Microfiber platform for oligodendrocyte myelination

Ong, William

2019

Ong, W. (2019). Microfiber platform for oligodendrocyte myelination. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/136941>

<https://doi.org/10.32657/10356/136941>

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Downloaded on 26 Apr 2023 20:29:39 SGT

Microfiber Platform for Oligodendrocyte Myelination

William Ong

**Interdisciplinary Graduate School
NTU Institute for Health Technologies (HealthTech NTU)**

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 plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

19th Nov 2019

.....

Date

.....

William Ong

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 research and writing 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.

19th Nov 2019

.....

Date

.....

Assoc. Prof. Chew Sing Yian

Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals where I was an author. This thesis also contains material that is partially contributed by a fellow lab member as indicated below.

Part of Chapter 2 is published as William Ong, Coline Pinese and Sing Yian Chew. Scaffold-mediated sequential drug/gene delivery to promote nerve regeneration and remyelination following traumatic nerve injuries. DOI: 10.1016/j.addr.2019.03.004.

The contributions of the co-authors are as follows:

- Assoc. Prof. Chew provided the initial review direction and edited the manuscript drafts.
- I prepared the manuscript drafts with the help of Dr Coline Pinese

Part of Chapter 3 is published as William Ong, Junquan Lin, Marie E. Bechler, Kai Wang, Mingfeng Wang, Charles ffrench-Constant and Sing Yian Chew. Microfiber drug/gene delivery platform for study of myelination. DOI: 10.1016/j.actbio.2018.06.011.

The contributions of the co-authors are as follows:

- Assoc. Prof. Chew provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by all other authors.
- I co-designed the study with Assoc. Prof. Chew and performed all the experiments at the School of Chemical and Biomedical Engineering and Lee Kong Chian School of Medicine. I also analysed the data.

- All confocal microscopy imaging was conducted by me in the Advanced Microscopy Facility at Experimental Medicine Building, LKC School of Medicine and SCBE Atomic Nano Imaging Microscope at School of Chemical and Biomedical Engineering.
- Junquan Lin designed the microfiber platform.
- Dr. Marie E. Bechler assisted in the myelin culture.
- Dr. Kai Wang synthesized the DPP-PCL.

Part of Chapter 3 is published as Ulla Milbreta, Junquan Lin, Coline Pinese, William Ong, Jiah Shin Chin, Hitomi Shirahama, Ruifa Mi, Anna Williams, Marie E. Bechler, Jun Wang, Charles French-Constant, Ahmet Hoke and Sing Yian Chew. Scaffold-mediated sustained, non-viral delivery of miR-219/miR-338 promotes CNS remyelination. DOI: 10.1016/j.ymthe.2018.11.016.

The contributions of the co-authors are as follows:

- Assoc. Prof. Chew provided the initial project direction and edited the manuscript drafts.
- I performed all the experiments for Figure 1 at the School of Chemical and Biomedical Engineering and Lee Kong Chian School of Medicine. I also analysed the data for Figure 1.
- Junquan Lin prepared the manuscript drafts and analysed most of the data. The manuscript was revised by all other authors.
- Dr. Ulla Milbreta performed most of the experiments, with the help from Dr. Coline Pinese, Jiah Shin Chin, Hitomi Shirahama, Ruifa Mi.

Effect of structural stiffness on YAP expression and actin turnover in Chapter 4 (4.3.4, 4.3.5) were contributed partially by Dr Nicolas Alexis Marinval.

The contributions of Dr Nicolas Alexis Marinval and me are as follows:

- I engineered the tunable stiffness scaffolds and performed the isolation of rat OPCs for all experiment. I also maintained the OPCs in culture before and after seeding on tunable stiffness substrates until fixation.
- Dr Nicolas Alexis Marival and I shared the responsibility of performing confocal imaging of OL after culture.
- Dr Nicolas Alexis Marival quantified the changes in YAP expression and actin turnover with my input. Dr Nicolas Alexis Marival also prepared the figures on YAP expression and actin turnover.

19th Nov 2019

.....
Date

.....
William Ong

Acknowledgement

This thesis would not be possible without the support of funding agencies. Funding support from the National Medical Research Council, Ministry of Education Tier 1 and Rehabilitation Research Institute of Singapore are acknowledged. I would also like to extend my gratitude to Nanyang Technological University Interdisciplinary Graduate Program for its graduate scholarship.

Next, I would like to acknowledge the support of fellow members at Chew Lab. I would like to thank my supervisor, Associate Professor Chew Sing Yian, for her guidance throughout my PhD journey. Prof Chew established collaboration with myelin experts from The University of Edinburgh, who have helped to lay the cornerstone for the work presented in this thesis. My appreciation also goes to my post-doctoral colleagues: Dr. Diao Huajia, Dr. Ulla Milbreta, Dr. Chooi Wai Hon, Dr. Nicolas Alexis Marinval, Dr. Zhang Kunyu and Dr. Coline Pinese for their scientific advice. Sincere gratitude also goes to my fellow PhD candidates at Chew Lab: Lin Junquan, Zhang Na and Chin Jiah Shin for the fun we had despite the stressful research environment. I would also like to acknowledge our project officers Peng Yanfen and Vincent Lin Po Hen for their unwavering help in running the mundane day-to-day tasks in the laboratory. All Chew Lab members, past and present, were integral to my PhD journey.

This thesis would also not been possible without support from my collaborators. I would like to thank Dr. Marie Bechler and Professor Charles ffrench-constant from The

University of Edinburgh for their expertise in oligodendrocyte myelination; Dr. Brenda Nai and Professor Lim Chwee Teck from National University of Singapore for their expertise in atomic force microscopy. Advice and guidance from co-supervisors Assistant Professor Albert Chen and Professor Jerry Chan Kok Yen are acknowledged. All scientific supports from collaborators are essential for successful completion of this thesis.

Last but not least, this doctoral thesis is wholly dedicated to my lovely partner and family.

Contents

Acknowledgement	7
Abstract.....	15
List of Abbreviations	18
List of Tables	21
List of Figures	22
Chapter 1: Introduction	33
1.1 Statement of Problems	33
1.2 Hypothesis and Objectives.....	34
1.3 Organization of this thesis	36
1.4 Background and significance	39
1.4.1 Remyelination following traumatic injuries and demyelinating diseases..	39
Notch Signaling	41
LINGO1 Signaling.....	42
1.4.2 Neural Tissue Engineering Platform for Oligodendrocytes Myelination ...	45
1.4.2.a Synthetic Fibers for Myelination	46
1.4.2.b Micropillars for Myelination	48
1.4.2.c Microsphere for Myelination	49
1.4.3 Mechanotransduction in Myelination	49
1.4.3.a Mechanical Cues that Influence Cellular Behavior	50
1.4.3.b Mechanotransduction Pathways	52

Chapter 2: Microfiber Drug/Gene Delivery Platform for the Study of Oligodendrocyte

