM-6390 LA) at an accelerating voltage of 10 kV. The average fiber diameter was determined using ImageJ (NIH, USA) by measuring 100 fibers per sample from high magnification (5000x) SEM images.

4.2.7. Scaffold degradation

Scaffolds (2.35 ± 0.15 mg) ($n = 8$) were completely immersed in 1 ml of PBS and incubated at 37 °C. At each time point, the weight of the hydrated scaffold was measured. The percentage of collagen degradation was then calculated using the following equation:

Percent of collagen degradation (%)

$$= \frac{\text{Initial mass of scaffold (mg)} - \text{Mass of scaffold measured at each time point (mg)}}{\text{Initial mass of scaffold (mg)}} \times 100\%$$

4.2.8. Drug release kinetics

The release kinetics of miR and NT-3 from the scaffolds was evaluated under static conditions. Neg miR- and NT-3-incorporated scaffolds were incubated in 1.2 ml of 1x TE buffer and PBS respectively at 37 °C. Three samples (2.08 ± 0.15 mg) were used. At each time point, 600 µl of buffer was collected from each sample and replaced with an equal volume of fresh buffer. The amount of NT-3 released over time was determined using a NT-3 ELISA assay. For miR quantification, to each of the supernatant collected, 100 µl of 100 mg/ml heparin was added and thoroughly mixed at 130 rpm for an hour to decomplex Neg miR from TKO. The concentration of miR that was released into the supernatant was then quantified using Quant-iTTM RiboGreen assay following manufacturer's protocol.

In order to retrieve the remaining Neg miR and NT-3 from the scaffolds at the end of the release kinetics study, the scaffolds were submerged in collagenase for 1 h at 37 °C. Upon complete dissolution of the collagen, the supernatant tested for NT-3 using ELISA whilst miR samples were decomplexed with heparin as described above and analysed using Quant-iTTM

RiboGreen RNA reagent kit. The loading efficiency of the drugs were then computed respectively using the following equation:

$$\text{Loading Efficiency} = \frac{\text{Total drug released} + \text{Total drug extracted}}{\text{Total theoretical drug loaded}} \times 100\%$$

4.2.9. Animals

All animals were obtained from In Vivos Pte Ltd (Singapore). The animal care and experimental procedures were carried out in accordance to the Institutional Animal Care and Use Committee (IACUC, NTU) guidelines. Animals were housed under temperature controlled conditions, with a normal 12:12 h light/dark cycle with ad libitum access to water and food.

4.2.10. Spinal cord injury surgical procedures

Thirty-eight adult female (7-9 weeks, 180-230 g) Sprague Dawley rats were used as outlined in Table 4.1. Buprenorphine (0.05 mg/kg) was administered to each animal subcutaneously 30 min before surgery. Anaesthesia was then induced using 4-5% mixture of isoflurane in air followed by intraperitoneal injection of Ketamine (75 mg/kg) and Xylazine (10 mg/kg). Once deeply anaesthetized, the dorsal region of the rats (cervical to pectoral) were shaved and cleaned with 70% ethanol and betadine. A midline incision was made at the disinfected area and the overlying muscles were bluntly dissected to expose the C5-C6 vertebrae. A laminectomy procedure was further performed to reveal the underlying spinal cord. Following that, the dura was sliced open and a one-third right lateral incision was made on the spinal cord. The scaffold (1 mm in length) was then implanted into the lesion site once the bleeding had subsided. After the implantation, a piece of fat tissue was used to cover the injury, the muscles were sutured using Prolene 4/0 single stitches and the skin was closed

using wound clips. Post-operative treatments included subcutaneous injection of Buprenorphine (0.05 mg/kg) and addition of Meloxicam (5 mg/ml) into the drinking water.

Group	Number of animals		
	Week 1	Week 2	Week 4
Neg miR + NT-3	n =3	n = 8	n =7
miR-219/miR-338 + NT-3	n =4	n = 5	n =7
NT3 only		n=4	

Table 4.1. Experimental groups.

4.2.11. Immunohistochemical analysis

At designated time points post-implantation, animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) before being transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA). The implanted scaffolds and some of the surrounding spinal cord tissues were then harvested and further transferred into 4% PFA for 2 h, followed by 15% sucrose solution for 24 h and finally 30% sucrose solution. Thereafter, the tissues were embedded in Tissue-Tek[®] O.C.T. Compound and cut into 20 µm thick horizontal sections using a cryostat (Leica CM1950). Immunofluorescent staining was performed to evaluate the effects of the treatment on various cell types of interest. Sections were permeabilized with 0.3% Triton X-100, blocked with 10% goat serum for 1 h and incubated with primary antibodies overnight at room temperature. Primary antibodies used included mouse anti-CC-1 (1:200 dilution), rabbit anti-Olig2 (1:200 dilution), rabbit anti-PDGFR α (1:3000 dilution; gift from Prof Stallcup), rat anti-MBP (1:200 dilution), chicken anti-NF200 (1:1000 dilution), mouse anti-CNPase (1:1000

dilution), and rabbit anti-GFAP (1:1000 dilution). After primary antibody incubation, the samples were washed thrice with PBS before secondary antibody incubation for 1 h at room temperature, using the following: goat anti-mouse Alexa Fluor 555 (1:1000 dilution), goat anti-rat Alexa Fluor 555 (1:1000 dilution), goat anti-rat Alexa Fluor 633 (1:500 dilution), goat anti-rabbit Alexa Fluor 488 (1:7000 dilution) and goat anti-chicken Alexa Fluor 488 (1:700 dilution). Finally, nuclei were labelled with DAPI (1:1000 dilution) and all slides were coverslipped with Mowiol.

Quantification for the number of DAPI, Olig2, PDGFR α and CC-1 positive cells in the tissue samples was conducted using fluorescently-labelled stitched collage consisting of several 10x magnified images (Leica DMI8). The region of interest was defined to be 100 μ m around the lesion site (Figure 3A) and cells were manually counted using ImageJ cell counter (NIH, USA). To quantify PDGFR α fold change between different time points, the following equation is used:

PDGFR α Fold Change Between Time points

$$= \frac{\text{Density of PDGFR}\alpha \text{ cells in Week X} - \text{Density of PDGFR}\alpha \text{ cells in Week Y}}{\text{Density of PDGFR}\alpha \text{ cells in Week X}}$$

where X and Y is 1,2 or 4; X $\neq$ Y

To quantify myelination index in the implant interface and within the scaffold, 40x magnified z-stacks obtained from Zeiss LSM800 along with a colocalization software from ImageJ (NIH, USA) were used. A total of three spinal cord sections were used for quantification per animal. The myelination index is defined by the following equation:

$$\text{Myelination Index (\%)} = \frac{\text{Number of overlapping NF200 and MBP pixels}}{\text{Number of NF200 pixels}} \times 100\%$$

4.2.12. Statistical Analysis

Quantification data was tested for normality and homogeneity of variances using Shapiro-Wilk and Levene's test respectively. Data with homogenous variances were analysed using one-way ANOVA followed by Tukey's post-hoc test. Kruskal-Wallis test followed by Mann-Whitney post-hoc test were used instead for non-parametric data. Error bars represent standard error of the mean, unless otherwise stated.

4.3. RESULTS

4.3.1. Fiber-mediated non-viral delivery of miR-219/miR-338 enhanced OPC differentiation and myelination *in vitro*

The efficacy of using a fiber platform to transfect primary rat OPCs non-virally with miR-219/miR-338 and the corresponding enhanced cellular differentiation and maturation were demonstrated *in vitro* previously.¹⁹⁴ However, the functionality of an OL is determined by its ability to wrap and myelinate. Hence, extending from our recent work, where initial OL myelin ensheathment was evaluated at Day 7,¹⁸⁰ I assessed the myelination outcomes at an extended culture period of 14 days in this work. As shown in Figures 4.2A to 4.2C, miR-219/miR-338 treatment significantly enhanced OPC differentiation and maturation when cells were cultured in myelin medium, resulting in larger numbers of myelin basic protein positive (MBP⁺) mature OL per Olig2⁺ oligodendroglial lineage cells as compared to negative scrambled miR (Neg miR) transfection ($85.1 \pm 5.1\%$ vs $59.1 \pm 9.4\%$, $p < 0.01$). This reinforced the efficacy of miR-219/miR-338 in enhancing OPC differentiation and maturation under both proliferative¹⁹⁴ and pro-myelinogenic conditions. Besides that, miR-219/miR-338 also markedly increased the myelin sheath length on the fibers as compared to the Neg miR control (Figure 4.2A and 4.2D). Further quantification of myelin sheath formation revealed

that miR-219/miR-338 led to more complete-looking myelin structures per OL (on average 2.4 ± 1.5 number of sheaths per cell, Figure 4.2E) as opposed to Neg miR treatment, which resulted in only 0.5 ± 0.9 number of sheaths per cell. Additionally, 88.6% of MBP⁺ cells in miR-219/miR-338 group had complete myelin sheath formation whereas only 34.3% of MBP⁺ cells in scrambled Neg miR group produced complete sheaths. As I did not detect the formation of complete sheaths from either samples at earlier time points (Day 7),¹⁸⁰ it is likely that the sheaths in Neg miR group failed to form properly instead of breaking down during the course of the experiment.

Figure 4.2. Scaffold-mediated delivery of miR-219/miR-338 promoted OPC differentiation and myelination *in vitro*. (A) Representative confocal images show that cocktail of miRs promoted differentiation of OPCs into MBP⁺ OL after 2 weeks. (B) Representative confocal images show that cocktail of miRs increased the length and number of complete myelin sheath per OL after 2 weeks (C) Quantitative data shows miR-219 and miR-338 treatment resulted in more MBP⁺ cells per Olig2⁺ cells. (D) Quantitative data shows miR-219 and miR-

338 treatment resulted in significant differences between the average sheath length (E) Quantitative data shows miR-219/miR-338 treatment resulted in higher average number of compact myelin sheath per OL. Each dot represents one cell. ** $p < 0.001$ (Student's t-test), * $p < 0.01$ (ANOVA). Data are represented as mean $\pm$ SD.

4.3.2. Sustained release of NT-3 and miRs from fiber-hydrogel scaffolds

A sustained availability of drugs may be desirable since OPCs require at least 14 days to differentiate, ensheath and form compact myelin.²⁰⁴ Hence, a scaffold was designed to deliver drugs in a sustained and localized fashion. In particular, neurotrophin-3 (NT-3), a neurotrophic factor that is commonly used for enhancing neuronal survival,²²² was delivered together with miR-219 and miR-338.²²⁰ In addition, the scaffold was designed to provide topographical guidance to regenerating axons through the means of aligned fibers, as shown in Figures 4.3A and 4.3B. Using characterization methods described previously,^{194, 220} I observed that both NT-3 (loading efficiency of 6.23%) and miRs-loaded (loading efficiency of 28.91%) scaffolds displayed burst release profiles within the first 1 h, where $\sim 10\%$ of the actual experimental amounts were released (Figure 4.3C). For the next 20 days, 70% of NT-3 and 65% of miRs that were successfully loaded were released. Thereafter, the release of NT-3 and miRs started to plateau at $\sim$ Day 60 (Figure 4.3C and Figure 4.4). Scaffold degradation study revealed a steady mass loss of the entire scaffold over time, up to approximately 60% in 30 days (Figure 4.3D).

Figure 4.3. Fiber-hydrogel scaffold for sustained non-viral drug/gene delivery. (A) Scanning electron microscopy images of cross-section of scaffold showing aligned electrospun fibers and collagen matrix. **(B)** Magnified image of aligned electrospun fibers **(C)** Cumulative release of miR and NT-3 over time. **(D)** Mass loss of scaffold over time. Data are represented as mean $\pm$ SD.

Figure 4.4. Cumulative release profile of Neg miR and NT-3. Data represented as mean $\pm$ SD.

4.3.3. Scaffold-mediated delivery of miR-219/miR-338 preserved number of oligodendroglial lineage cells around injury site after SCI

Having established that scaffold-mediated non-viral delivery of miR-219/miR-338 could enhance OL differentiation and myelination *in vitro*, I next assessed the *in vivo* therapeutic efficacy of this approach using a hemi-incision SCI model as a proof-of-concept. In our opinion, this C5 hemi-incision model was most suitable as it is well-established and surgically reproducible. I first evaluated if scaffold-mediated delivery of miR-219/miR-338 would elicit effects on the total cell numbers around the lesion site (Figure 4.5A). Specifically, I quantified the number of DAPI⁺ cells and found that there was no significant difference in the total cell number between the two treatment groups at all time points (Figure 4.5B-4.5C). Next, I asked if miR-219/miR-338 would affect the number of oligodendroglial lineage cells,

which would include OPCs and OLs, around the lesion site through the analysis of Olig2 expression.²²³ I found that miR-219/miR-338 treatment maintained a consistent number of Olig2⁺ cells at Week 1, 2 and 4 post implantation. On the contrary, significant changes were observed in animals that received non-functional, negative scrambled miR treatment. Specifically, at Week 1 post implantation, the densities of Olig2⁺ cells around the perilesion site were similar between both groups (2410 cells/mm² in miR-219/miR-338 group and 2520 cells/mm² in Neg miR group) (Figure 4.5B and 4.5D). However, by Week 2 post implantation, the number of Olig2⁺ cells decreased significantly in the Neg miR group and this number remained consistently low after 4 weeks of recovery. For both of these time points, I observed significantly higher Olig2⁺ cell densities in miR-219/miR-338 group as compared to the Neg miR group ($p < 0.001$) (Figure 4.5B and 4.5D).

Figure 4.5. Scaffold-mediated non-viral delivery of miR-219/miR-338 preserved oligodendroglial lineage cell numbers after SCI. (A) Representative image of scaffold implanted in injury site. Double white lines around the scaffold delineate the perilesion region where quantifications were conducted. **(B)** Olig2⁺ cells (green) in miR-219/miR-338 treatment group (left) and Neg miR group (right). **(C)** DAPI⁺ cell density remained consistent at perilesion

area after SCI regardless of scaffold treatment. **(D)** Olig2⁺ cell density remained consistent at perilesion area in the presence of miR-219/miR-338 but decreased significantly under treatment with Neg miR. *** p<0.001, ** p<0.01, * p<0.05; N.S.- Not Significant (ANOVA). Data are represented as mean ± SEM.