Myelination 56

Summary	56
2.1 Introduction	57
2.2 Materials and Methods.....	60
2.2.1 Materials	60
2.2.2 Fabrication and characterization of suspended microfiber substrate	61
2.2.3 Scanning Electron Microscopy	62
2.2.4 Preparation of miR-loaded Fibers.....	63
2.2.5 OPCs Isolation and Myelination Culture	63
2.2.6 Immunofluorescent staining.....	64
2.2.7 Evaluating Surface Charges with Anton Paar Surpass	66
2.2.8 Statistical Analysis.....	67
2.3 Results.....	67
2.3.1 Characterizations of Suspended PCL Microfiber Substrates	67
2.3.2 Scaffold-mediated miR-219 / miR-338 Delivery Promoted OPC Differentiation in the Absence of Mitogen.....	70
2.3.3 Scaffold-mediated miR-219 / miR-338 Delivery Promoted the formation of myelin-like extensions on fiber.....	72
2.3.4 Scaffold-mediated miR-219 / miR-338 Delivery Promoted Longer Fiber Ensheathment	74

2.3.5 Scaffold-mediated miR-219 / miR-338 Delivery Promoted the Formation of More Myelin-like Extensions per Cell	75
2.3.6 Scaffold-mediated miR-219 / miR-338 Delivery Enhanced <i>in vitro</i> Long Term Differentiation and Myelination	76
2.3.7 Synergistic Effect of miR-219 / miR-338 and Fiber Diameter on Myelination ..	79
2.3.8 Effect of surface chemistry/charges on Myelination.....	81
2.4 Discussions	83
2.5 Conclusion	88
Chapter 3: Evaluating Potential of Mesoporous Silica Nanoparticles as RNA Transfection Reagent for Neural Cells	
Summary	89
3.1 Introduction	89
3.2 Materials and Methods.....	92
3.2.1 Materials	92
3.2.2 Fabrication and characterization of suspended microfiber substrate	92
3.2.3 Synthesis of PEI-modified mesoporous silica nanoparticles (MSN-PEI).....	93
3.2.4 Preparation of MSN-PEI Complexes and Reverse Transfection Fibers.....	94
3.2.5 Neuronal and Glia Cell Culture	95
3.2.6 Immunofluorescent staining.....	96
3.2.7 <i>In vitro</i> Gene Silencing and qPCR	97
3.3 Results	98

3.3.1	Characterizations of MSN-PEI complexes	98
3.3.2	Cellular Uptake of MSN-PEI Complexes	100
3.3.3	Gene Silencing by MSN-PEI Complexes	103
3.4	Discussions	105
3.5	Conclusion	108
Chapter 4: Effect of Structural Stiffness of Oligodendrocyte Myelination		109
	Summary	109
4.1	Introduction	109
4.2	Materials and Methods.....	111
4.2.1	Materials	111
4.2.2	Fabrication of tunable stiffness platform	112
4.2.3	Scanning Electron Microscopy	112
4.2.4	Atomic Force Microscopy	113
4.2.5	OPCs Isolation and Myelination Culture	113
4.2.6	Immunofluorescent staining.....	114
4.2.7	Statistical Analysis.....	115
4.3	Results	115
4.3.1	Engineering of Tunable Stiffness Microfiber Platform	115
4.3.2	Effect of Structural Stiffness on OPC Differentiation.....	120
4.3.3	Effect of Structural Stiffness on OPC Myelination	123

4.3.4	Effect of Structural Stiffness on YAP Signaling.....	129
4.3.5	Effect of Structural Stiffness on Actin Turnover	130
4.4	Discussion.....	132
4.5	Conclusion	136
Chapter 5: Effect of Intrinsic Stiffness of Oligodendrocyte Myelination		137
Summary		137
5.1	Introduction	137
5.2	Materials and Methods.....	139
5.2.1	Materials	139
5.2.2	Synthesis of Poly-lactic Acid.....	140
5.2.3	X-ray Diffraction and Differential Scanning Calorimetry	141
5.2.4	Fabrication of tunable stiffness platform with PLA	142
5.2.5	Scanning Electron Microscopy	142
5.2.6	Atomic Force Microscopy Bending and Indentation	142
5.2.7	Nanotensile	143
5.2.8	OPCs Isolation and Myelination Culture	144
5.2.9	Immunofluorescent staining.....	145
5.2.10	Statistical Analysis	145
5.3	Results.....	146
5.3.1	Characterization of PLA Fibrous Structure.....	146

5.3.2	Effect of Intrinsic Stiffness on OPC Differentiation.....	153
5.3.3	Effect of Intrinsic Stiffness on OPC Myelination	154
5.4	Discussion.....	159
5.5	Conclusion	162
Chapter 6:	Conclusion and Future Work	164
6.1	Summary	164
6.2	Future Directions	166
Chapter 7:	References	171

Abstract

Injuries or disorders in the central nervous system disrupt crucial sensory and motor functions. This critical system has limited regenerative capability, often contributes directly to permanent disabilities in afflicted adults. Limited regenerative capability is likely a result of the disruption of the intrinsic nervous system environment following injuries and diseases. The lack of treatment options stems from our lack of understanding of nervous tissue regeneration and alteration in nerve cellular microenvironment. As such, recent studies focused on unveiling the cellular biology of nervous system, zooming in on events following distress to uncover specific mechanisms that impede tissue regrowth and functional recovery. In this thesis, we developed platforms to help enhance our understanding of the nervous system with clever engineering. Specifically, we developed an artificial fiber-based platform for oligodendrocyte myelination. Our systems deliver simple solutions to study the complex process of multilamellar wrapping by oligodendrocyte, which is crucial in maintaining the functions of the central nervous system. We model intrinsic axons and the nervous system microenvironment with suitable materials and structures, to study the mechanobiology of oligodendrocyte myelination.

We first optimized the oligodendrocyte myelin-artificial axons culture in vitro to evaluate the feasibility of attaining physiologically relevant myelination with electrospun fibers. With appropriate biophysical and chemical cues, oligodendrocytes produced robust

multilamellar myelin sheaths surrounding artificial axons engineered with synthetic polymers. Morphologically, we observed concentric wrapping around our electrospun fibers, mimicking closely the physiological central nervous system myelination. We developed a drug/gene screening platform designed as a quick, simple and cheap alternative to animal testing. Using molecules known to promote oligodendrocyte myelination, we showed that our physiologically relevant system produced readouts similar to animal experiments, thereby highlighting it as an efficient platform to screen potential therapeutics targeting remyelination.

To further enhance the utility of our artificial axons drug/gene screening platform, we evaluated the efficacy of incorporating engineered mesoporous silica nanoparticles in our system for neuronal cells. We achieved highly efficient uptake of RNA by all major neuronal and glia cells through optimization of particles to RNA ratio. With the particles-RNA complex, we performed gene silencing experiments to highlight the efficacy of incorporated nanoparticles. In comparison to commercial transfection reagents, our engineered particles displayed similar silencing efficacy on our screening platform.

We further developed our artificial axons platform to study the mechanobiology of oligodendrocyte myelination. Using three-point bending theory, we established a tunable structural stiffness platform, which decouples stiffness from other biophysical cues, to investigate the effect of pathological stiffening of microenvironment on oligodendrocyte myelination. We demonstrated that increasing structural stiffness promotes oligodendrocyte

precursor differentiation, which was validated with conventional two-dimensional tunable stiffness hydrogels. Moreover, for the first time, we demonstrated with our artificial axon-based tunable stiffness, that an increase in structural stiffness impedes oligodendrocyte myelination. We investigated the role of YAP signaling in the mechanotransduction of oligodendrocyte and observed translocation of YAP into oligodendrocyte nucleus in high stiffness microenvironment. When examined the crucial actin turnover process required for myelination, we observed significantly higher F-actin ratio in high stiffness microenvironment, suggesting an obstructed actin disassembly process.