4.3.4. Scaffold-mediated delivery of miR-219/miR-338 promoted OPC differentiation after SCI

We next examined the maturity of the Olig2⁺ cell population around the lesion site as miR-219/miR-338 over-expression has been reported to enhance OPC differentiation *in vitro*^{194, 216} and *in vivo*, albeit in a development model.^{217, 218} The expression of platelet-derived growth factor receptor alpha (PDGFR α), a cell surface marker that is expressed by OPCs but not mature OLs,¹⁸⁷ was assessed at Week 1, 2 and 4 post-implantation to track the number of OPCs over time. Quantification results showed significant reduction in PDGFR α ⁺ cell density from Week 1 to Week 4 for both treatment groups (440 cells/mm² to 180 cells/mm² for miR-219/miR-338 group and 410 cells/mm² to 200 cells/mm² for Neg miR group, p < 0.001) (Figure 4.6A and 4.6B). When comparing miR-219/miR-338 and Neg miR treated animals within a specific time point, no significant difference was observed (Figure 4.6B).

In order to validate these findings, I next evaluated the expression of CC-1, a protein marker that is predominantly found in mature OLs but absent in OPCs.²²⁴ Complementary to the trend that was observed in PDGFR α expression, CC-1⁺ cell density around the injury area increased from Week 1 to Week 4 for both treatment groups (1050 cells/mm² to 1770 cells/mm² in miR-219/miR-338 group and 1050 cells/mm² to 1380 cells/mm² in Neg miR group) (Figure 4.6C and 4.6D). However, the degree of OL differentiation appeared more extensive

in the presence of miR-219/miR-338. In particular, significantly more CC-1⁺ mature OLs were observed when animals received miR-219/miR-338 after 2 and 4 weeks of treatment (Figure 4.5D, $p < 0.05$ and 0.01 respectively vs. Neg miR). Besides enhancing the extent of OL differentiation, miR-219/miR-338 treatment also appeared to increase the rate of OL differentiation. Specifically, significantly larger numbers of mature CC-1⁺ OLs were detected starting 2 weeks post implantation of miR-219/miR-338 scaffolds. This number of mature OLs continued to increase after 4 weeks of recovery. In contrast, animals that received Neg miR treatment showed significant increase in the number of mature CC-1⁺ OLs only after 4 weeks post implantation. Taken together, the results suggest that PDGFR α ⁺ cells lost their viability over time following SCI. However, as indicated by the CC-1⁺ cell numbers, those cells that survived could be aided by the miR treatment, hence resulting in enhanced OL differentiation and maturation.

Previously, NT-3 was demonstrated to stimulate OPC proliferation²²⁵⁻²²⁷ and maturation.²²⁷ Since all our scaffolds contained NT-3 along with miRs, to confirm that the results obtained were indeed due to the effects of miR-219/miR-338, I included another treatment group that received only NT-3, without the addition of miRs, for comparison at Week 2 post implantation. The results showed that NT-3 did not lead to any significant changes in both Olig2⁺ and CC-1⁺ cell populations as compared to Neg miR treatment (Figure 4.7). Instead, for both of these cell populations, significant differences were only detected between miR-219/miR-338 and NT-3 groups as well as between miR-219/miR-338 and Neg miR groups.

Figure 4.6. Scaffold-mediated non-viral delivery of miR-219/miR-338 promoted greater extent and rate of OPC differentiation. **(A)** PDGFR α ⁺ cells (red) in miR-219/miR-338 treatment group (left) and Neg miR group (right). **(B)** Density of PDGFR α ⁺ cells per perilesion area. **(C)** PDGFR α ⁺ cell fold change decrease between different time points. **(D)** CC-1⁺ mature OLs (red) in miR-219/miR-338 treatment group (left) and Neg miR group (right). **(E)** Density of CC-1⁺ cells per perilesion area. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Kruskal-Wallis, Mann-Whitney U test). Data are represented as mean $\pm$ SEM.

Figure 4.7. NT-3 alone did not prevent loss of oligodendroglial lineage Olig2⁺ cells nor induced OPC maturation after SCI. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (ANOVA). Data represented as mean $\pm$ SEM.

4.3.5. Scaffold-mediated delivery of miR-219/miR-338 enhanced myelin formation after SCI

The microenvironment of the injured area within the spinal cord is very inhibitive for myelin formation.²²⁸ To determine the extent of myelin regeneration after injury, I stained the samples with MBP, along with 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) for validation at 4 weeks post implantation. Under the fluorescence microscope, MBP and CNPase signals were identifiable in the tissues and within the implants as shown in Figure 4.8A. By quantifying the percentage of area of the scaffold that possessed MBP⁺ signals, I found that MBP expression was more extensive in scaffolds that incorporated miR-219/miR-

338 (MBP⁺ area = $45.73 \pm 4.74\%$) as compared to Neg miR samples (MBP⁺ area = $20.92 \pm 2.77\%$) (Figure 4.8B).

I next proceeded to evaluate the extent of myelination within the scaffolds by quantifying the myelination index (defined as the percentage of colocalization of MBP with axonal NF200). In particular, I found that miR-219/miR-338-treated animals ($60.51 \pm 4.99\%$) displayed significantly higher myelination index than Neg miR treated animals ($32.06 \pm 7.54\%$, $p < 0.05$) (Figure 4.8C and 4.8D). As OLs were also able to myelinate electrospun fibers *in vitro*, I wanted to know if the fibers within the scaffold were myelinated in an *in vivo* setting. Surprisingly, from the transmission electron microscopy (TEM) images, I was unable to identify any compact myelin structures around the electrospun fibers in both of the treatment groups (Figure 4.9).

Additionally, I also analyzed the host-implant interface for potential signs of myelination. At Week 4 post implantation, I observed MBP⁺ tubular structures that surrounded NF⁺ axons at the host-implant interface for both miR-219/miR-338 and Neg miR treated animals (Figure 4.10A). I then quantified the myelination index and found a trend that suggested better myelination outcomes in the presence of miR-219/miR-338 ($13.65 \pm 1.77\%$ vs. $9.49 \pm 2.99\%$ for Neg miR treatment, Figure 4.10B). Finally, further analyses using TEM showed that compact myelin formation (Figure 4.10C) could be detected in animals that received miR-219/miR-338. In contrast, these structures were absent in animals that received negative, scrambled miR treatment (Figure 4.10D).

Figure 4.8. Scaffold-mediated non-viral delivery of miR-219/miR-338 supported myelin regeneration and axon ensheathment within scaffold (A) Representative images comparing different miR-219/miR-338- and Neg miR-loaded scaffolds. Relatively prominent MBP and CNPase signals were observed within scaffolds with miR-219/miR-338 at Week 4. White dotted lines demarcate the region occupied by the scaffold. **(B)** Percent of fiber-scaffold area that is MBP⁺. **(C)** Representative images from Week 4 samples showing MBP⁺ signals in

scaffold. White arrows indicate axons that appeared to be partially ensheathed. **(D)** Myelination index obtained within the scaffolds. ** $p < 0.01$, * $p < 0.05$ (Student's t-test).

Figure 4.9. Representative transmission electron microscopy images within the scaffold of both miR-219/miR-338 and Neg miR group. Black asterisk denotes a cross sectional view of an individual electrospun PCLEEP fiber. No compact myelin sheaths were observed around the electrospun fibers screened in both groups.

Figure 4.10. Scaffold-mediated non-viral delivery of miR-219/miR-338 showed a trend of enhanced myelination at host-implant interface. (A) Representative images obtained at Week 4 depicting MBP⁺ tubular structures surrounding NF⁺ axons. **(B)** Myelination index obtained at host-implant interface. **(C, D)** Representative transmission electron microscopy images showing the (C) presence and (D) lower degree of myelinated axons in (C) miR-219/miR-338 and (D) Neg miR groups respectively. N.S. – Not Significant (Student's t-test).

4.3.6. Scaffold-mediated delivery of miR-219/miR-338 did not affect neurite outgrowth or exacerbate glial scarring after SCI

Although the fiber-hydrogel scaffold provided localized drug delivery,²²⁰ the transfection of miRs was still indiscriminatory at the implant/injury site. Hence, we proceeded to investigate the effects of the scaffolds on neurite outgrowth and glial scarring.

I observed that regardless of miR treatment, no significant difference was detected in NF200 signals at the host-implant interface (Figure 4.11A and 4.11B), as well as within the scaffolds (Figure 4.11A and 4.11C). These observations were consistent with our *in vitro* results where the well-established and robust DRG neuronal culture was used. Specifically, *in vitro* transfection of DRG neurons showed that the non-viral transfection of miR-219/miR-338 had no significant impact on the extents of neurite outgrowth as compared to untreated cells, cells treated with negative scrambled miR or the transfection reagent, TKO, alone (Figure 4.12).

In the case of glial scarring, when the intensity of GFAP (a positive indicator of glial scar formation by activated astrocytes²²⁹) was analyzed at the perilesion region, no significant difference was detected between miR-219/miR-338 and Neg miR treated groups at all time points (Figure 4.13).

Figure 4.11. Scaffold-mediated non-viral delivery of miR-219/miR-338 did not affect neurite regeneration *in vivo*. **(A)** NF200 expression (red) at injury sites and light micrograph of fiber-hydrogel scaffolds in miR-219/miR-338 (left) and Neg miR group (right). **(B)** Percentage of host-implant interface area occupied by neurofilament ingrowth. **(C)** Percentage of scaffold area occupied by neurofilament ingrowth. N.S.- not significant (ANOVA). Data represented as mean $\pm$ SEM. Scale bars represent 500 μ m.

Figure 4.12. miR-219/miR-338 has no effect on neurite growth *in vitro*. **(A).** Representative images of Untreated, TKO, Neg miR and miR-219/miR-338. **(B)** Average total length of neurites. **(C)** Average longest length of neurite. N.S.- not significant (ANOVA). Data represented as mean $\pm$ SEM.

Figure 4.13. Scaffold-mediated non-viral delivery of miR-219/miR-338 did not affect glial scar formation. (A) GFAP expression (green) at injury sites and light micrograph of fiber-hydrogel scaffolds in miR-219/miR-338 (left) and Neg miR group (right). **(B)** Area fraction of glial scar formed at perilesion region. N.S.- not significant (ANOVA). Data represented as mean $\pm$ SEM. Scale bars represent 500 μ m.

4.4. DISCUSSION

Axon demyelination within the CNS, regardless through mechanical injuries or pathological consequences, usually results in functional impairments that can severely affect a patient's quality of life. Yet, effective remyelination strategies remain limited. Besides being a keystone cell type for restoring energy-efficient signal conduction in axons, OLs are also

crucial in providing metabolic support for axonal survival. Unfortunately, the injured CNS is often not permissive for myelin regeneration due to the plethora of inhibitory factors that are present.^{96, 228} Despite that, it may be possible to bypass this restriction if a potent method to drive robust myelin regeneration exists. Correspondingly, this study highlights the use of scaffold-mediated non-viral delivery of miR-219/miR-338 to promote *in vivo* CNS remyelination, using SCI as a proof-of-concept.

Previous works by several groups have identified miR-219 and miR-338 to be responsible for regulating the maturation and myelination of cells from the oligodendroglial lineage.^{217, 218, 230} Moreover, a recent work by Lu *et al.* showed that miR-219 and miR-338 have additive effects on OPC differentiation and are required for the full extent of myelination.²¹⁹ Mechanistically, these two miRs exert their effects by inhibiting a set of OPC differentiation-inhibiting genes such as PDGFR α , SRY-box containing gene (Sox6), forkhead box J3 (FoxJ3), zinc finger protein 238 (ZFP238) and Hes5.²¹⁷⁻²¹⁹ Hes5, in particular, is a critical downstream effector within the Notch signaling pathway and has also been shown to be related to the expression of myelin markers.^{231, 232} From developmental studies, it was observed that Hes5 levels were elevated in naive OPCs and progressively decreased as they differentiated and started to express myelin genes.^{231, 232} The rest of the inhibitory genes mentioned were demonstrated to be similarly regulated in our past studies, which investigated the effects of sustained fiber-mediated delivery of miR-219 and miR-338 on OPC differentiation.^{194, 216}

Here, I hypothesized that a combination of scaffold topography and RNA interference would prime the endogenous population of OPCs to engage in myelination after injuries within the CNS. I first explored the effects of miR-219/miR-338 cocktail on OPC

differentiation and myelination *in vitro* using electrospun PCL fibers. These fibers were fabricated to be suspended,¹⁸⁰ unlike those used in our previous work¹⁹⁴ which were condensed to form a fibrous mat. However, as the miR loading strategy was similar, we would also anticipate a burst release within the first week and subsequent sustained release for another week.¹⁹⁴ Consistent with previous *in vitro* findings, OPCs matured faster when transfected with miR-219/miR-338 as indicated by the increased percentage of cells expressing MBP at Week 2. The effect of these miRs' gene silencing, using this platform, has also been confirmed in those studies.^{194, 216} Besides that, I also demonstrated that this miR cocktail could greatly enhance the ensheathment length and number of sheaths that were formed by MBP⁺ OLs after 2 weeks of differentiation. The quantification method for counting the number of sheaths per cell in this paper was adopted from a previous study by Bechler *et al.*²⁰⁴ Compared to their measurements, the number of complete sheaths I obtained were 2 to 3 folds lesser. This observation may be due to the process of miR transfection, which may be more cytotoxic to sensitive cells like OPCs and OLs.

The scaffold utilized in this *in vivo* study contained two important features, aligned electrospun fibers and collagen hydrogel. This design was adopted from our previous study where aligned electrospun fibers were employed to provide topographical guidance for axon ingrowth.^{220, 221} These fibers were also arranged in a 3D conformation so as to imitate the architecture of the natural extracellular matrix and allow infiltrating cells to maintain their viability.²³³ To keep these fibers intact during and after implantation, collagen hydrogel was added for physical support. Importantly, another role played by collagen hydrogel was the non-viral and localized sustained delivery of drugs, which has been shown in our previous works^{220, 221} and in this study. However, unlike previous work,²²⁰ I decreased the

concentration of the collagen hydrogel and also varied the transfection reagent for miR delivery in this study. Of note, I found that oligonucleotides were taken up by cells in the surrounding tissues, up to a distance of 300 μm .²²⁰ Hence, I chose a distance of 100 μm away from the borders of our scaffold for analysis of cellular response as I would expect an increase of myelin under the influence of the miRs. This analysis of cellular response at the perilesion region is also consistent with the literature.²³⁴

In our attempt to characterize the density of oligodendroglial lineage cells around the perilesion site, I observed notable differences in the number of Olig2⁺ cells between miR-219/miR-338 and Neg miR treatment groups from 2 weeks post implantation onwards. When comparing the perilesional density of Olig2⁺ cells between miR-219/miR-338 and Neg-miR treatment groups, some differences were observed during Week 2 and Week 4 post injury. Based on the consistent density of DAPI⁺ cells during the entire duration of the experiment, these results indicated that miR treatment increases the quality of differentiation but not the viability of the cells. Due to limited literature investigating the effects of miR-219/miR-338 on OPC cell survival, further confirmation of this observation would require lineage tracing of OPCs.²³⁵

Axon myelination within the injured CNS is an important element of regeneration.²³⁵ Consistent with a recent study by Lu *et al.*,²¹⁹ albeit in a non-SCI model, I observed similar trends where miR-219/miR-338 treatment led to higher number of axons being myelinated as compared to Neg miR treatment. Besides examining OPCs/OLs, I also assessed the effects of miR-219/miR-338 on neurons and astrocytes. Previously, I showed that this scaffold platform could deliver other miRs, such as miR-222, to enhance axonal regeneration *in vivo*.²²⁰

Therefore, the lack of significant differences in neurite length between miR-219/miR-338 and Neg miR group *in vivo* and *in vitro* indicated that it was most likely due to the insensitivity of neurons/axons towards miR-219/miR-338 and not due to the failure of miR uptake/delivery. The same conclusion can be drawn for results observed in astrocytes where glial scarring was not significantly induced in both miR-219/miR-338 and Neg miR treated groups. However, as GFAP is only indicative of astrocyte activation,²²⁹ there might be other secretory factors that led to a more permissive environment for myelin regeneration. Future studies could be conducted to investigate this hypothesis.

OLs were shown to be able to myelinate electrospun fibers *in vitro*.^{204, 236} However, when I sectioned the scaffolds and examined them under the TEM, I did not observe any fiber ensheathment *in vivo*. Based on a recent study by Assinck *et al.*, I speculate that one possible reason could be that the OPCs within the scaffolds were differentiating into Schwann cells instead of OLs.²³⁵ Contrary to OLs, there are not many studies demonstrating the potential of Schwann cells in ensheathing electrospun fibers *in vitro* and *in vivo*. Moreover, it has been shown previously that Schwann cell myelination is regulated by signaling molecules, such as Neuregulin 1 Type III.^{198, 237} As our fibers were not coated with myelination cues, it was unlikely to be myelinated. Hence, future works could explore the effects of miR-219/miR-338 on OPC differentiation into Schwann cells vs. OLs. Importantly, I would also seek to elucidate the relative potential of OLs and Schwann cells in inducing myelin regeneration and subsequent functional recovery after SCI.

RNAi is widely adopted in treatments related to cancer therapy and genetic diseases. However, it presents much wider implications and is potentially useful in regenerative

medicine. Unfortunately, one of the major stumbling blocks to the translational use of RNAi is the development of proper delivery systems for effective and efficient *in vivo* delivery of these molecules with minimal off-target effects. Traditional approaches of systemic delivery for cancer and genetic disease therapy may not be directly beneficial to traumatic tissue injuries since the latter often involves confined injuries that also require the reestablishment of *in vivo* tissue structures. Scaffold-mediated delivery, as highlighted here, represents a potential approach since scaffolds can provide the necessary structural support and directional cues for tissue regrowth and restructuring. Furthermore, scaffold-mediated delivery of drugs provides localized delivery to the site of injury,²²⁰ hence minimizing off-target systemic effects. Therefore, results from this work will highlight the importance of RNAi and scaffold-mediated gene silencing to regenerative medicine. This study further enforces the potential of RNAi in directing the differentiation of endogenously recruited progenitor cells. Such aspects have not been demonstrated to date.

Clinically, the implantation of similar biocompatible scaffolds for SCI treatment has already gained momentum^{122, 238} and results have, thus far, shown great promise. I believe that in due time as the regulatory bodies are convinced of scaffold implantation as a treatment option for SCI or even demyelinating diseases, this work can be the next step forward to attain a higher resolution therapeutic avenue.

4.5. CONCLUSION

Our current study reflects the potency of scaffold-mediated delivery of miR-219/miR-338 in affecting the fate of oligodendroglial lineage cells to differentiate from OPCs to

matured OLs. Furthermore, I also demonstrated that such non-viral, localized and sustained delivery of miR-219/miR-338 could promote myelination within the injured CNS.

CHAPTER 5

REGENERATIVE REHABILITATION: EXPLORING THE SYNERGISTIC EFFECTS OF REHABILITATION AND IMPLANTATION OF BIO-FUNCTIONAL SCAFFOLD IN ENHANCING NERVE REGENERATION

ABSTRACT

Clinically, rehabilitation is the one of the most common treatment option for traumatic injuries. Despite that, recovery remains suboptimal and recent breakthroughs in regenerative approaches may potentially improve clinical outcomes. To date, there have been numerous studies on the utilization of either rehabilitative or regenerative strategies for traumatic injury treatment. However, studies that document the combined effects of rehabilitation with regenerative tissue engineering options remain scarce. Here, in the context of traumatic nerve injury treatment, I use a rat spinal cord injury (SCI) model as a proof of concept to evaluate the synergistic effects of regenerative tissue engineering and rehabilitation. Specifically, I implanted a pro-regenerative hybrid fiber-hydrogel scaffold and subjected SCI rats to intensive rehabilitation. Of note, the rehabilitation session was augmented by a novel customized training device that imparts normal hindlimb gait movements to rats. Morphologically, more regenerated axons were observed when rats received rehabilitation (~ 2.5 times and ~ 2 times enhancement after 4 and 12 weeks of recovery respectively, $p < 0.05$). Besides that, I also observed a higher percentage of anti-inflammatory cells ($36.1 \pm 12.9\%$ in rehab rats vs. $3.31 \pm 1.48\%$ in non-rehab rats, $p < 0.05$) and perineuronal net formation in rehab rats at Week 4. Physically, rehab animals were also able to exert higher ankle flexion force (~ 0.779 N vs. ~ 0.495 N at Week 4 and ~ 1.36 N vs. $\sim$

0.647 N at Week 12 for rehab vs. non-rehab rats, $p < 0.001$) and performed better than non-rehab rats in the open field test. Taken together, I conclude that coupling rehabilitation with regenerative scaffold implantation strategies can further promote functional recovery after traumatic nerve injuries.

5.1. INTRODUCTION

Rehabilitation is currently the clinical gold standard for improving the functions of patients following traumatic injuries^{239, 240} as it is by far the most effective treatment for preventing further deterioration of tissue structures and functions. While this approach is widely adopted and allows patients to regain function over time, recovery for those severely injured remain suboptimal. Besides improving the rehabilitative regime, it is recently recognized that tissue regeneration, coupled with rehabilitation, would be the path to achieve better recovery¹⁶⁶.

Termed 'regenerative rehabilitation', this paradigm involves the synergistic integration of principles and approaches from both the regenerative medicine and rehabilitation fields in order to maximize the recovery of the patients in form and function¹⁶⁶. Significant advances in regenerative medicine might be able to improve survival from catastrophic injuries, but often fail to fully restore function^{131, 241-243}. Similarly, treatment with rehabilitation alone is insufficient^{244, 245}. Accordingly, this implies that in many trauma cases, neither regenerative medicine nor rehabilitation alone can result in complete recovery, especially for patients who sustained more complex traumatic injuries¹⁶⁶.

In the context of traumatic nerve injuries, currently available clinical treatments mainly rely on preserving and preventing further decline of the injured nerve²⁴⁶. Fortunately, tissue engineering approaches for attaining neural regeneration have shown promising outcomes in the last few decades amidst the immense technical hurdles. One example is the groundbreaking work by Lu *et al.* where they demonstrated the efficacy of using neural stem

cells embedded in fibrin matrices containing growth factor cocktails for promoting spinal cord repair¹²¹. Besides that, Li *et al.* also had considerable success with neural regeneration by implanting Neurotrophin-3 (NT-3) loaded chitosan scaffolds in fully transected rats¹²⁹ and in hemi-transected monkeys¹²⁸.

However, enhanced morphological regeneration does not equate to enhanced functional recovery. This was evident in the study by Lu *et al.* where even though axonal growth was extremely robust, the animals did not show weight bearing capabilities¹²¹. Moreover, while these studies were able to elegantly improve spinal cord regeneration, none evaluated rehabilitation as a treatment option for promoting motor function recovery. Conversely, works that conducted rehabilitation on spinally injured animals²⁴⁷⁻²⁴⁹ did not explore scaffolds for tissue regeneration purposes. Since spinal cord injury (SCI) is a complex phenomenon, combinatorial therapies that include both regeneration and rehabilitation are most likely required. As such, this work explored this strategy by using a rat SCI model as a proof of concept. Specifically, the effects of rehabilitation were evaluated on spinally injured rats with an implanted pro-regenerative hybrid scaffold.

Here, an established NT-3 loaded fiber-hydrogel scaffold^{181, 220, 221, 250} was implanted into T9-T10 fully transected (2 mm gap) spinal cords of Sprague-Dawley rats. Thereafter, the effects of body weight-supported treadmill training (BWSTT) on nerve regeneration was explored. Dollaporn Anopas, our collaborator, custom-designed and built a rehabilitative apparatus for intensive gait rehabilitation. This construct was armed with a pair of hindlimb manipulation device which could be clamped onto the rats' ankle to actuate pre-programmed normal rat hindlimb gait during training²⁵¹. I show that administration of NT-3, a reputed

neurotrophin for enhancing neuronal survival²²², coupled with rehabilitation for spinally injured rats promoted axonal ingrowth into scaffolds, enhanced perineuronal nets formation within the thoracic region and improved plantarflexion in the rat hindlimbs. Our results demonstrate the potential of combining neural rehabilitation with regenerative medicine to provide a unique solution to improve tissue regrowth following traumatic nerve injuries.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Rat tail collagen type 1 was purchased from Corning, USA. Neurotrophin-3 (NT-3) was procured from PeproTech, USA. Heparin and bovine serum albumin (BSA) were obtained from Sigma, USA. Chicken anti-NF200 (822601) was procured from Biolegend, USA. Rat anti-MBP (MCA409S) and mouse anti-CD68 (ED1, MCA341GA) was purchased from Biorad, USA. Rabbit anti-CD206 (ab64693) was obtained from Abcam, UK. Mouse anti-iNOS/NOS type II (iNOS, 610329) was purchased from BD Biosciences, USA. Fluorescein-conjugated *Wisteria floribunda agglutinin* (WFA, FL1351) was bought from Vector Laboratories, USA. Goat anti-chicken Alexa Fluor 555, goat anti-rat Alexa Fluor 488, goat anti-mouse Alexa Fluor 555, goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 633 and 4',6-diamidino-2-phenylindole (DAPI) were acquired from Life Technologies, USA.

5.2.2. NT-3 loaded PCLEEP scaffold fabrication

The scaffold was fabricated using a procedure described previously²²⁰. Briefly, PCLEEP electrospun fibers, obtained using an air gap electrospinning technique, were rolled into a bundle and placed within a mold. Thereafter, 200 µl of rat tail collagen type 1 was prepared at a concentration of 3 mg/ml following manufacturer's protocol. Following that, 1 µl of NT-3

from a stock concentration of 2 µg/µl (2 µg per scaffold) constituted in 0.1% BSA and heparin (400 µg/µl) (BSA : heparin v/v ratio = 1:1) was mixed with the collagen before adding into the mold (4 mm in diameter). After gelation, the scaffolds were frozen at - 20 °C for 3 h before being lyophilized overnight. Finally, the scaffolds were cut into 2 mm long pieces and sterilized under UV light for 45 min before *in vivo* implantation.

5.2.3. Animals

A total of 26 adult female Sprague Dawley rats (7-9 weeks, 180-230 g)(n = 4 each for rehab and non-rehab group at Week 4 and n = 9 each for rehab and non-rehab group at Week 12) were obtained from In Vivos Pte Ltd (Singapore). Animal care and experimental procedures were carried out in accordance to the Institutional Animal Care and Use Committee (IACUC, NTU) guidelines. Animals were housed under temperature controlled conditions, with a normal 12:12 h light/dark cycle with ad libitum access to water and food.

5.2.4. Surgical procedures for severe spinal cord injury

Buprenorphine (0.05 mg/kg) was first administered to each animal subcutaneously 30 min before surgery. They were then placed in a chamber consisting 4-5% mixture of isoflurane in air and injected with Ketamine (75 mg/kg) and Xylazine (10 mg/kg) intraperitoneally. Once the animals were well anaesthetized, the dorsal region of the rats (thoracic) were shaved and cleaned with 70% ethanol and betadine. A midline incision was made at the disinfected area and the overlying muscles were bluntly dissected to expose the vertebrae. A laminectomy procedure was further performed to reveal the underlying spinal cord. Following that, the dura was sliced open and a 2 mm full transection was made on the spinal cord (T9-T10). The scaffold (2 mm in length) was then implanted into the lesion site once the bleeding had

subsided. After the implantation, the muscles were sutured using Prolene 4/0 single stitches and the skin was closed using wound clips. Post-operative treatments and care included addition of Meloxicam (5 mg/ml) into the drinking water for a duration of ten days post-surgery and expression of bladder twice a day until recovery.