We extended our tunable structural stiffness platform to study the effect of intrinsic axonal stiffness on oligodendrocyte myelination. By controlling the crystallinity of artificial axons, we demonstrated tunable intrinsic stiffness in individual fibers. Similar to tunable structural stiffness platform, our intrinsic stiffness platform decouples the changes in intrinsic stiffness from other biophysical parameters that could influence myelination. Using this novel platform, we demonstrated that high intrinsic stiffness of individual artificial axons hinders oligodendrocyte myelination.

Taken together, this thesis advanced the utility of an electrospun polymer-based artificial axons platform for oligodendrocyte myelination. Crucially, platforms introduced in this thesis help accelerate the drug discovery process for myelin degenerative diseases and improve our understanding of myelination.

List of Abbreviations

A

Atomic Force Microscopy (AFM)

C

Central Nervous System (CNS)

Cyclophilin B (PPIB)

D

Differential Scanning Calorimetry (DSC)

Di(thiophene-2-yl)-diketopyrrolopyrrole (DPP)

E

Extracellular Matrix (ECM)

F

Fetal Bovine Serum (FBS)

Fibroblast Growth Factor (FGF)

L

Lg-like domain-containing Nogo receptor interacting protein 1 (LINGO-1)

L-Thyroxine (T4)

M

Megakaryocytic acute leukaemia (AMKL)

Mesenchymal Stem cells (MSCs)

Mesoporous Silica Nanoparticle (MSN)

MicroRNA (miR)

Molecular Weight (M_n)

Multiple Sclerosis (MS)

Myelin Basic Protein (MBP)

N

N-Acetyl-L-Cysteine (NAC)

Neural Precursor Cell (NPC)

O

Oligodendrocyte Precursor Cell (OPC)

Oligodendrocyte (OL)

Oligodendrocyte Transcription Factor 2 (OLIG2)

P

Paraformaldehyde (PFA)

Peripheral Nervous System (PNS)

Platelet-derived Growth Factor (PDGF)

Polyacrylamide (PAA)

Polycaprolactone (PCL)

Polydopamine (PD)

Poly-D-Lysine (PDL)

Poly-Lactic Acid (PLA)

R

RE-1 Silencing Transcription Factor (REST)

RNA interference (RNAi)

S

Scanning Electron Microscopy (SEM)

Small-interfering RNAs (siRNA)

Spinal Cord Injury (SCI)

T

Two Dimensional (2D)

Three Dimensional (3D)

Transcriptional co-activator with PDZ-binding motif (TAZ)

Trifluoroethanol (TFE)

Tri-iodothyronine (T3)

X

X-ray Diffraction (XRD)

Y

Yes-associated Protein (YAP)

List of Tables

Table 3.1: Sequences of primer used for qPCR

Table 3.2: Particle sizes and zeta potential data of RNA/MSN-PEI Complexes from Dynamic Light Scattering. Presence of RNA increases the overall complexes size initially. Higher RNA to MSN-PEI ratio decreased the overall particle size thereafter. RNA adsorbed on MSN-PEI also decreased the zeta potential of complexes.

List of Figures

Figure 2.1: Graphical Summary of Chapter 2. We engineered a suspended microfiber platform for in vitro myelination. Microfibers could be surface modified with polydopamine to delivery promyelogenic molecules to enhance myelination.

Figure 2.2: Confocal Images show more than 95% pure OPCs following isolation.

Figure 2.3: Electrospun microfiber platform for OL functional drug testing. (A) The workflow of suspended microfiber substrate. (B) SEM image showed that electrospun microfiber has a diameter of $2.0 \pm 0.3 \mu\text{m}$ (100 fibers were measured). (C) OPCs differentiated into OL and extended myelin-like MBP⁺ structures along the suspended fibers as shown by lines of green MBP signals.

Figure 2.4: SEM images of the cross-section of the microfiber substrate. With repeated melting of PCL film and repeated fiber deposition, suspended fibers remained taut in layers. (A) Low magnification view shows fibers suspended in layers above glass coverslip. (B) Higher magnification view shows taut fibers.

Figure 2.5: MBP⁺ wrapping on fluorescent PCL-DPP-PCL fibers. (A) Confocal image shows MBP signals ran along red coloured fibers. (B) 3D-reconstruction image shows MBP⁺ tubular structures surrounding red fluorescent PCL-DPP-PCL fibers. (C) $95.3 \pm 1.6 \%$ of the cells expressed OPCs NG2 marker 1-day post seeding.

Figure 2.6: Scaffold-mediated delivery of miR-219/miR-338 promoted OPCs differentiation in the absence of mitogen. (A) Representative confocal images show that cocktail of miRs promoted differentiation of OPCs into MBP⁺ OL. (B) Quantitative data shows miR-219 and miR-

338 treatment resulted in more MBP⁺ cells per DAPI. (C) Quantitative data shows miR-219 and miR-338 treatment resulted in more MBP⁺ cells per OLIG2⁺ cell. # indicates $p < 0.05$; two-tailed t-test, with an average of more than 140 DAPI and 110 OLIG2⁺ cells quantified from each experimental group per experiment (n=3).

Figure 2.7: Scaffold-mediated delivery of miR-219/miR-338 promoted the formation of MBP⁺ myelin-like extensions on fiber instead of membrane on a glass coverslip. (A) Confocal images showing the formation of both myelin-like extensions on fiber and myelin membrane on coverglass by the same cell. (B) Representative confocal images show miR-219/miR-338 promoted the formation of myelin-like wrappings around fibers. (C) Quantitative data shows miR-219 and miR-338 treatment allowed a higher percentage of MBP⁺ cells to form myelin-like extensions only. * indicate $p < 0.01$; two-tailed t-test, with at least 50 MBP⁺ cells from each group per experiment (n = 3).

Figure 2.8: Scaffold-mediated delivery of miR-219/miR-338 increased myelin-like extension length of OL. (A) Representative confocal images show miR-219 and miR-338 treatment resulted in longer myelin-like extension length on suspended fiber platform. (B) A Frequency distribution shows a shift of extension length distribution towards higher values when OPCs were treated with miR-219 and miR-338. (C) Log transformation of lengths shows the distribution of length of myelin-like extensions, with a significant difference between the mean of miR-219/miR-338 and NEG miR treated group. * indicate $p < 0.01$; two-tailed t-test, 196 extensions measured from 3 independent experiments.

Figure 2.9: Scaffold-mediated delivery of miR cocktail increased the number of myelin-like extensions per OL. (A) Representative confocal images showing miR-219/miR-338 enhanced formation of more myelin-like extensions per OL. (B) Distribution of the number of myelin-like

extension per OL. Quantitative data shows miR-219/miR-338 treatment resulted in a higher average number of myelin-like extensions per OL. Each dot represents one cell * indicate $p < 0.01$; two-tailed t-test, 35 cells in the miR-219/miR-338 group and 30 cells in NEG miR group, from 3 independent experiments.