5.2.5. Animal rehabilitation

One week before surgery, tested animals were acclimatized, once every day, following a progressive duration (5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 30 min), to run on a motor driven treadmill belt at a speed of 12 cm/s (speed was determined based on the endurance of the rats). Body weight-supported treadmill rehabilitation training for the rats was conducted on a custom-made rehabilitation machine²⁵¹ (Figure 5.1) starting one week after surgery to allow for sufficient recovery. Rehab rats adopted a quadrupedal position while training and were imparted with a pre-programmed normal hindlimb gait by the manipulation devices, which, to the best of our knowledge, have not been implemented in other neural rehabilitation studies. Training was conducted on every Monday, Wednesday and Friday for 30 min at a treadmill belt speed of 3 cm/s²⁵¹. Rehabilitation was conducted every two days because each session is quite strenuous for the rats. Over-exercise may lead to more injuries instead of recovery. The reference was our previous preliminary work where I tested the suitability of these conditions for both the rats and the trainer. A 30 min training session would sufficiently train the rat while a belt speed of 3 cm/s was used as it synchronizes with the hindlimb manipulator in the custom-built rehabilitation device.

Figure 5.1. Rehabilitation machine was customized to impart healthy gait in injured rats.

Complete view of the rehabilitation machine with the various labelled components. A magnified view of the body weight support and the manipulation device is shown in the box with the green and blue frame respectively. A magnified view of the rehab rat fitted onto the rehab machine and effector powered by the manipulation device is depicted in the box with red frame.

5.2.6. Immunohistochemical analysis

At designated time points post-implantation (Week 4 and 12), animals were euthanized in CO₂ before being transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA). The implanted scaffolds and some of the surrounding spinal cord

tissues were then harvested and further transferred into 4% PFA for at least 3 h, followed by 15% sucrose solution for 24 h and finally 30% sucrose solution. Thereafter, tissues were embedded in Tissue-Tek® O.C.T. Compound and cut into 20 µm thick horizontal sections using a cryostat (Leica CM1950). Immunofluorescent staining was performed to evaluate the effects of the treatment on various cell types of interest. Sections were first permeabilized with 0.3% Triton X-100, followed by blocking with 10% goat serum for 1 h and incubating with primary antibodies overnight at 4 °C. Primary antibodies used for recognizing neurofilaments and myelin basic protein (MBP) included chicken anti-neurofilament 200 (NF200, 1:1000 dilution) and rat anti-MBP (1:200 dilution) respectively. After primary antibody incubation, the samples were washed thrice with PBS before secondary antibody incubation for 1 h at room temperature using goat anti-chicken Alexa Fluor 555 (1:700 dilution) and goat anti-rat Alexa Fluor 488 (1:700 dilution) respectively.

For macrophage staining, a sequential staining protocol was adopted. Specifically, sections were first post-fixed briefly for 10 min with 4% PFA, washed with 1 x PBS thrice and placed in 50 ml of 1 x citrate buffer (pH 6) in the oven at 85 °C for 45 min for antigen retrieval. Thereafter, the samples were then removed from the oven and left to cool at room temperature for 30 min before blocking with 10% goat serum for 1 h. After blocking, mouse anti-ED1 (1:50 dilution) and rabbit anti-CD206 (1: 200 dilution) were added to the sections and incubated at 37 °C for 30 min. Following that, sections were washed thrice with 1 x PBS before secondary antibody incubation for 1 h at 37°C using goat anti-mouse Alexa Fluor 555 (1:700 dilution) and goat anti-rabbit Alexa Fluor 488 (1:700 dilution). After the first round of primary and secondary antibody incubation, samples were washed thrice with 1 x PBS and further incubated with mouse anti-iNOS (1:100 dilution) at 37 °C for 30 min. Samples were

then subsequently washed thrice with 1 x PBS and incubated with goat anti-mouse Alexa Fluor 633 (1:500 dilution) for 1 h at 37 °C.

For perineuronal nets (PNNs) staining, sections were first blocked with 10% goat serum for 1 h, stained with *Wisteria floribunda agglutinin* (WFA, 1:1000 dilution) for 4 h, washed thrice with 1 x PBS and incubated with mouse anti-NeuN (1:500) at 4 °C overnight. Thereafter samples were washed thrice with 1 x PBS before incubating with goat anti-mouse Alexa Fluor 555 (1: 700) for 1 h at room temperature. Nuclei were labelled with DAPI (1:1000 dilution) for all immunohistochemically stained samples during secondary antibody incubation and all slides were coverslipped with Fluoromount-G.

The area percentage of NF200 staining within the scaffold was quantified with ImageJ (NIH, USA) using fluorescently-labelled stitched collage consisting of several 10x magnified images (Leica DMI8). A total of three sections per animal were used for the quantification. The area percent of NF200 staining was calculated using the following equation:

$$\text{Area percent of NF200 in scaffold (\%)} = \frac{\text{Area occupied by NF200}^+ \text{pixels}}{\text{Area of scaffold}} \times 100\%$$

To quantify myelination index within the scaffold, 40x magnified confocal z-stack images (Zeiss LSM800) were analyzed using ImageJ (NIH, USA) and a customized colocalization plugin. For each sample section, five images were taken at different regions of the scaffold (top left, top right, middle, bottom left and bottom right). A total of three sections were quantified per animal and the myelination index was defined by the following equation:

$$\text{Myelination Index (\%)} = \frac{\text{Number of colocalized NF200 and MBP pixels}}{\text{Number of NF200 pixels}} \times 100\%$$

Macrophages were manually quantified using 40x magnified images obtained from the confocal microscope (Zeiss LSM800). One image was acquired at each pre-defined region per section. In total, three sections were quantified per animal. The pre-defined regions were namely, the rostral implant interface (Interface (Rostral)), the rostral side within the scaffold (In Scaffold (Near Rostral)), the middle of the scaffold (In Scaffold (Middle)), the caudal side within the scaffold (In Scaffold (Caudal)) and the caudal implant interface (Interface (Caudal)). The density of ED1⁺ cells was calculated using the following equation:

$$\text{Density of ED1}^+ \text{ cells (cells/mm}^2\text{)} = \frac{\text{Number of DAPI}^+ \text{ED1}^+}{\text{Area of ROI (mm}^2\text{)}}$$

The percentage of ED1⁺ cells was calculated using the following equation:

$$\text{Percent of ED1}^+ \text{ cells (\%)} = \frac{\text{Number of DAPI}^+ \text{ED1}^+ \text{ cells}}{\text{Number of DAPI}^+ \text{ cells}} \times 100\%$$

The percentage of ED1⁺iNOS⁺ or ED1⁺CD206⁺ cells was calculated using the following equation:

$$\begin{aligned} &\text{Percentage of ED1}^+ \text{iNOS}^+ \text{ or ED1}^+ \text{CD206}^+ \text{ cells (\%)} \\ &= \frac{\text{Number of DAPI}^+ \text{ED1}^+ \text{iNOS}^+ \text{ or ED1}^+ \text{CD206}^+ \text{ cells}}{\text{Number of DAPI}^+ \text{ED1}^+ \text{ cells}} \times 100\% \end{aligned}$$

In the spinal cord, perineuronal nets (PNNs) were identified as a halo surrounding NeuN⁺ cells²⁵². Quantification was conducted using 20x magnified images (Leica DMI8) acquired at increasing distances (1 mm, 2 mm and 3 mm) from the scaffold interface rostrally and caudally. In total, three sections were quantified per animal. The percent of NeuN⁺ cells with PNN was calculated with the following equation:

$$\text{Percent of NeuN}^+ \text{ cells with PNN (\%)} = \frac{\text{Number of NeuN}^+ \text{ cells encircled by WFA}}{\text{Number of NeuN}^+ \text{ cells}} \times 100\%$$

5.2.7. Behaviour test

The hindlimb recovery of animals was evaluated through a weekly open field test using the Basso, Beattie, Bresnahan (BBB) scoring system²⁵³. Animals were placed within a circular enclosure (diameter of 1.5 m) and allowed to move around freely. Each rat was filmed for a duration of 3 min and their scores were assessed by two independent observers blinded to group identity.

The plantarflexion strength of animals was measured using a custom-made device that imparts dorsiflexion on the rats' hindlimb. The amount of force exerted by the rats when they resist through plantarflexion was detected through a force-sensing resistor placed underneath the hindlimb. In order to conduct the test, rats were first secured onto the harness with one of their hindlimbs strapped to a base plate, which was then further slotted in place above a force sensor. At the default starting position, the base plate is completely parallel to the ground (0°). Once the motor is activated, the base plate will be pivoted to a 45° angle in 2 s (inclination phase). This position is then maintained for 10 s (steady phase) before pivoting back to the normal default position in 2 s (declination phase). Measurements of the force (N) as a function of time throughout the entire cycle were obtained for both hindlimbs (three measurements per limb) of the rehab and non-rehab rats at Week 6 and Week 12. These values were also converted to a frequency plot using Gaussian distribution for statistical analysis.

5.2.8. Statistical analysis

Data was first tested for normality and homogeneity of variances using Shapiro-Wilk and Levene's test respectively. Those with homogenous variances were analysed using one-way ANOVA followed by Tukey's post-hoc test. For non-parametric data, Kruskal-Wallis test followed by Mann-Whitney post-hoc test were used instead. All error bars represent standard deviation.

5.3. RESULTS

5.3.1. Rats that underwent rehabilitation had better axonal infiltration into site of injury

I observed that at Week 4, animals that received rehabilitation had better axonal ingrowth into the injury site as shown by the increased amount of NF200 expression within the scaffolds (Figure 5.2, $2.00 \pm 0.79\%$ for rehab rats vs. $0.79 \pm 0.14\%$ for non-rehab rats). By Week 12, this difference became more significant (Figure 5.2, $4.05 \pm 0.65\%$ for rehab rats vs. $2.07 \pm 0.45\%$ for no rehab rats, $p < 0.05$). However, quantification of the myelin index yielded no prominent difference between rehab and non-rehab animals at both time points (Supplemental Figure 5.3A and B).

Figure 5.2. Rats that underwent rehabilitation had better axonal infiltration into scaffold.

(A) Representative images of spinal cord sections stained with NF200 and images of scaffolds taken under polarized light to identify aligned fibers. **(B)** Quantification of the area percent of NF200 staining in scaffold. ANOVA, **: p-value < 0.01; *: p-value < 0.05. All data are shown as mean $\pm$ SD; n = 4 for both rehab and non-rehab rats at Week 4; n = 7 and 9 for rehab and non-rehab rats respectively at Week 12.

Figure 5.3. Rehabilitation did not enhance myelination of axons in scaffold. (A) Representative images of NF200 and MBP staining in scaffold at both Week 4 and 12. White arrows denote region of axon that is co-localized with myelin, suggesting myelination. **(B)** Quantification of the myelination index within the scaffold. ANOVA, N.S. = Not Significant. All data are shown as mean $\pm$ SD; $n = 4$ for both rehab and non-rehab rats at Week 4; $n = 7$ and 9 for rehab and non-rehab rats respectively at Week 12.

5.3.2. Density and percentage of pan-macrophages within the injury site were unaffected by rehabilitation

Macrophages are instrumental in reflecting the immunological state within the injured region. As such, I wanted to know if rehabilitation will bring about any changes in their relative abundance within and outside the scaffold. Through ED1 staining (a pan-macrophage marker in rats), I observed that there was no significant difference in the density of ED1⁺ cells within the scaffold and at both the rostral and caudal implant interfaces at Week 4 between rehab

and non-rehab rats (Figure 5.4A and 5.4B). This trend was consistent as well when I looked at the percentage of cells that were ED1⁺ (Figure 5.4A and 5.4C). Although there was no notable disparity in the amount of macrophages between rehab and non-rehab rats in each region quantified, there were, however, significant differences between the regions analyzed. Specifically, higher percentage of cells were ED1⁺ at the centre of the scaffold as compared to the caudal implant interface for both rehab ($23.1 \pm 4.72\%$ at middle of scaffold vs. $12.9 \pm 2.87\%$ at caudal interface, $p < 0.05$) and non-rehab animals ($26.6 \pm 7.04\%$ at middle of scaffold vs. $15.3 \pm 3.82\%$ at caudal interface, $p < 0.05$) (Figure 5.4A and 5.4C).

Figure 5.4. Density and percentage of pan-macrophages within the injury site were unaffected by rehabilitation. (A) Representative images of ED1⁺ cells around and within the injury site at Week 4. Quantification of the **(B)** density and **(C)** percentage of ED1⁺ cells.

ANOVA, **: p-value < 0.01; *: p-value < 0.05; N.S. = Not Significant. All data are shown as mean $\pm$ SD; n = 4 for both rehab and non-rehab rats.

5.3.3. Injury region in rehab rats had relatively lower percentage of ED1⁺iNOS⁺ cells and higher percentage of ED1⁺CD206⁺ cells.

Next, I wanted to know if rehabilitation would lead to the skewing of macrophage phenotype (pro-inflammatory (M1-like) and anti-inflammatory (M2-like)) within the scaffold as well as the rostral and caudal regions of the host-implant interface. I observed that, in all the regions surveyed around the injury, there was a higher percentage of ED1⁺iNOS⁺ (M1-like) cells in non-rehab animals (Figure 5.5A and 5.5B). When comparing M2-like cells, there appeared to be a significantly larger percentage of ED1⁺CD206⁺ cells in the rostral and caudal implant interface of rehab rats as compared non-rehab rats ($p < 0.05$)(Figure 5.5A and 5.5C). Moreover, the percentage of ED1⁺CD206⁺ cells at the caudal implant interface were also significantly higher in rehab rats ($36.1 \pm 12.9\%$) than non-rehab rats ($3.31 \pm 1.48\%$)($p < 0.05$)(Figure 5.5A and 5.5C).

Figure 5.5. Injury region in rehab rats had relatively low and high percentage of ED1⁺iNOS⁺ and ED1⁺CD206⁺ cells respectively. (A) Representative images of ED1⁺iNOS⁺ and ED1⁺CD206⁺ cells around and within the injury site at Week 4. White and green arrows denote ED1⁺iNOS⁺ and ED1⁺CD206⁺ cells respectively. Graph depicting the percentage of **(B)** ED1⁺iNOS⁺ cells and **(C)** ED1⁺CD206⁺ cells. ANOVA, *: p-value < 0.05. All data are shown as mean ± SD; n = 4 for both rehab and non-rehab rats.

5.3.4. Scaffold implantation did not affect rehabilitation-induced perineuronal nets formation

I next proceeded to stain for plasticity-associated perineuronal nets (PNNs) using WFA, a broad PNN marker²⁵⁴, with the hypothesis that its expression would be increased due to exercise training-induced regulation within the spinal cord²⁵², regardless of the presence of the scaffold. As expected, I observed a clear trend where the percentage of NeuN⁺ cells with

PNN increased as the distance from the injury site increased rostrally and caudally. This was especially significant at the caudal end of rehab animals comparing distances at 3 mm ($15.4 \pm 3.41\%$) vs. 1 mm ($6.93 \pm 3.94\%$) ($p < 0.05$) (Figure 5.6A and 5.6B). Additionally, there was a trend where animals that underwent rehabilitation had a larger percentage of NeuN⁺ cells that were surrounded by PNNs at all distances away from the injured area rostrally and caudally as compared to non-rehab rats.

Figure 5.6. Perineuronal nets formation were enhanced rostrally and caudally away from the injured region in rehab rats at Week 4. (A) Representative images comparing percent of NeuN⁺ cells surrounded with a perineuronal net (PNN) at 1mm, 2mm and 3mm away from

the injured area rostrally and caudally in both rehab and non-rehab rats. White arrows denote the characteristic halo encircling NeuN⁺ cells when stained with *Wisteria Floribunda* agglutinin (WFA). **(B)** Graph depicting the percentage of NeuN⁺ cells enclosed by a PNN. Kruskal-Wallis, *: p-value < 0.05. All data are shown as mean $\pm$ SD; n = 4 for both rehab and non-rehab rats.

5.3.5. Rats that underwent rehabilitation had better motor recovery than rats that did not undergo rehabilitation

The custom-made device that exerts dorsiflexion on the rats' hindlimbs and subsequently measure the force of plantarflexion is shown in Figure 5.7. At Week 6, I observed that rehab rats (0.779 ± 0.113 N) had significantly stronger plantarflexion strength as compared to non-rehab animals (0.495 ± 0.0673 N, $p < 0.001$) (Figure 5.8A). In addition, rehab rats were more able to maintain this plantarflexion over the duration of the steady phase, as seen by the various higher value blue shades (rehab animals) of lines depicted as compared to the red shades (non-rehab animals) of lines, where each line corresponded to the measurement of an animal (Figure 5.8A). This trend remained consistent at Week 12 (1.36 ± 0.133 N by rehab rats vs. 0.647 ± 0.111 N by non-rehab rats, $p < 0.001$) (Figure 5.8B). Comparing the compiled plantarflexion test results for both rehab and non-rehab rats at Week 6 and 12, I observed that rats which underwent rehabilitation had improved hindlimb strength as compared to rats that did not undergo rehabilitation ($p < 0.001$ for plantar flexion) (Figure 5.8C). Moreover, in just 6 weeks, rehab rats were already able to outperform non-rehab rats from Week 12. Although the BBB results validated that rehab animals performed better than non-rehab animals at Week 12, this was not reflected at Week 6 (Figure 5.8D).

Figure 5.7. Rats were successfully mounted onto the ankle flexion device for assessment.

(A) Setup of ankle flexion device. **(B)** Magnified view of the rat's hindlimb before and after inclination. **(C)** Simplified diagram depicting the components of the device. **(D)** Timeline of assessment protocol. **(E)** Graph showing the conversion of the force value from the force detector (Flexiforce) into grams. All data are shown as mean $\pm$ SD.

Figure 5.8. Rats that underwent rehabilitation had better motor recovery than rats that did not undergo rehabilitation at Week 6 and Week 12. Graphs showing the profile of vertical force exerted with respect to time during steady phase (2-12s) at **(A)** Week 6 and **(B)** Week 12. Blue and red shaded measurements belong to rehab and non-rehab rats respectively. Each line represents the measurements from an animal. **(C)** Comparison of the force exerted in the vertical direction between rehab and non-rehab rats during steady phase (2-12s) at Week 6 and Week 12. **(D)** Comparison of the BBB locomotor score between rehab and non-rehab rats over a period of 12 weeks. Kruskal-Wallis test, **: p-value < 0.01; *:p-value < 0.05 between rehab and non-rehab rats. All data are shown as mean $\pm$ SD; n = 6 for both groups at Week 6 and n = 3 for both groups at Week 12.

5.4. DISCUSSION

Currently, rehabilitation is the most widely utilized treatment option for patients with traumatic injuries. However, this strategy only serves to prevent the decline of tissue integrity and function and has limited success in more severe cases, which reflects the reality that rehabilitation alone cannot completely restore functions. On the other hand, regenerative medicine may offer new options, but on its own, results are not optimal as well. In response to these gaps, it has recently been proposed that regenerative rehabilitation, the marriage of regenerative medicine and rehabilitative efforts for restoring tissue form and function respectively, is the potential way to go for an effective and all-rounded clinical treatment¹⁶⁶.

This practice is particularly applicable in the context of traumatic nerve injury as similar trends were observed in this field. For example, while sizeable morphological improvements were achieved through cellular^{121, 255} or biomaterial scaffolding^{128, 129} methods for regenerative purposes, locomotor functions remained disappointing^{121, 129}. Importantly, even though there have been many studies exploring regenerative medicine^{121, 128, 129, 255} and neural rehabilitation²⁴⁷⁻²⁴⁹ for treating traumatic nerve injuries, not many have applied both of these principles concurrently in their work.

Therefore in this study, I opted for the combinatorial approach of neuroregeneration and rehabilitation, capitalizing on the advancement of biomaterial scaffolds and body weight-supported treadmill training (BWSTT) concept, to promote nerve regeneration and functional recovery using SCI model as a proof of concept. Additionally, to further improve the rehabilitative process, our customized treadmill also contained a pair of pre-programmed manipulators for actuating the injured rat's hindlimb according to the normal gait.

Experimentally, animal models for traumatic SCI, such as contusion and incomplete laceration, are most relevant in clinical settings as injuries in humans are typically caused by physical impact²⁵⁶. Although rare, patients with a completely lacerated spinal cord can survive but their chances of regaining motor function below the injured region is extremely slim²⁵⁶. Correspondingly, in order to demonstrate the efficacy of our treatment strategy, we opted for the most stringent full spinal cord transection model. Animals were first spinally transected at the T9-T10 region with a 2 mm gap before implantation with a NT-3 loaded hybrid fiber-hydrogel scaffold between the rostral and caudal stumps to facilitate axonal growth across the injury. This platform was established in our previous study^{181, 220, 221, 250} and was also demonstrated to be effective for sustained drug and gene delivery after SCI^{220, 250}. The scaffold used in this work was exactly the same as the previous oligodendrocyte work. The only difference is that it only contained Neurotrophin-3 (NT-3) and did not contain any miRs. As such, we would expect the release profile of the NT-3 to be similar; a burst release within the first few days followed by a sustained release over a few weeks. As NT-3 has been shown to be a survival and growth factor for neurons, its administration with the scaffold would serve as a neuroprotective agent. With more spared axons present, it would increase the chance of regeneration and subsequent functional recovery. Without a scaffold implant into the spinally injured rats, axonal growth may still occur. However, without topographical guidance from the scaffold, the axons will regrow in a haphazard manner. Additionally, without the delivery of NT-3 for neuroprotection, axons may also take a longer duration to regenerate. Consistent with these studies, I also observed neurofilament signals penetrating into the interior of the scaffold. Surprisingly, I also noticed that in one of the rehab rats, a bundle of neurofilaments were able to traverse across the entire length of the scaffold at

week 12 (Figure 5.2A), suggesting rehabilitation-induced regeneration. Although crossing of neurofilaments across the scaffold in full spinally transected animals have been reported¹²⁹, due to the lack of similarity in the experimental methodology and limited literature, it is not known exactly how rehabilitation might have led to such robust axonal regeneration. Additionally, while enhanced axonal regeneration was seen in rehab animals, I did not observe significant differences in myelinated axon formation between the two groups within the scaffold (Figure 5.3). Taken together, future studies could explore how rehabilitation might induce axonal regeneration, where the axonal projections originate and also if the inclusion of pro-myelinogenic factors, such as miR-219/miR-338,²⁵⁰ could further enhance functional recovery.

The combinatorial approach mentioned in this work refers to both the regenerative aspect, where we add an electrospun fiber/collagen hybrid scaffold containing a neurotrophic factor NT-3 to enhance axonal ingrowth as well as the rehabilitation aspect, where after regenerating, the exercise regime will spur the newly regenerated axons to form functional connections with existing motor networks, allowing motor recovery. Fundamentally, rehabilitation serves to affect the entire animal and not the tissue scaffolds as they are inanimate objects and does not respond directly to the effects of rehabilitation. However, it may be possible for rehabilitation to indirectly affect the tissue scaffolds through factors secreted by the infiltrating cells in response to rehabilitation. Some possible outcomes may include a faster or slower degradation of the tissue scaffold. If the tissue scaffold is designed to contain certain pro-regenerative factors that are released only when it degrades, and if rehabilitation does promote the degradation of the tissue scaffold, then this may result in further enhanced nerve regeneration.

The CNS is an immune privileged region²⁵⁷ and as a result, it is ill-equipped to resolve the protracted immune responses mounted following traumatic SCI.²⁵⁸ While part of the reason for these prolonged reactions can be attributed to the continual recruitment and activation of leukocytes and glia, the persistence (dwell time) of various leukocyte subpopulations, such as macrophages/microglia, around the lesion site (up to months) are responsible as well.²⁵⁸ This slow rate of clearance was also observed in our results where ED1⁺ macrophages were still present within and outside the scaffold 4 weeks (chronic time point²⁵⁹) following SCI. Additionally, regardless of rehabilitation, the density and percentage of macrophages present remained unaffected. On the other hand, rehabilitation might have affected macrophages functionally, which was evident from the reduction of pro-inflammatory ED1⁺iNOS⁺ cells within and outside the scaffold, and the increment of anti-inflammatory ED1⁺CD206⁺ cells outside the scaffold. As anti-inflammatory macrophages have been shown to improve the morphological aspects of the injured spinal cord (reduce axonal dieback, reduce demyelination, promote tissue repair),²⁶⁰ such skewing elicited through rehabilitation might be related to the enhanced axonal ingrowth into the scaffold in rehab animals.

Perineuronal nets (PNNs) are structures formed by the extracellular matrix (ECM) which enwrap the soma and proximal dendrites of neurons²⁶¹ and is highly associative with neuronal plasticity, a key mechanism for functional recovery following SCI. Within the spinal cord, the expression of PNNs increases with exercise in both injured²⁴⁹ and uninjured rats²⁵², albeit in the absence of scaffolds. As such, we proceeded to evaluate if rehabilitation-induced modulation of PNNs will be dysregulated in this model when an implanted pro-regenerative

scaffold is present. Not surprisingly, our results are in agreement with the literature²⁵² where rehab animals had a higher percentage of neurons with PNNs as compared to non-rehab animals in the thoracic spinal region since the effects induced by exercise training are far-reaching and could even evoke beneficial responses in the brain^{262, 263}.

We did not evaluate a set of 'rehabilitation only' animals. However, based on literature, rats that had a contusion injury and received rehabilitation without drug treatments scored around 4 points (maximum of 21 points) for their open field test²⁴⁷. This is very similar to the score that our rehab rats with NT-3 treatment obtained. However, as our injury model (full transection) is much more severe than the contusion model, it can be expected that the actual score for a spinally transected rat with rehabilitation but without treatment to be much lower than 4. This is also justified because in the contusion model, there are spared spinal cord tissues available that can establish new functional networks with damaged axons through axonal sprouting, enabling some degree of motor recovery. This indicates that both regeneration and rehabilitation are required for enhancing functional recovery following injury.

Although rhythmic hindlimb activity induced by ascending sensory inputs can be achieved even when the adult spinal cord is isolated from the brain, it is generally unable to provide weight bearing functions²⁶⁴, except in cats that have undergone intensive training^{265, 266}. This meant that the BBB test, which utilizes a non-linear, ordinal grading scale, might not be fully compatible for certain injury models^{267, 268}, such as an extremely severe SCI (e.g. 2 mm complete transection injury gap) in animals. Specifically, the inability of the animals to bear their own weight would have limited their attainable score to a maximum of 7, and

consequently resulted in reduced resolution for accessing recovery²⁶⁷. Therefore, to further augment the functional assessment, our collaborators developed a customized device for quantifying the animals' plantarflexion strength based on the resistive force exerted during forced dorsiflexion of the hindlimb. Similar method of measurements have also been obtained from humans clinically^{269, 270} and more recently, animals²⁷¹⁻²⁷³, for studying maximum muscle contractility through electrical stimulation. Although I do not incorporate electrical stimulation, the range of rat plantarflexor muscles' force values obtained in our study (~ 0.5 N to ~ 1.4 N) were consistent with those animal studies^{272, 273}, verifying that these measurements could be an indicator of hindlimb recovery.

5.5. Conclusion

In conclusion, I demonstrated that there are beneficial effects by combining regenerative medicine and rehabilitation. The implantation of a pro-regenerative NT-3 loaded scaffold and intensive gait-controlled exercise training could enhance tissue regeneration (axonal growth), PNNs formation and functional recovery in spinally transected rats. Furthermore, I found that the immunological state within the scaffold and implant interface at chronic time points are drastically different and that rehabilitation might play a role in modulating the presence of anti-inflammatory macrophages for better recovery. Taken together, this study provides a proof of concept of the benefits of regenerative rehabilitation.