Figure 2.10: Scaffold-mediated delivery of miR-219/miR-338 promoted long-term 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. Anova with Tukey Post-Hoc test; at least 100 cells quantified per sample group in each experiment repeats ($n = 3$ independent experiments) * $p < 0.01$. (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 a higher average number of compact myelin sheath per OL. Each dot represents one cell ** $p < 0.001$; two-tailed t-test, 40 cells in miR-219/miR-338 group and 35 cells in NEG miR group, from 3 independent experiments.

Figure 2.11: Electrospun fiber of varying diameter. (A, B, C) Representative SEM images show a distinct electrospun fiber diameter. (D) Distribution of electrospun fiber diameter with significant differences between groups. A: $0.460 \pm 0.095 \mu\text{m}$, B: $2.231 \pm 0.382 \mu\text{m}$, C: $3.219 \pm 0.294 \mu\text{m}$. ** $p < 0.001$; ANOVA, at least 95 fibers measured in all groups.

Figure 2.12: Myelination response to bolus miR transfection on fiber of varying diameter (A) Representative confocal images show miR-219/miR-338 promote OL myelination when the biophysical requirement is met. (B) Quantitative data shows miR-219 and miR-338 treatment

resulted in rightward shift of ensheathment length distribution (C) Log transformation of lengths shows the distribution of length of myelin sheaths. $**p<0.001$; $\#p<0.05$ ANOVA, at least 33 sheaths were measured from each group.

Figure 2.13: Surface chemistry and charges influence oligodendrocyte myelination response.

(A) Zeta potential data of unmodified, PDL-coated, dopamine-coated PCL fibers. PDL coating creates slightly more positive charges in comparison with uncoated fiber. Dopamine coating induced negative potentials regardless of coating time. (B) Dopamine-coated fibers left at room temperature has more negative potential as time progresses. (C) Longer durations of dopamine coating resulted in more positive potential. (D) Transfection reagents on PCL fibers resulted in highly positive potential. (E) Surface chemistry of PCL fiber influences the degree of in-vitro myelination. $*P<0.05$, $**P<0.01$, $***P<0.001$, One-way ANOVA with Tukey Post-hoc.

Figure 3.1: Gel electrophoresis data of RNA/MSN-PEI complexes. Negative control microRNA was used as a model RNA for complexation study. A lower amount of free-RNA escaped down the gel with an increasing mass of MSN-PEI. At least 20 times the weight of MSN was required to fully adsorbed all RNA.

Figure 3.2: Neuronal and glia lineage cell culture viability following MSN-PEI transfection. Neuronal and glia cells remain viable on suspended fiber substrates and expressed its marker (GFAP for Astrocytes, Nestin for NPCs, Olig2 for OPCs and Tuj1 for Neurons). Since Tuj1 labels the tubulin of neurons, no cytoskeleton stain with phalloidin was necessary.

Figure 3.3: Cy5-ODN/MSN-PEI complexes uptake efficiency. (A) Representative confocal images show the uptake of complexes by post-mitotic and mitotic cells. Post-mitotic and matured astrocytes and neurons show limited uptake at low MSN-PEI ratio. Mitotic OPCs and

NPCs show efficient uptake of complexes in most conditions. (B) Quantitative data show the percentage of cells with cy5-ODN/MSN-PEI complexes uptake. Post-mitotic cells have significantly lower uptake of complexes with neurons being the most difficult to transfect. Nonetheless, high MSN-PEI to RNA ratio still enables sufficiently high cy5-ODN uptake through bolus transfection. At least 30 cells were imaged at high confocal magnification in each group.

Figure 3.4: siRNA/MSN-PEI complexes gene silencing efficacy in neural cells. (A) Quantitative data showing fold change in PPIB expression obtained through $\Delta\Delta C_t$ approach following transfection. Matured astrocytes show inefficient knockdown of PPIB regardless of transfection reagent used while NPCs show significant PPIB knockdown with both lipofectamine and MSN-PEI with bolus transfection. MSN-PEI show similar efficiency as lipofectamine in gene silencing outcome. $**P<0.01$, $***P<0.001$, One-way ANOVA. Error bar showing S.E.M. with 3 biological repeats for astrocytes and 2 biological repeats for NPC. (B) Quantitative data showing fold change in REST expression obtained through $\Delta\Delta C_t$ approach following transfection. Bolus delivery of siREST with MSN-PEI achieved the same level of gene silencing as commercial lipofectamine. Gene silencing through scaffold-mediated delivery of siREST with MSN-PEI remained poor. Error bar showing S.E.M. with 2 biological repeats.

Figure 4.1: Fabrication of artificial axons with tunable structural stiffness to mimic environmental signals experienced by oligodendrocytes. (A) Axon structural stiffness (i.e. ability of axons to deflect due to stiffness of tissues and extracellular microenvironment that surrounds axons) affects oligodendrocyte myelination. (B) Schematic showing principle of tunable axon structural stiffness. By assuming each artificial axon as a simple supported beam, structural stiffness along the midline of the supported axon is related to the ratio of axon radius, r , and length of support, l .

Figure 4.2: Engineering of variable stiffness platform. 50 μm thick, 8 cm long PCL film was mounted onto the collector wheel to collect the electrospun fibers. 0.5 mm thick PCL frames of various sizes were cut out by customized punchers and used to secure the fibers on PCL films with silicon glue. Individual scaffolds were cut and utilized for downstream experiments.

Figure 4.3: Fibers from the same source have consistent diameter and topography in different PCL frame dimension. (A) Representative SEM images showing 1 μm diameter fibers in 3 PCL frames length have similar fiber diameter and alignment. Scale bar represents 50 μm (B) Quantitative data showing consistent fiber diameter in structures of different dimension in 1 μm fiber source. (C) Quantitative data showing consistent fiber alignment in structures of different dimension in 1 μm fiber source. (D) Representative SEM images showing 3 μm diameter fibers reservoir in 3 PCL frames length have similar fiber diameter and alignment. Scale bar represents 50 μm (E) Quantitative data showing consistent fiber diameter in structures of different dimension in 3 μm fiber source. (F) Quantitative data showing consistent fiber alignment in structures of different dimension in 3 μm fiber source.

*Figure 4.4: Changes in structural stiffness of artificial axons microenvironment by altering PCL frame dimension. Higher structural stiffness promoted OPC differentiation. (A) Scanning Electron Microscopy images of fiber of 4 artificial axon source, structural stiffness and fiber density data with Atomic Force Microscopy. Scale bar represents 50 μm . Quantitative AFM data shows a decreasing trend in structural stiffness of artificial axons with increasing PCL frame dimension regardless of artificial fiber source. Density of artificial axons were kept constant. *** $P < 0.001$, ** $P < 0.01$. Kruskal-Wallis (B) Representative confocal images show the number of MBP+ cells on structures with the same artificial axon source but different PCL*

frame length. Scale bar represents 50 μm . Quantitative data show increasing structural stiffness of microenvironment with shorter PCL frame dimension increased the percentage of MBP+ cells. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ One-way ANOVA with Tukey post-hoc.

Figure 4.5: High scaffold structural stiffness impedes OPC myelination. (A) Representative confocal images showing less complete myelin sheaths and shorter sheath length with stiffer artificial axons. Scale bar represents 20 μm . (B) Quantitative data and representative images show lower average myelin sheath number per OL with increasing scaffold stiffness.

*** $P < 0.001$, One-way ANOVA, with an average of more than 90 cells pooled from 9 animals quantified per sample group. (C-D) Quantitative data showing the distribution of myelin sheath length in 3 sample groups. High artificial axon structural stiffness resulted in shorter average myelin sheath length. ** $P < 0.001$, One-way ANOVA, with a total of 494 sheaths quantified from cells pooled from 9 animals.

Figure 4.6: High structural stiffness impedes OPC myelination within the same cell population on the same substrate. (A) Atomic force microscopy data showing V-shaped trend in variation of artificial axon structural stiffness across the platform. *** $p < 0.001$ One-way ANOVA, with an average of 15 randomly selected points per sample length in 3 distinct set of scaffolds. (B) Confocal images showing more complete myelin sheaths and greater sheath length on artificial axons, where structural stiffness was lowest, i.e. along the midline of 3.0 cm PCL frame structure. Scale bar represents 20 μm . (C-E) Quantitative data showing lower myelin sheath number per oligodendrocyte and shorter average myelin sheath length at structurally stiff ends vs structurally soft midpoint of artificial axon platform. *** $p < 0.001$, Kruskal-Wallis. A total of 487 sheaths quantified from cells pooled from 9 animals.

Figure 4.7: High artificial axons calibre promotes OL myelination independently from structural stiffness. (A) Representative confocal images show the enhanced myelination on structures with the same structural stiffness but higher fiber calibre. Scale bar represents 20 μm . (B) Quantitative AFM data showing the ability to modulate axon calibre and suspended length l to obtain platforms with similar axon structural stiffness. Samples indicated in blue were used for understanding axon calibre effect on myelination, independent of axon stiffness effects. $**p < 0.01$, $***p < 0.001$, One-way ANOVA with Tukey post-hoc. Quantitative data shows significantly higher artificial axon diameter formed with the same PCL polymer. (C-D) Quantitative results showing larger axon calibre and similar fiber density of artificial axons. $***P < 0.001$, independent t-test. (E-F) Higher axonal calibre promotes formation of significantly longer myelin sheaths and slightly more sheaths per cells with similar axon structural stiffness, $***p < 0.001$, Independent T-test.

Figure 4.8: YAP relay mechanotransduction signals in myelinating oligodendrocytes. (A) Confocal images show structurally stiff axons promoted YAP nuclear translocation throughout oligodendrocyte differentiation (D3-D7) and myelination (D10). Scale bar represents 20 μm . (B) Quantitative data show consistent enhanced YAP nuclear translocation on structurally stiff axons throughout OPC differentiation and myelination. $*p < 0.05$, one-way ANOVA with Tukey post-hoc test. (C) Schematic showing enhanced YAP nuclear translocation on structurally stiff axons.

Figure 4.9: Myosin II relay mechanotransduction signals in myelinating oligodendrocytes. (A) Confocal images showing decreased F-actin expression by differentiated oligodendrocytes cultured on structurally compliant artificial axons. (B) Quantification showing a significant reduction in F/G actin ratio by oligodendrocytes cultured on structurally compliant artificial

axons. ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Tukey post-hoc test. (C) Schematic showing enhanced F-actin expression on structurally stiff axons.

Figure 5.1: Fabrication of artificial axons with tunable intrinsic stiffness to mimic environmental signals experienced by oligodendrocytes. (A) Axon intrinsic stiffness (i.e. how easily an axon can be compressed due to changes in axon viscosity and/or cytoskeletal changes) affects oligodendrocyte myelination. (B) Schematic showing principle of tunable axon intrinsic stiffness by altering the degree of crystallinity of artificial axons.

Figure 5.2: Physical characterization of PLA artificial axons. (A) ^1H NMR data shows the predicted PLA protons with a ratio of 1:3 in both PLA-50 and PLA-94 (B) Differential Scanning Calorimetry data comparing the effect of heat on the melting characteristics of PLA-50 and PLA-94. PLA-50 shows single peak correlated to an amorphous structure while PLA-94 shows an additional peak representing crystalline structure. Higher heat capacity of PLA-50 suggests a large proportion of amorphous structure. (C) X-ray diffraction data shows a more intense peak for PLA-94 fiber, indicating a more crystalline structure than PLA-50 fiber.

*Figure 5.3: A higher degree of crystallinity increases PLA artificial axons intrinsic stiffness. (A) Scanning electron microscopy images show similar polymeric fiber formed from electrospinning with PLA-50 and PLA-94 polymer. Scale bar represents 50 μm . (B) Quantitative data shows both electrospun fibers from both polymers has similar fiber diameter. (C) Quantitative data shows similar fiber density in suspended structure engineered by the two PLA polymer. (D) Atomic force microscopy indentation of the fibrous structure shows similar structural stiffness between structures of the same suspended length. Increasing suspension length decreases structural stiffness. ** $P < 0.01$, *** $P < 0.001$, One-way ANOVA. (E) Atomic force microscopy indentation of single fiber confirms PLA-94*

*has higher Young's modulus and thus, stiffer fiber. ***P<0.001, Independent T-test. (F) Single fiber nanotensile test shows PLA-94 electrospun fiber has higher Young's modulus, indicating a stiffer fiber in comparison to PLA-50 fiber. **P<0.01, Independent T-test.*

*Figure 5.4: High structural and intrinsic stiffness of PLA promote OPC differentiation. (A) Representative confocal images show the number of MBP+ cells on structures with the same artificial axon source of PLA-50 but different PLA frame length. Scale bar represents 50 μ m. Quantitative data show increasing structural stiffness of microenvironment with shorter PCL frame dimension increased the percentage of MBP+ cells. (B) Representative confocal images show the number of MBP+ cells on structures with the same artificial axon source of PLA-94 but different PLA frame length. Scale bar represents 50 μ m. Quantitative data show increasing structural stiffness of microenvironment with shorter PCL frame dimension increased the percentage of MBP+ cells. **P<0.01, *P<0.05 One-way ANOVA with Tukey post-hoc.*

*Figure 5.5: High PLA artificial axons structural stiffness impedes oligodendrocyte myelination. (A) Representative confocal images show the extent of oligodendrocyte myelination on PLA-50 artificial axons. Scale bar represents 20 μ m. (B) Quantitative data shows shorter suspension and thus, higher structural stiffness impedes the formation of myelin sheath per cell on PLA-50 fibers. ***P<0.001, T-test. (C) Quantitative data shows shorter suspension length and higher structural stiffness also decrease the length of myelin sheath formation on PLA-50 fibers. (D) Representative confocal images show the extent of oligodendrocyte myelination on PLA-94 artificial axons. Scale bar represents 20 μ m. (E) Quantitative data shows longer suspension and thus, lower structural stiffness impedes the formation of myelin sheath per cell on PLA-94 fibers. ***P<0.001, T-test. (F) Quantitative*

data shows shorter suspension length and higher structural stiffness also decrease the length of myelin sheath formation on PLA-94 fibers.