CHAPTER 6

NON-VIRAL DELIVERY OF MICRORNAS TO MODULATE HOST-IMPLANT INTEGRATION BY REGULATING FOREIGN BODY REACTION

ABSTRACT

Biomedical implant failure due to the host's response remains a challenging problem. In particular, the formation of the fibrous capsule is a common barrier for the normal function of medical devices. Currently, there is mounting evidence indicating that the polarization state of macrophages has an important role to play in effecting the foreign body reaction (FBR). This opens up a potential avenue for improving host-implant integration. Here, I hypothesize that controlling macrophage polarization will modulate implant fibrosis and may be achieved through scaffold-mediated RNA interference. Specifically, I utilized electrospun poly(caprolactone-co-ethyl ethylene phosphate) nanofiber scaffolds to deliver microRNAs (miRs) to induce macrophage polarization in order to modulate FBR. C57BL/6 mice that were treated with M2-inducing miRs, namely Let-7c and miR-124, displayed relatively thinner fibrous capsule formation around the scaffold at both Week 2 ($10 \pm 3.5 \mu\text{m}$ and $7.4 \pm 2.9 \mu\text{m}$ respectively) and Week 4 ($11.5 \pm 1.6 \mu\text{m}$ and $7 \pm 1.3 \mu\text{m}$ respectively). In contrast, Anti-Let-7c (M1-inducing miR) treated animals developed relatively thicker fibrous encapsulation at Weeks 2 ($17 \pm 4.5 \mu\text{m}$) and 4 ($21 \pm 4.3 \mu\text{m}$). Histological analysis showed that the density of blood vessels in the scaffolds were the highest in miR-124 treatment group (101.4 ± 20.4 blood vessels/ mm^2), followed by Anti-Let-7c (71.8 ± 14 blood vessels/ mm^2) and lastly, Let-7c treatment group (35.9 ± 28.8 blood vessels/ mm^2). Based on immunohistochemical quantifications, these miR-encapsulated nanofiber scaffolds were useful for localized,

sustained delivery of miRs and able to modulate macrophage polarization during the first 2 weeks of implantation.

6.1. INTRODUCTION

Foreign body reaction (FBR) is the host's response towards surgically implanted biomaterials, prostheses or medical devices¹⁶⁹. It is a multistage phenomenon, which is initiated when an implant comes into contact with blood and usually ends with a layer of dense collagenous deposition that surrounds the implant, a phenomenon known as fibrous encapsulation. The formation of these collagenous structures are evidence of wound healing and has been recognized to be a consequence of the reciprocal interactions between macrophages and fibroblasts¹⁷⁰. Although it is perceived by the body to be beneficial, encapsulation of the implant will isolate it from the surrounding tissues²⁷⁴, thereby hindering its intended functions, especially for drug delivery systems²⁷⁵. For the past few decades, efforts to prevent implant fibrosis have varied and encompassed the alteration of material properties such as porosity and shape^{276, 277} and the regulation of cellular aspects such as collagen production in fibroblasts²⁷⁸. Unfortunately, despite these endeavours, success remains limited due to the complexity of this physiological mechanism.

Currently, there is a general consensus that macrophages play a critical and diverse role in mediating FBR and dictating implantation outcomes¹⁷³ due to its persistent presence and reactivity to its microenvironment. Macrophages, unlike lymphocytes, are dynamic and can adopt distinct functional phenotypes within a spectrum (M1 and M2). In this context, macrophages at the M1 spectrum are considered pro-fibrotic while those at the M2 spectrum are anti-fibrotic²⁷⁹. The acquisition of these phenotypes, otherwise known as polarization, occurs depending on the factors to which macrophages are being exposed²⁷⁹. Apart from conventional signalling molecules (e.g. LPS, IL-4, etc), RNA interference (RNAi) technology, in particular microRNAs (miRs), are also good alternatives for inducing macrophage

polarization²⁸⁰. MiRs are small, non-coding RNA molecules that are known to be involved in gene and protein regulation²⁸¹ and have found useful applications in cancer diagnosis and treatment²⁸². One of the main advantages presented by miRs over conventional signalling molecules is their ability to target multiple genes and pathways²⁸³. Correspondingly, they could potentially elicit more pronounced biological effects.

Recently, Banerjee *et al.* identified Let-7c and its corresponding miR inhibitor, Anti-Let-7c to be involved in regulating macrophage polarization²⁸⁴. In particular, the overexpression of Let-7c resulted in macrophages having a more M2 phenotype while the administration of Anti-Let-7c led to macrophages possessing a more M1 phenotype. Similar to this work, Ponomarev *et al.* identified another miR, miR-124, to be implicated in skewing macrophages' activation towards the M2 phenotype²⁸⁵. While both studies provided comprehensive information on the mechanics of the signalling pathways, there was a lack of therapeutic applications of these miRs.

In this study, I fabricated a scaffold made of randomly-oriented poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP) nanofibers with miRs encapsulated to evaluate its influence on fibrous capsule formation as well as host-implant integration through influencing macrophage phenotypes. PCLEEP scaffolds and not PCLEEP-collagen scaffolds were used because the latter is specially customized for spinal cord implantations. PCLEEP scaffolds are relatively flat and can fit within the subcutaneous region of the rodent's skin as compared to the PCLEEP-collagen scaffolds, which has more height. Additionally, nanofiber scaffolds were the preferred choice due to its architecture, which mimics the extracellular matrix (ECM). Moreover, their topographical orientation were also shown to affect host-implant

integration¹³. Based on previous studies, I have shown that this scaffold platform is well-suited for delivering small non-coding RNAs, such as miRs and small interfering RNAs (siRNAs) both *in vitro*²⁸⁶⁻²⁸⁹ and *in vivo*^{276, 290}. As such, I hypothesize that nanofibers, along with the augmentation of miRs reported to regulate macrophage phenotype, may potentially ameliorate fibrous capsule formation and enhance implant acceptance by the host.

6.2. MATERIALS AND METHODS

6.2.1. Materials

AlexaFluor488-tagged oligoribonucleotide (AF488-RNA) was purchased from Integrated DNA Technologies, USA. 2,2,2-trifluoroethanol (TFE), heparin and Tween-20 were obtained from Sigma, USA. TE buffer (pH 8.0) and PBS (pH 7.4) were purchased from 1st Base, Singapore. TransIT-TKO (TKO) reagent was purchased from Mirusbio, USA. Negative miR (Neg-miR), Let-7c, Anti-Let-7c and miR-124 were purchased from Ambion, USA. RiboGreen reagent and Anti-Rat HRP (Catalog number: 31470) were purchased from Invitrogen, USA. Superfrost Plus glass slides and DAPI were purchased from Thermo Fisher Scientific, USA. Rat F4/80 (clone CI:A3-1, Catalog Number: ab6640) and Rabbit CD206 primary antibodies (Catalog Number: ab64693) were obtained from Abcam, UK. Mouse iNOS/NOS Type II (iNOS) primary antibody (Catalog Number: 610328) was purchased from BD Biosciences, USA. Fluoromount-G mounting medium and citrate buffer were bought from eBioscience, USA. Anti-Rabbit HRP (Catalog number: K4003) and Anti-Mouse HRP (Catalog number: K4001) were procured from Agilent Technologies, USA. OPALTM kit (contains TSA Cy3, TSA Cy5 and TSA FITC) was bought from PerkinElmer, USA.

6.2.2. Electrospinning of miR-encapsulated PCLEEP nanofibers

AF488-RNA, having a similar number of base pairs as miRs, was used to assess fiber morphology and oligonucleotide distribution following electrospinning and drug encapsulation. PCLEEP fibers encapsulating AF488-RNA complexed with TKO transfection reagent (AF488-RNA complexes) were fabricated using a working solution that was prepared in the following order. First, 0.5 g of PCLEEP was dissolved in 1.232 ml of 2,2,2-Trifluoroethanol (TFE). Concurrently, 38 μ l of TE buffer (pH 8.0) was mixed with 90 μ l of 50 μ M AF488-RNA and 180 μ l of TKO (1:2 v/v RNA:TKO ratio) and the mixture was incubated at room temperature for 15 minutes to form the complexes. The resulting AF488-RNA/TKO mixture was then added to the initial PCLEEP solution and mixed thoroughly. The resulting solution contains 32% (w/v) of PCLEEP and has a theoretical loading of 60 μ g of RNA. A flow rate of 0.6 ml/h (KD Scientific, USA) was utilized for all electrospinning processes. High DC voltage (Gamma High Voltage Research, USA) was applied to the polymer mixture at a positive voltage of 7-11.5 kV (the voltage was increased as time passed to stabilize the polymer jet stream). A rotating (230 rpm), negatively charged strip of aluminium foil (-4 kV) was used as the collector whereas a 22G needle was used as the spinneret. To obtain miR-encapsulated PCLEEP fibers, AF488-RNA was replaced with either Neg-miR, Let-7c, Anti-Let-7c or miR-124. The electrospun fibers were allowed to accumulate on the aluminium foil for 2 to 3 hours to form a scaffold strip, which was then further processed for *in vivo* implantation.

6.2.3. Evaluation of scaffold morphology and miR distribution in fibers

A glass slide was held in front of the collector during the electrospinning process for 20 s to collect some AF488-RNA encapsulated PCLEEP fibers for visual evaluation of miR distribution using a fluorescence microscope (Olympus IX71) at 60x magnification. Fibers were also sputter coated with platinum for 60 s at 20 mA and analyzed under a scanning

electron microscope (SEM; JOEL, JSM-6390LA, Japan) at an accelerating voltage of 10 kV. The average fiber diameter was then determined using ImageJ (NIH, USA) by measuring 100 fibers per sample from high magnification (5000x) SEM images.

6.2.4. Analysis of miR release kinetics

From each of the scaffold strip that encapsulated a different miR, (namely Neg-miR, Let-7c, Anti-Let-7c and miR-124), a 1 x 2 cm piece (average mass of 88.6 ± 3.7 mg) was cut out and used for the release kinetics study. The various 2 cm² pieces were completely submerged in 2 ml of PBS (pH 7.4) using Teflon rings and incubated at 37 °C in a 12-well plate. At fixed time points, 1 ml of supernatant was retrieved and replenished with 1 ml of fresh PBS (pH 7.4). For each of the 1 ml supernatant, 4 µl of heparin (5 mg/ml) was added to de-complex the miR from the TKO reagent. The concentration of miR that was released into the supernatant was then quantified using RiboGreen assay following manufacturer's protocol. Fluorescence intensity measurements were then carried out using a microplate reader (Tecan, Infinite 200, Austria). The percent of cumulative release, based on experimental loading, was averaged using measurements from all 4 types of scaffolds (Neg-miR, Let-7c, Anti-Let-7c and miR-124). The experimental loading efficiency of miR was evaluated based on previous protocols²⁸⁷. Briefly, Neg-miR, Let-7c, Anti-Let-7c and miR-124 encapsulated PCLEEP scaffolds (2 cm²) used for the release assay were each dissolved in 3 ml of chloroform at the end of the release kinetics study. Thereafter, 500 µl of PBS (pH 7.4) was added and the aqueous phase was extracted. Similarly, the miRs within the extracted aqueous solution were decoupled from TKO, using 4 µl heparin (stock concentration of 5 mg/ml), before running RiboGreen assay. The efficiency of miR extracted from the dissolved scaffold solution was also accounted for by doping PCLEEP solutions with a known amount of miRs/TKO complex, after which, the

polymer solution was vacuum-dried overnight to remove the solvents. The dry polymer constructs containing miRs/TKO were then dissolved in 3 ml of chloroform and added with 500 μ l of PBS (pH 7.4). The aqueous phase was extracted and 4 μ l of heparin (stock concentration of 5 mg/ml) was used to de-complex miR from the TKO reagent. The concentration of miRs extracted was quantified as described above to obtain extraction efficiencies. The experimental loading efficiencies were then computed using the following equation:

$$\text{Loading Efficiency} = \frac{\text{Total amount of miRNA released} + \text{Actual amount of miRNA extracted}}{\text{Experimental miRNA loading}} \times 100\%$$

6.2.5. Implantation of scaffolds

Five types of PCLEEP scaffolds, namely plain, Neg-miR, Let-7c, Anti-Let-7c and miR-124 were subcutaneously implanted into 10-12 weeks old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). All mice were monitored for signs of toxicity, including significant weight change, grooming, irritability and respiratory rate. All animal procedures were performed in accordance with an Institutional Animal Care and Use Committee (IACUC) protocol approved by Duke University. The scaffolds were cut into discs of 8 mm diameter using a biopsy punch and UV irradiated for 30 minutes prior to implantation. Mice were anesthetized using gas anesthesia (O₂, 2 L/min, isoflurane, 2%). After induction of anesthesia, the surgical site was shaved and sterilized. An 8 mm incision on the back of the mouse was made and a subcutaneous pocket was created at the incision by blunt dissection. A scaffold was then inserted into each pocket. The incision was closed with 4-0 monofilament absorbable suture and the mice were monitored until full recovery from anesthesia. For each time point, a total of three animals for each treatment group were used and each animal was implanted with only one scaffold.

6.2.6 Histological and immunohistochemical analysis

At 2 and 4 weeks post implantation, the implant specimen along with some of the surrounding tissues were removed and fixed in 10% neutral buffered formalin (NBF) for at least 24 hours. The samples were then cut into two halves and embedded in paraffin, sectioned (3 μ m thickness) and mounted onto Superfrost Plus glass slides. For each animal, a total of 3 sections were obtained and mounted separately onto 3 glass slides in an adjacent fashion for histological comparisons after staining. One complete set of samples were sent for routine Elastic-Van Gieson (EVG) staining, while the other two sets were stained with Masson's Trichrome and Hematoxylin and Eosin (H & E). The thickness of fibrous capsule was quantified using Masson's Trichrome stained samples and were further confirmed using EVG stained samples. Quantification was conducted using a brightfield stitched collage consisting of several 10x magnified images (Leica DMI8) of Masson's Trichrome stained samples. Each of the stitched images was then overlaid, using Adobe Photoshop CS6 (Adobe Systems, USA), with a smartgrid containing 8 equidistance divisions (Supplemental Figure 1). Following that, measurements were taken at regions where the divisions intersected with the fibrous capsule using ImageJ (NIH, USA). The density and size of blood vessels within the scaffolds were evaluated with H & E stained images. Images were taken using a light microscope (Leica DMI8) at 40x magnification. A total of 8 images were taken along the entire length of the scaffold. Blood vessels can be identified as structures possessing a lumen with red blood cells within. The total number of blood vessels were quantified using the Cell Counter plugin (ImageJ, NIH) and used for comparison. The area of a blood vessel was quantified by tracing out its periphery and measuring it using ImageJ (NIH, USA).