Figure 5.6: High PLA artificial axons intrinsic stiffness impedes oligodendrocyte myelination.

*(A) Representative confocal images show the extent of oligodendrocyte myelination on 2 cm structure PLA artificial axons. Scale bar represents 20 μ m. (B) Quantitative data shows more crystalline and thus, higher intrinsic stiffness PLA-94 fiber decreases the number of myelin sheath per cell. *** $P < 0.001$, T-test. (C) Quantitative data shows high crystallinity and higher intrinsic stiffness resulted in the formation of shorter myelin sheath on PLA-94 fibers. (D) Representative confocal images show the extent of oligodendrocyte myelination on 3 cm structure PLA artificial axons. Scale bar represents 20 μ m. (E) Quantitative data shows more crystalline and thus, higher intrinsic stiffness PLA-94 fiber decreases the number of myelin sheath per cell on more compliance structure. *** $P < 0.001$, T-test. (F) Quantitative data shows high crystallinity and higher intrinsic stiffness resulted in the formation of shorter myelin sheath on PLA-94 fibers on more compliance structure.*

Chapter 1: Introduction

1.1 Statement of Problems

Oligodendrocytes and the precursors are a population of neuroglia in the central nervous system (CNS) that form myelin sheaths around axons. Oligodendrocytes and the myelin are essential for efficient saltatory signal conduction in axons and providing metabolic support for neurons. As such, damage to the vital CNS myelin could lead to loss of neurological cognitive and motor functions. These impairments are often irreversible and permanent. Demyelination, or the loss of myelin, is a chronic neural degenerative disease, which posts significant economic burdens on families and countries due to induced disabilities ¹. Thus, there is an urgent need to develop new therapeutics to protect and regenerate the myelin to achieve recovery of neurological functions ². Neural tissue engineering is developed for the purpose of promoting nerve tissue repair using biomolecules, nerve grafts or biological scaffolds ³. Despite the advancement of the field, promoting remyelination, particularly in the CNS, is still a daunting task.

A classical experiment by Dr. Albert Aguayo elegantly proved that CNS tissues do not lose the intrinsic regenerative capability. Rather, the inhibitory environment of the CNS after an injury is instrumental to the lack of regeneration following CNS degenerative diseases and traumas ⁴. Fast forward to almost four decades after the ground-breaking work, we have had glimpses of the details on the inhibitory environment and much less on how to overcome them to promote regeneration. Thus, neural tissue engineering should provide a platform to better understand the environmental factors involved in inhibiting CNS tissue regeneration and perform effective drug-testing.

By mimicking the size scale and architecture of extracellular matrices or even cells, electrospun fibers have been utilized in neural tissue engineering. For instance, synthetic polymer fibers have been employed as artificial axons to replace neurons in conventional neuron-oligodendrocytes co-cultures for studies on myelination ⁵. This strategy helps to remove influences of axonal signaling and reveals the intrinsic myelination capabilities of oligodendrocytes in response to physical signals ⁶. However, cellular-substrate interaction was not optimized for the purpose of neural tissue engineering ⁷. Fiber platforms that were engineered previously do not prevent neuronal cells from mechanically sensing the stiff underlying substrate ⁵, which could affect cellular responses in migration, proliferation or differentiation due to the dissimilarity between substrate stiffness from the native tissues ⁸. Therefore, electrospun fiber artificial axons platforms could be better designed and optimised for the purpose of neural tissue engineering.

1.2 Hypothesis and Objectives

In this thesis, we hypothesize that re-engineered, suspended electrospun fiber artificial axons could better replicate the intrinsic mechanical microenvironment of oligodendrocytes to promote myelination in culture. Robust myelination on suspended fiber platform could be effectively translated for drug-testing purposes and investigated for the oligodendrocyte response towards changing mechanical microenvironment. Accordingly, the overall objective of this project is to expand the applications of synthetic electrospun fiber in neural tissue engineering by conceptualizing and developing a biomimetic artificial axon

platform capable of delivering mechanical and topographical cues. In this regard, we propose to pursue the following specific aims:

1. Optimize and characterize re-engineered electrospun fiber artificial axon platform

- Establish a robust system to engineer reproducible suspended fiber platforms using the electrospinning technique.
- Characterize physical properties of electrospun fiber scaffolds, such as fiber diameter, topography and morphology.
- Characterize mechanical properties of suspended fiber platform, such as fiber intrinsic stiffness, platform structural stiffness.

2. Evaluate glial and neuronal cell response towards microRNA-incorporated suspended electrospun fibers

- Evaluate oligodendrocytes *in-vitro* myelination on suspended electrospun fibers.
- Evaluate the effect of surface chemistry and charge on oligodendrocytes *in-vitro* myelination on artificial axons.
- Evaluate the potential of suspended fiber platform as a drug-testing platform by investigating the effect of microRNA-delivery on oligodendrocytes differentiation and myelination.
- Evaluate the potential of incorporating engineered nanoparticles for siRNA delivery on suspended microfiber platform for glia and neuronal cell transfection.

3. Evaluate the effect of mechanical stiffness on glial cell response on suspended electrospun fibers

- Evaluate the efficacy of suspended fiber platform in decoupling fiber diameter, fiber intrinsic stiffness and structural stiffness.
- Evaluate the effects of mechanical stiffness on oligodendrocytes differentiation and myelination by decoupling fiber diameter, fiber intrinsic stiffness and structural stiffness.
- Investigate the mechanotransduction pathways on suspended fiber platform.

1.3 Organization of this thesis

Chapter 1 is instrumental in framing the direction of this thesis by introducing the current state and limitation of neural tissue engineering. We then presented our hypothesis and objectives of the project. Following which, we discuss relevant background information that is essential to the appreciation of this work. Specifically, we review the importance of remyelination in demyelinating diseases and traumatic injuries. We also discuss the current state of biomimetic scaffolds for remyelination research and the need for a novel neural tissue engineering tool to investigate possible therapeutics. Last but not least, we review the mechanotransduction signaling, and how these pathways are relevant to oligodendrocyte differentiation and myelination.

Chapter 2 focuses on the development of an electrospun fiber-based artificial axon platform for oligodendrocyte myelination. We discuss the current limitations of *in vitro*

myelination assays, which hinder our effort to improve understanding of the complex CNS myelination process. We hypothesize that optimal mechanical microenvironment and appropriate chemical cues are required for robust *in vitro* myelination. We show that by amalgamating the necessary physical and chemical cues, oligodendrocyte produces concentric wrappings on an axon-free scaffold that mimics closely the intrinsic environment. Moreover, we demonstrate the versatility of our platform as a drug/gene testing assay for oligodendrocyte myelination.

Chapter 3 investigates the potential of incorporating engineered particles for drug/gene delivery. The preceding chapter illustrates the importance of chemical cues such as microRNA in attaining robust *in-vitro* myelination. However, commercially available transfection reagent employed in chapter 2 lacks the flexibility in its design to deliver ribonucleic acids to notoriously difficult neural cells. Thus, chapter 3 accentuates the versatility of mesoporous silica nanoparticles (MSN) as a ribonucleic acid transfection reagent for neural cells. We evaluate the potential of engineered MSN in replacing conventional transfection reagents to achieve gene silencing. Moreover, we examine how MSN complexes could be distributed from our fibrous platform to have localized delivery.

Chapter 4 studies the effect of mechanical microenvironment on oligodendrocyte myelination. Diseases or injuries alter intrinsic mechanical properties of the CNS. Such modifications of the mechanical microenvironment are known to influence the behavior of mechanosensitive cells. These cells adapt their rate of migration, viability, proliferation and

differentiation according to the stiffness of their physical environment. However, how such changes in mechanical environment influence oligodendrocyte myelination is not known. The lack of robust *in vitro* myelination assay and the inability to control mechanical stiffness independently render such study difficult. We engineered a microfiber-based platform with tunable mechanical stiffness to investigate the effect of structural stiffness on oligodendrocyte myelination. This work demonstrates that our platform could effectively decouple other factors influencing myelination. We further show mechanical stiffness influences oligodendrocyte differentiation and myelination differently.

Chapter 5 built upon the findings in prior chapter to investigate the effect of artificial axons intrinsic stiffness on myelination. Axonal intrinsic stiffness could be affected by changes in axon cytoskeleton during aging and neural diseases. In this chapter, we employed a unique crystallinity property to mimic changes in axonal cytoskeleton, which influence the intrinsic stiffness of axons. When built into suspended artificial axons, we continued to observed impairment in OL myelination with high structural stiffness regardless of intrinsic stiffness of artificial axons. Similarly, we observed that high artificial axon intrinsic stiffness impairs OL myelination.

Chapter 6 draws conclusions based on the above-discussed work and suggests possible future directions.

1.4 Background and significance

1.4.1 Remyelination following traumatic injuries and demyelinating diseases

Axonal sprouting and neurite regrowth after SCI often serve as indicators of regeneration. While equally important, remyelination, however, is less extensively examined and poorly understood in the field of SCI. Remyelination restores multilamellar, lipid-rich myelin to demyelinated axons, which is essential for axonal metabolic support and conduction of action potential^{9,10}. Following mild contusion injuries, spontaneous remyelination ensues with some degree of long-term remodeling and plasticity^{11,12}. However, robust remyelination was never attained. Chronically injured axons were characterized by significantly lower myelin sheaths length and higher g-ratio as illustrated by *Franklin et al* (see below)¹¹. Crucially, this small degree of spontaneous remyelination enables only minimal functional recovery¹³. Although further disablement of spontaneous remyelination did not aggravate the small recovery following trauma, it is not currently known if enhancement of remyelination to pre-injury level would permit better functional outcome¹³. Thus, detailed remyelination mechanism following spinal cord trauma has to be thoroughly examined¹⁴.

Adapted, credit to aforementioned authors

Much remyelination mechanistic insight can be drawn from studies dealing with CNS demyelinating diseases model. In particular, toxins lysolecithin-induced demyelination see differentiating oligodendrocyte precursor cells (OPCs) after 7 days-post injection, while the onset of remyelination began quickly after 14 days post injection ¹⁵. Similarly, virus infection-induced demyelination has active remyelination at 4 weeks post-infection ¹⁶. In both cases, successful remyelination requires repopulation of demyelinated lesions by the OPCs, followed by differentiation of these cells to mature oligodendrocytes (OL), and finally the restoration of myelin cytoarchitecture on axons ¹⁷. Thus far, overwhelming evidence has shown that remyelination failure is largely associated with OPCs failing to differentiate into myelinating OL ¹⁷, due to the presence of an inhibitory environment in the injury sites, the lack of neurotrophic support and/or biochemical cues ¹⁸. Failure to remyelinate axons leads to further axonal degeneration (illustrated below). Two key inhibitory pathways, Notch and LINGO, were identified to perturb remyelination.

Adapted, credit to aforementioned author

Notch Signaling

NOTCH signaling is a negatively regulator of OPC differentiation that impedes remyelination^{19,20}. Developmentally, axon contact-mediated NOTCH signaling inhibits OPC maturation to promote their migration along the white matter tracts of the CNS. Correspondingly, NOTCH expression declines as OPCs differentiate and myelinate the axons. In spinal cord injuries (SCI) or autoimmune demyelination disease, NOTCH is re-expressed in the lesion sites^{21,22}. In these inflamed lesions, reactive astrocytes express Jagged1 in response to the inflammatory cytokine, TGF- β 1. Jagged1 binds to Notch1 receptor that is expressed by immature OL to induce Hes5 protein expression. Hes5 in turn block the expression of other helix-loop-helix factors, thereby hindering OL maturation²¹. Thus, intercepting the re-establishment of NOTCH signaling should make the lesion environment more permissible to OPC differentiation and remyelination.

For instance, delivering γ -secretase inhibitor (MW167) to block pathological NOTCH and Jagged1 expression was attempted to remyelinate the CNS²³. MW167 interferes with presenilin protein activity, which produces the NOTCH intracellular domain that translocates to the nucleus to regulate gene transcription²⁴. Intraventricular injection of 1 mM MW167 completely abolished the generation of the intracellular domain of Notch in OL from murine brains and spinal cords. More importantly, suppressing NOTCH expression was instrumental in giving more remyelinated axons in an EAE demyelination model after 11 days²³. However, this strategy was effective only if NOTCH was knocked out early in the OPC lineage^{19,25}. Ablating NOTCH from Olig1⁺ OPCs and OL lineage cells in Cre-LoxP mice promoted OPCs

differentiation during CNS remyelination ¹⁹. However, eliminating NOTCH from PLP⁺ matured OL in Cre-LoxP recombinant mice had no pro-remyelination effect in toxin-induced demyelination model ²⁵. Together, these results suggest that NOTCH expression in progenitors has important implications in their regenerative capability during the adult phase.

LINGO1 Signaling

The arguably most crucial contributor of inhibitory environment to remyelination is leucine-rich repeat and Ig-like domain-containing Nogo receptor interacting protein 1 (LINGO-1). LINGO-1 expression is upregulated in both SCI and autoimmune demyelinated lesion that see chronic demyelination ²⁶. Although LINGO-1 has a role to play in impeding neurite outgrowth ²⁷, it is most prominently known to hinder remyelination by stalling OPC differentiation ²⁸⁻³¹. Specifically, LINGO-1 signaling pathway reduces gelsolin expression, an intracellular actin-binding protein, to reduce the motility of actin microfilaments that is essential for OPCs differentiation ³¹. LINGO-1 antagonist has been consistently shown to promote remyelination in both toxin-induced and experimental autoimmune demyelination models ²⁹⁻³¹. In particular, 10 µg/ml of anti-LINGO-1 antibody, which is a LINGO-1 antagonist, dramatically increased OPCs and pre-OL differentiation into OL by 40-fold and 10-fold respectively in an axonal co-culture ³⁰. Most excitingly, anti-LINGO-1 antibody promotes remyelination in MOG-induced autoimmune and toxin-induced demyelinating model ^{29,30}, which is likely attributed to robust OPCs differentiation. It also promotes nerve regrowth after SCI ²⁶.