Immunofluorescent staining using OPAL™ was performed to characterize macrophage polarization in response to the different treatments. Following deparaffinization, the 3 µm thick sections were placed in citrate buffer and microwaved for antigen retrieval. Following that, non-specific antibody binding was reduced by incubating the samples in a 10% goat serum blocking solution for 10 min at room temperature. Sections were then decanted without washing and incubated with primary antibodies at room temperature for 30 min. Primary antibodies against the pan-macrophage marker rat F4/80 (1:50 dilution), the M1 macrophage marker mouse iNOS (1:100 dilution) and the M2 macrophage marker rabbit CD206 (1:200 dilution) were used. Samples were then washed thrice with PBS before secondary antibody incubation for 10 min at room temperature: anti-rat HRP (1:500 dilution), anti-mouse HRP (1:500 dilution) and anti-rabbit HRP (1:500 dilution). Lastly, TSA FITC (1:100 dilution) was added to anti-rat HRP, TSA Cy5 (1:100) dilution was added to anti-mouse HRP and TSA Cy3 (1:100 dilution) was added to anti-rabbit HRP. Nuclei were labelled with DAPI (1:1000 dilution) and slides were mounted with Fluoromount-G mounting medium.

Confocal images were obtained with LSM800 (Zeiss, Germany) at 40x magnification using the pan-macrophage marker F4/80 staining as the reference channel. Images were taken at the z-level where F4/80 staining was the most prominent. A total of 10 images along the entire length of the scaffold were procured for each sample (5 images at the implant interface and 5 images outside the implant interface). Quantification and analysis of M1 and M2 macrophages were conducted using TissueQuest analysis software. The percentage of M1 and M2 macrophages over the total cell population was determined by dividing the number of DAPI⁺F4/80⁺iNOS⁺ or CD206⁺ cells by DAPI⁺F4/80⁺ cells. The equation to obtain M1:M2 cell ratio with a log scale is described by:

$$M1:M2 \text{ cell ratio (log scale)} = \log_{10} \frac{\text{Number of DAPI}^+F4/80^+iNOS^+ \text{ cell}}{\text{Number of DAPI}^+F4/80^+CD206^+ \text{ cell}}$$

Values larger than 0 indicate a predominance of pro-inflammatory macrophages while values smaller than 0 indicate a predominance of anti-inflammatory macrophages. The equation to obtain M1: M2 expression fold difference with a log scale is described by:

$$M1:M2 \text{ fold difference (log scale)} = \log_{10} \frac{\text{Total area of } iNOS^+ \text{ pixels in Cell } X}{\text{Total area of } CD206^+ \text{ pixels in Cell } X}$$

where X refers to all the cells detected by the TissueQuest software

Values larger than 0 indicate that the macrophage expressed more iNOS than CD206 while values smaller than 0 indicate that the macrophage expressed more CD206 than iNOS.

6.2.7. Statistical Analysis

A one-way analysis of variance (ANOVA) was used to determine statistical significance of different treatments within each time point while independent-samples t-test was used to evaluate statistical differences between similar treatments for different time points. Tukey post-hoc test was conducted for one-way ANOVA. Shapiro-Wilk test was used to test for data normality while Levene's test was used to test for homogeneity of variances. Kruskal-Wallis test followed by the Mann-Whitney post-hoc test were used instead for data with unequal variances or non-parametric distribution. All analyses were conducted using the SPSS software. A p-value < 0.05 was considered statistically significant. All correlation analyses were conducted with linear regression using Prism Graphpad software. Error bar represents standard deviation (SD), unless otherwise stated.

6.3. RESULTS

6.3.1. RNA-loaded electrospun fibers were successfully fabricated

To evaluate the drug distribution of miR throughout the scaffold, AF488-RNA, consisting of similar number of base pairs as miR, was encapsulated within nanofibers using electrospinning as shown in Figure 6.1A, 6.1B and 6.1C. In addition to the uniform distribution of miR throughout the scaffolds, bead-free miR-encapsulated fibers were also observed (Figure 6.1D). The average diameters of the scaffolds remained similar regardless of drug encapsulation, as shown in Table 6.1.

Treatment Groups	Fiber Diameter (Average $\pm$ SD; nm)
Plain	391 $\pm$ 7
Neg-miR	412 $\pm$ 9
Let-7c	426 $\pm$ 9
Anti-Let-7c	398 $\pm$ 6
miR-124	386 $\pm$ 5

Table 6.1. Average fiber diameter of the various treatment groups

The encapsulated miRs within each 1 x 2 cm piece of scaffold were released in a sustained manner for at least 30 days after an initial burst release upon immersion into PBS (Figure 6.1E). A total of 52.5% of miRs were released over the span of 30 days of which around 84.5% were released between Day 0 and 7. Thereafter, the remaining 15.5 % accounted for the sustained release up to Day 30. The experimental loading efficiency of miRs into electrospun PCLEEP scaffolds was 26.4 $\pm$ 7.3 %.

Figure 6.1. RNA-loaded electrospun fibers were successfully fabricated. (A) Fluorescence image depicting uniform distribution of AF488-RNA throughout PCLEP fibers. **(B)** Phase Contrast image of RNA-loaded PCLEP fibers. **(C)** Merged image of A and B. **(D)** SEM image of miRNA-encapsulated fibers showing uniform fiber diameter, $f = 403 \pm 3$ nm. **(E)** Cumulative release of miRNA/TKO complexes from electrospun PCLEP fibers. Data are represented as mean $\pm$ SEM; $n = 4$.

6.3.2. Mice implanted with scaffolds containing M2 macrophage-inducing miRs possessed thinner fibrous encapsulate

The fibrous capsule is defined as the dense, relatively acellular collagenous structure that surrounds the scaffold implant (collagen stains blue in Masson's trichrome staining)(Figure 6.2). Since the regions around the scaffolds also consisted of endogenous collagen, EVG stained images (Figure 6.3A and 6.3B) were also used to confirm the identification of the fibrous capsule based on the absence of elastin (elastin stains black in EVG staining). As seen from the Masson's trichrome stained images, scaffolds that encapsulated Let-7c, an M2-inducing miR, led to thinner fibrous encapsulation ($10 \pm 3.5 \mu\text{m}$ at Week 2 and $11.5 \pm 1.6 \mu\text{m}$ at Week 4) as compared to Anti-Let-7c, an M1-inducing miR ($17 \pm 4.5 \mu\text{m}$ for Week 2 and $21 \pm 4.3 \mu\text{m}$ for Week 4)(Figure 6.3A to 6.3C. To confirm the effects of M2-inducing miR on fibrous capsule formation, I also included another previously reported M2-inducing miR, miR-124, in our study. Similar trends of reduction in capsule thickness were obtained for miR-124 treatment (Figure 6.3A to 6.3C; $7.4 \pm 2.9 \mu\text{m}$ at Week 2 and $7 \pm 1.3 \mu\text{m}$ at Week 4). For the control groups, plain scaffolds resulted in a relatively thin capsule ($13.8 \pm 4.4 \mu\text{m}$) at Week 2 (Figure 6.3A and 6.3C). However at Week 4 (Figure 6.3B and 6.3C), the capsule increased in thickness ($14.6 \pm 3.8 \mu\text{m}$) and was comparable to the measurement from Neg-miR encapsulated scaffolds (Figure 6.3B and 6.3C) ($14.6 \pm 4.7 \mu\text{m}$). Overall, statistical analysis showed that there was a significant difference between Anti-Let-7c and miR-124 treatment groups at Week 4 ($p < 0.05$)(Figure 6.3C).

Figure 6.2. Image depicting the skin region where the scaffold was implanted. The fibrous capsule is defined as the light blue layer surrounding the scaffold which is demarcated by the two black dotted lines as shown in the image with a green border. The red boxes correspond to the region where images are taken. Region outside the scaffold is denoted by an asterisk. Black lines that intersect the fibrous capsule are where the fibrous capsule thickness measurements were made.

Figure 6.3. Mice implanted with scaffolds containing M2 macrophage-inducing miRNAs possessed thinner fibrous encapsulate. Representative Masson's Trichrome and Elastic Van Gieson stained images of scaffold-tissue interface at **(A)** Week 2 and **(B)** Week 4 for determining fibrous capsule thickness. The pair of black arrows demarcate the thickness of the fibrous capsule while the black asterisk represents the region outside the scaffold. **(C)** Bar graph comparing the thickness of fibrous capsules between the various treatment groups at Weeks 2 and 4. *: p-value < 0.05; data are represented as mean $\pm$ SD; n = 3 animals per group.

6.3.3. Scaffolds containing miR-124 show more extensive blood vessel formation

The density of blood vessels in plain scaffolds remained rather consistent at both Week 2 (59.4 ± 67.7 blood vessels/mm²) and Week 4 (34.3 ± 35.4 blood vessels/mm²) (Figure 6.4A and 6.4B) as compared to scaffolds encapsulated with M2-inducing Let-7c miR, where the highest number of blood vessels per area were observed at Week 2 (87.2 ± 63.4 blood vessels/mm²) (Figure 6.4A and 6.4B). However, by Week 4, Let-7c treatment resulted in the lowest blood vessel density (35.9 ± 28.8 blood vessels/mm²) relative to all the other treatment groups (Figure 6.4A and 6.4B). Neg-miR loaded scaffolds had a slight decline in blood vessel density as well from Week 2 (78.8 ± 24.1 blood vessels/mm²) to Week 4 (57.8 ± 8.6 blood vessels/mm²) (Figure 6.4A and 6.4B). Conversely, there was a modest increase in blood vessel density with time in M1-inducing Anti-Let-7c group at Week 2 (59.5 ± 60.4 blood vessels/mm²) vs. Week 4 (71.8 ± 14 blood vessels/mm²) (Figure 6.4A and 6.4B). In miR-124 samples, although it had the smallest blood vessel density at Week 2 (36.4 ± 22.7 blood vessels/mm²), there was a three-fold increase at Week 4 (101.4 ± 20.4 blood vessels/mm²) (Figure 6.4A and 6.4B) and was the highest as compared to the rest of the treatment groups. In addition, statistical analysis showed that between the treatment groups at Week 4, miR-124 led to significantly thinner capsule ($p < 0.05$)(Figure 6.4B) as compared to both plain and Let-7c. Of note, there was a clear indication that Anti-Let-7c and miR-124-encapsulated scaffolds contained the largest average blood vessel size at Week 2 (135.9 ± 158.7 μm^2 and 103.6 ± 105.4 μm^2 respectively) and Week 4 (108.7 ± 127.8 μm^2 and 130 ± 131.8 μm^2 respectively) (Figure 6.4C).

Figure 6.4. Scaffolds containing miR-124 show more extensive blood vessel formation. (A) Representative H&E images of blood vessels (black arrow) found within the scaffolds after Week 2 and Week 4. **(B)** Graph depicting the number of blood vessels per area. **(C)** Size of blood vessels formed in various treatment groups at Week 2 and Week 4. *: p-value < 0.05; data are represented as mean ± SD; n = 3 animals per group.

6.3.4. Macrophage expression is associative with fibrous capsule thickness at Week 2

Macrophage phenotype was determined via the coexpression of the pan-macrophage marker, F4/80, with the M1 marker, iNOS, or M2 marker, CD206. At Week 2, macrophages adopted a more anti-inflammatory phenotype and a more neutral phenotype within and outside the plain scaffolds respectively (Figure 6.5A, 6.5B and 6.5C). This profile was completely different in other treatment groups, suggesting that the miRs were indeed able to polarize the macrophages and was also consistent with the type of miRs that was used to

implanted. **(B)** Graph showing the polarization ratio of macrophages found in and outside different scaffolds. **(C)** Heat map depicting the polarization ratio of all macrophages ($n \geq 110$ cells counted for each animal) found in all the animals in and outside the different scaffolds. Red and green colour denote a predominance of iNOS and CD206 expression by each cell respectively. Each heat map bar represents one animal. Data are represented as mean $\pm$ SD; $n = 3$ animals per group.

A

	Encapsulate thickness	Macrophage phenotype (Outside scaffold)
Plain	Thin	Neutral
Neg-miR	Thick	Pro-inflammatory
Let-7c	Thin	Anti-inflammatory
Anti-Let-7c	Thick	Pro-inflammatory
miR-124	Thin	Anti-inflammatory

B

	Encapsulate thickness	Macrophage phenotype (Outside scaffold)
Plain	Thick	Pro-inflammatory
Neg-miR	Thick	Pro-inflammatory
Let-7c	Thin	Anti-inflammatory
Anti-Let-7c	Thick	Pro-inflammatory
miR-124	Thin	Pro-inflammatory

Table 6.2. Semiquantitative correlative analysis of fibrous encapsulate thickness to macrophage phenotype outside scaffold. (A) Analysis of Week 2 samples. (B) Analysis of Week 4 samples.

6.3.5. Macrophage expression is not associative with fibrous capsule thickness at Week 4

Compared to the macrophage expression profile at Week 2, the macrophages within and outside the various scaffolds at Week 4 were generally more pro-inflammatory, with the exception of macrophages found outside Let-7c loaded scaffolds (Figure 6.6A, 6.6B and 6.6C). In particular, the macrophages outside miR-124 loaded scaffolds were expressing much more iNOS relative to the macrophages residing outside the rest of the miR loaded scaffolds (Figure 6.6A, 6.6B and 6.6C). Despite that, the fibrous layer surrounding the miR-124 loaded scaffold at Week 4 was the thinnest (Figure 6.3C). Altogether, the results at Week 4 did not appear to maintain the trend that was observed in Week 2. Specifically, the pro-inflammatory phenotype at Week 4 did not correspond to the formation of thicker fibrous encapsulate and anti-inflammatory phenotype did not result in thinner fibrous encapsulate (Table 6.2).

Figure 6.6. Macrophage expression is not associative with fibrous capsule thickness at Week 4. (A) Representative images of macrophages present in and outside different scaffolds. **(B)**

Graph showing the polarization ratio of macrophages found in and outside different scaffolds.

(C) Heat map depicting the polarization ratio of all macrophages ($n \geq 269$ cells counted for each animal) found in all the animals in and outside different scaffolds. Red and green colour denote a predominance of iNOS and CD206 expression by each cell respectively. Each heat map bar represents one animal. Data are represented as mean $\pm$ SD; $n = 3$ animals per group.

6.4. DISCUSSION

Foreign body reaction (FBR) remains a significant problem for biomedical implants. The formation of dense collagenous structures not only physically isolates the implant from the host tissues but also limits the diffusivity of loaded drugs, resulting in hindered treatments. A logical approach to retain the functionality of the implant would thus encompass imparting it with the ability to mitigate FBR. Nanofibers have been found to be a better material than films in controlling implant fibrosis^{13, 278}. Nanofiber scaffolds are biomimicking due to their structural similarity to the extracellular matrix^{291, 292}. Furthermore, they provide topographical cues to direct cell fate²⁹²⁻²⁹⁴. Implant fibrosis is the cumulative undesirable outcome of the body's response, more specifically macrophages, to an implanted material. The implant, therefore, will intrinsically determine how these macrophages will react. Without any intervention, the result of the material-cell interaction or the 'fate' of the cells will remain consistent as long as the same material for the implant is used. By having the ability to control and/or direct cell fate, these nanofiber scaffolds used in this work are able to trick the cells into responding differently during the material-cell interaction. This change in response may thus prevent the formation of fibrosis. Han N. and co-workers showed that nanofibers when introduced by the versatile electrospinning technique, could also be adapted as surface modifications on neural prostheses to moderate FBR^{294, 295}. Apart from providing

biomimicking architecture, I have demonstrated sustained non-viral delivery of small non-coding RNAs (e.g. siRNAs) using nanofiber platform both *in vitro*^{286, 287, 289, 290} and *in vivo*^{276, 290}. In these studies, siRNAs were delivered with the intention of affecting a specific gene. However, since biological systems frequently contain many redundancies, the suppression of a single gene may not be sufficient to elicit significant changes in biological outcomes. Therefore, miRs, which could regulate several genes at once^{283, 284} were explored in this work. Here, the target cell of interest is the macrophages as they have reciprocal interactions with fibroblasts and are also the main contributors to fibrous capsule formation. A system that targets the inflammatory response can also have wider implications in the amelioration of other diseases that involve macrophages, such as cancer and atherosclerosis^{295, 296}.

Here, we established a platform that delivers miR non-virally to enhance host-implant integration by decreasing fibrous capsule formation. Scaffolds were electrospun from PCLEEP, a synthetic polymer that has enhanced biodegradability as compared to PCL^{296, 297} due to its hydrophilicity. As such, when miRs are encapsulated within the PCLEEP nanofibers instead of PCL nanofibers, it will be released at a faster rate. The encapsulation of small non-coding RNAs also served to protect these labile molecules from biodegradation while enabling long term gene silencing due to the sustained availability of the drugs. In order to obtain bead-free miR-encapsulated fiber formation, the most important factor to consider is the viscosity of the electrospinning solution. As long as the solution has sufficient viscosity (at the very least it should not be like water), the fibers formed will not contain beads. The second most important factor to consider is the polymer used. A lower molecular weight polymer (eg. 10k Da MW PCLEEP) will tend to form beaded fibers as compared to its 100k Da MW PCLEEP counterpart because the electrospinning solution is harder to stabilize for low molecular

weight polymers. I have shown that by encapsulating miRs within PCLEEP fibers, a sustained release could be achieved for at least 30 days (Figure 1E). Besides that, as miR dosage is an important determinant in achieving favourable biological responses²⁹⁷⁻²⁹⁹, the maximum amount of miR that could be loaded into each scaffold was utilized. Neutrophils are the first responders to interact with the implant but they are short-lived (24 to 48 hrs) and quickly replaced by macrophages which can stay around for months³⁰⁰. As most miRs were released within the first week of implantation, which coincided with the infiltration of the macrophages, they can be uptaken to regulate the foreign body reaction. From my results, it appeared that this initial regulation is sufficient to significantly reduce fibrous capsule formation in the later stages. Apart from the chemical cues involved, topological presentation, in particular fiber diameter^{299, 301} and alignment^{13, 286}, also have a significant impact on cellular behaviour. Based on these studies, randomly-oriented PCLEEP nanofiber scaffolds with similar fiber diameter range comprising of different miRs were fabricated (Table 1). Using AF488-RNA as the model drug for ease of visualization, I could infer that these scaffolds contained a uniform distribution of miRs, which would allow cells that are in contact with the scaffolds to have equal accessibility to these biomolecules.

The miRs of interest in this study were initially Let-7c and anti-Let-7c as they had been reported to affect macrophage polarization. Specifically, the overexpression of Let-7c induced M2-like macrophage polarization whereas anti-Let-7c promoted M1-like macrophage polarization by inhibiting the function of endogenous Let-7c^{284, 285}. However, I also included miR-124, another M2 macrophage inducing miR²⁸⁵, in order to further validate our observed outcomes. Currently, it has been well-documented that macrophages are key mediators of fibrosis based on their interactions with fibroblasts during an inflammatory response³⁰¹.

Consequently, the regulation of the phenotypic state of macrophages may alter host-implant integration outcomes¹⁶⁹. The purpose of our study primarily revolves around managing fibrous capsule formation, which is a measure of how well the implanted biomaterial can be accepted by the host. From the Masson's trichrome stained samples, I observed that scaffolds encapsulated with Let-7c and miR-124, both M2-inducing miRs, resulted in thinner fibrous encapsulation as compared to the rest of the treatment and control groups throughout the duration of the implantation (up to 4 weeks). The opposite was true, where scaffolds containing Anti-Let-7c, an M1-inducing miR, had a relatively thicker fibrous capsule than the controls (plain and Neg-miR scaffolds at 4 weeks). This suggested a correlation between the effect of M1 and M2-inducing miRs and thickness of fibrous capsule formation. In order to establish this relationship, I proceeded to evaluate the phenotypic condition of the macrophages after being subjected to miR treatment.

Even though it is known that macrophages regulate foreign body reaction, there is currently no known exact macrophage polarization state that can lead to the most ideal biomaterial integration outcome. Attempting to solve this conundrum with a simple M1 or M2 macrophage skewing would also be undermining the complex processes that occur during this period of extensive ECM remodelling. Regardless, the use of both Let-7c and miR-124 appeared to result in thinner fibrous capsule formation. Taken together, these results seemed to imply that the initial macrophage response is able to determine the ultimate capsule thickness. Even though the expected macrophage polarization was not observed at the end of Week 4, the initial polarization at Week 2 appeared to be sufficient in altering the final thickness of the capsule (Figure 2). Additionally, sustained delivery of the miRs for tuning macrophage polarization might not be a necessary criteria for modulating fibrous capsule

thickness. However, more work is required to elucidate this apparent switch in the mechanism that regulates fibrosis. Future studies could include systematic gene screening of the macrophages present in miR-124 treated groups during Week 2 to Week 4 to identify potential factors that may reduce the extent of fibrous encapsulation and also utilize this platform as a drug depot for implantation to treat diseases.

Besides evaluating fibrous encapsulation, another measure of host-implant integration is the presence of blood vessels within the scaffold³⁰². Generally, an increased quantity of large-sized blood vessels suggests enhanced integration. Recent studies have reported that M2-polarized macrophages promoted angiogenesis³⁰³ as well as vasculogenesis³⁰⁴. Yet on the other hand, there are studies that showed M1-polarized macrophages to be able to promote angiogenesis as well, secreting VEGF and stimulating endothelial cell sprout formation both *in vitro* and *in vivo*^{305, 306}. Here, I observed that blood vessels were more abundant in animals that had Anti-Let-7c and miR-124 treatment at Week 4. In Let-7c treatment groups, a large number of blood vessels were found within the scaffolds at Week 2 instead. These groups corresponded to a higher number of macrophages expressing iNOS than CD206 at that particular time point, indicating that samples containing more M1-polarized macrophages led to more blood vessel formation. Besides blood vessel quantity, Anti-Let-7c and miR-124 samples also resulted in larger blood vessels being formed at both Week 2 and Week 4. Let-7c was able to decrease the extent of fibrous encapsulation as well but was not able to induce blood vessel formation. Lastly, although Anti-Let-7c was as potent as miR-124 in stimulating blood vessel growth, it fared the worst in regulating fibrosis. Taken together, miR-124 may be the preferred choice as compared to Let-7c and Anti-Let-7c for reducing fibrous capsule thickness and enhancing blood vessel formation.

Comparing the results from our study with previous related *in vivo* works, which quantified fibrous encapsulation^{13, 276, 307-310}, the combination of both electrospun fibers and miRs may be a useful strategy to modulate fibrous capsule formation. In these studies referenced, the scaffolding systems used included electrospun nanofibers^{13, 276}, hydrogels^{307, 308}, chemically crosslinked polymers³⁰⁹ and decellularized extracellular matrix³¹⁰. Among these systems, the fibrous capsule quantified in the non-treated groups for electrospun fibers^{13, 276} were never observed to be thicker than 100 μm . However, in other platforms³⁰⁷⁻³¹⁰, the fibrous encapsulation quantified was always greater than 100 μm . This implied that intrinsically, electrospun fibers alone might be less prone to FBR and thus might be a better alternative as compared to other systems for reducing fibrous capsule formation. This observation is consistent with our results where the plain and Neg-miR groups elicited relatively thin fibrous capsules at the end of the study ($14.6 \pm 3.8 \mu\text{m}$ and $14.6 \pm 4.7 \mu\text{m}$ respectively). With the addition of M2-inducing miRs, the thickness of the fibrous encapsulation dropped further ($11.5 \pm 1.6 \mu\text{m}$ in Let-7c treated samples and $7 \pm 1.3 \mu\text{m}$ in miR-124 treated samples (Figure 2) and the percent reduction, when compared to the controls, amounted to approximately 21 to 52 %. Statistically, these percentage reductions are comparable to a similar study conducted previously, where siRNA against Col1 (siCol1) was utilized for suppressing collagen secretion in fibroblast (25 to 57 % reduction when compared to the controls)²⁷⁶. Even so, the significance of using miRs appears to be greater since the thinnest fibrous capsule that was formed in the presence of siCol1 was around 25 μm whereas in this study, miR-124 treated samples had an average fibrous thickness of only $7 \pm 1.3 \mu\text{m}$.

6.5. CONCLUSION

This study demonstrates the viability of using a nanofiber platform for sustained, non-viral delivery of miRs to regulate foreign body reaction. The encapsulated miRs were released locally in a sustained manner for up to 30 days and led to phenotypic changes in macrophages at the initial time point (2 weeks) as well as reduced fibrous capsule formation. The pronounced variation in fibrous capsule and blood vessel formation between the different treatment groups suggested that regulating macrophage phenotype during the acute phase could be a potential avenue to affect implantation outcomes.

CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 CONCLUDING REMARK

Tissue engineering is a powerful tool for tissue replacement and regeneration due to the plethora of fabrication methods, materials and administering procedures available to work with. By sequentially decorating the surface of synthetic biocompatible PCL fibers with poly-DOPA, a mussel-inspired bioadhesive, followed by Protein A which allows the specific binding of recombinant proteins containing an Fc region (L1CAM and Nrg1 type III), primary neurons and OPCs can be induced to increase its neurite length and myelin sheath length respectively. In vivo, the implantation of a hybrid 3D scaffold delivering miR-219/miR-338 in an animal SCI model is able to maintain the viability of oligodendroglial lineage cells, promote their rate and extent of differentiation around the injury site as well as improve myelination within and outside the scaffold. Additionally, the combinatorial approach of rehabilitation and regeneration led to better axonal ingrowth, reduced inflammatory and increased anti-inflammatory immune cells, strengthened synaptic connections and importantly, enhanced motor recovery and hindlimb plantarflexion force. As immune cells always participate during injuries, immunomodulation of macrophages using miR-124, the most abundant miR in the brain, reduced fibrous encapsulation of scaffold and increased blood vessel formation, indicating enhanced host-implant integration. This presents another potential treatment for CNS nerve injuries by targeting not only the endogenous microglial but also the infiltrating macrophages from the peripheral circulation.

7.2 FUTURE DIRECTIONS

This thesis just demonstrated that there are still much more to explore and discover before traumatic nerve injury in the CNS can be fully treated. While trivial, the work compiled here forms a fundamental block in the climb towards better outcomes in CNS nerve regeneration. Notably, given the relative abundance of all the glial cells (OLs, microglial and astrocytes) combined, their role in maintaining the functionality of the CNS cannot be further overemphasized.

The platform that utilizes Protein A as a molecular orientor for presenting bound proteins to cells is useful for large-scale protein screening processes. Future work may include the screening of known or unknown protein enhancers on all the neural cells and elucidating the effects of these proteins on each cell type. These studies serve to isolate the effects of individual protein and may provide information on their relative importance for inducing certain cellular behaviours. Cell-wise, this platform can also provide crucial data on the necessity of orienting various proteins of interest for affecting cellular function and behaviour in a specific cell type. Useful proteins screened can then be decorated on scaffolds and delivered in tandem with other therapeutics to enhance anatomical recovery during CNS nerve injuries.

After knowing that miR-219/miR-338 is able to enhance the functions of oligodendroglial lineage cells at all stages (from immature OPCs to mature OLs) with no side effects to other cell types, as well as the benefits of rehabilitation with a pro-regenerative scaffold, future work should be to incorporate these miRs, along with the growth and survival factors targeting neurons and axons, with rehabilitation for potentially enhancing better

functional outcomes. Although not fully established, we are also observing some promising results through the use of miR-124, particularly in its macrophage polarization ability and promotion of blood vessel formation. As this miR is also overexpressed during neurogenesis, it will not only be useful in modulating the immunoenvironment within the CNS following a traumatic injury but may also promote the differentiation of endogenous neural stem cells to towards the neuronal cell lineage. Additionally, since there are tons of immune cells during the early stages of the injury, the administration of miR-124 may potentially be able to ward off any undesirable chronic inflammatory response that may lead to further tissue damage. Future work can seek to investigate the effect of miR-124 on the immune cell population during the acute and sub-acute stages of spinal cord injury and possibly relate their polarization states to the response of surrounding glial and neuronal cells. As immune cells also specialize in secretion, prudent manipulation may allow us to harness their presence as tiny bioreactors for secreting useful molecules to enhance anatomical and subsequently functional recovery.

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