Other promising therapeutics targeting these pathways have been reported. For instance, MicroRNA (miR) is a novel remyelination therapeutics with large potential due to its ability to regulate multiple mRNA expressions ³². OPCs differentiation is regulated by the inhibitory NOTCH and LINGO signaling pathways independently. Antagonizing a specific pathway may have limited efficacy due to presence of other remaining inhibitory signal. Thus, new therapeutics such as miR that could interfere multiple inhibitory signals are highly valuable. In particular, miR-219 and miR-338 have been presented as key regulators for OPCs differentiation, myelination and remyelination by targeting the inhibitory pathways.

For instance, Hes5 is a critical downstream effector of NOTCH signaling that inhibits OPCs differentiation. miR-219 and miR-338 suppress Hes5 expression in this NOTCH pathway to promote OPC maturation. In particular, 50 nM of miR-219 and miR-338 mimics downregulated Hes5 gene expression by 10-fold while 25 nM of LNA that inhibits miR-219 and miR-338 expression resulted in 2-fold higher Hes5 expression and 4-fold lower number of Myelin Basic Protein (MBP⁺) OL *in vitro* ³³. Viral vector-induced miR-219 and miR-338 overexpression accelerated OL maturation in the development of chick neural tube and mouse cortex. Conversely, inhibition of miR-219 and miR-338 with antisense LNA blocked OL differentiation in zebra fish ³³. Furthermore, miR-219 directly inhibited LINGO-1 transcript and OPCs differentiation transcriptional regulator (*Nfia*, *Nfib*, *Etv5*) to promote OPCs differentiation and remyelination in LPS-induced demyelination model and functional recovery in EAE mice ³⁴.

Thus, through the understanding of the inhibitory signals that impede remyelination, therapeutics could be established to direct remyelination following traumatic injuries or demyelinating lesions. This was evident in the use of miR-219 and miR-338 for remyelination in the demyelinating model ³⁴. However, despite the advancement in the field, development of experimental medicines is still very much in its infancy ¹⁰. While the majority of our understanding of remyelination comes from demyelinating diseases, differences between these lesions and other injuries could render some therapeutics ineffectual. An example is in promoting inflammatory responses in chronic inactive demyelinating lesions. Chronic inactive plaque from demyelinating CNS is characterized by minimal inflammation and macrophage infiltration, and is correlated with the absence of active remyelination ^{35,36}. On the other hand, remyelination is expected in the rim of chronic active lesions, where macrophages and other leukocytes are present ³⁷. Enhancing the infiltration of M2-polarised macrophages, regulatory T-cells and expression of chemokine/chemokine receptor CXCL12/CXCR4 promoted remyelination through stimulating OPC differentiation in demyelinating lesions ³⁸⁻⁴⁰. However, the inflammatory response after SCI is fundamentally different from autoimmune inflammatory diseases. Specifically, neutrophils and macrophages continue to persist in SCI sites even in chronic injuries ⁴¹. Thus, inducing inflammatory response may not help with remyelination of trauma-induced injury.

In this thesis, we identified two major areas to address that could potentially help to accelerate drug development. Firstly, we aim to develop a rapid and cost-efficient way to screen for promising therapeutics for remyelination; Secondly, we strive to improve our

understanding of the mechanotransduction on myelination to build a physiologically relevant model for remyelination. We discuss these two areas of concern in the following sections.

1.4.2 Neural Tissue Engineering Platform for Oligodendrocytes Myelination

While *in vivo* models have been indispensable in aiding our understanding of myelination and remyelination, some aspects of these processes such as cytoskeletal changes of OL are difficult to observe and analyze. Thus, *in vitro* modeling of myelination or remyelination is a valuable tool in dissecting the mechanisms of myelin growth and repair ⁴². Early *In vitro* models developed by the field include dissociated CNS culture ⁴³, OL-Dorsal root ganglion co-culture ⁴⁴, OL-CNS neurons co-culture ⁴⁵. These myelinating co-cultures have live neurons as the permissive substrate for OL myelination, thereby preserving the physiological cell-cell interaction between neuron and OL during myelination. Thus, these *in vitro* cultures have been a staple in myelin research.

Neurons have a multitude of interactions with oligodendroglia. For instance, neurons could modulate OPCs proliferation and migration while conversely, OPCs could metabolically support neurons ⁴⁶. Moreover, axons produce inhibitory or inductive biophysical and biochemical signals that could alter the degree of myelination ⁴⁷. The complex crosstalk between two closely-related cell types makes it difficult to uncouple direct OL effect and axonal-induced effect on myelination ⁶. Thus, efforts have been made to engineer other substrates that are permissive to OL myelination to remove the crosstalk and potential confounding factors. This was enabled by the discovery that the initiation of myelination does

not require axonal signals as OL could myelinate paraformaldehyde-fixed axons ⁴⁸. Some ingeniously engineered platform include synthetic fibers ⁵, micropillars ⁴⁹, and microspheres ⁵⁰.

1.4.2.a Synthetic Fibers for Myelination

Synthetic fiber is the most popular non-axonal substrate to replace axons in a myelinating culture, due to their similarity in architecture and size scale ^{6,51}. These fibers were engineered through the process of electrospinning, where strong electrostatic forces of repulsion are used to extrude out micron to submicron fibers from a polymeric solution. Electrospun fibers were first employed by Lee *et al* to investigate fiber diameter-dependent effect of myelination ⁶. In this pioneering work, fibers with a diameter lower than 400 nm were shown not to be myelin permissive, in stark contrast with larger fiber diameter (illustrated below) ⁶. This established the biophysical threshold for myelination that is similarly observed *in vivo*. Subsequent work by Bechler *et al* investigated how myelination was affected by the regional differences of OL, as they elegantly showed that OPCs derived from the cortex and spinal cord could exhibit a varying response to laminin through changes in their myelin sheaths number and length ⁵¹.

The advantage of electrospun fiber in neural tissue engineering lies in its versatility⁵². First, electrospun fibers could provide topographical cues to direct neural cell fate. For instance, electrospun fiber substrate significantly enhanced neural progenitor cells (NPC) differentiation⁵³, and this differentiation is dependent on fiber diameter, with smaller fiber diameter favoring OL differentiation⁵⁴; Nanofibers topography was also found to significantly reduces astrocytic differentiation from embryonic stem cells⁵⁵. Second, electrospun fibers could be biofunctionalized through surface-mediated adsorption or encapsulation with pro-regenerative molecules⁵⁶⁻⁵⁸. Low *et al* surface-functionalized electrospun nanofibers with mussel-inspired polydopamine coating to deliver siRNA in a sustained manner. The RNA interference enhances NPC differentiation through RE-1 Silencing Transcription Factor (REST) knockdown⁵⁶. This was followed up with the encapsulation of retinoic acid and brain-derived neurotrophic factor in a co-polymer fiber to enhance the differentiation of the same cell types⁵⁷. Subsequently, Diao et al synergize the RNA interference methodology and nanofiber topography to promote OPCs differentiation⁵⁸. Third, electrospun fibers could be designed into implantable scaffolds for *in vivo* neural tissue engineering^{59,60}. Aligned fibers in a nanofiber-hydrogel scaffold could guide the neurite extension of axons severed by SCI⁵⁹. This could be coupled with non-viral drug/gene delivery to promote regeneration⁶⁰. Altogether, electrospun polymeric fiber is the leading candidate in neural tissue engineering to replace conventional neuron-OL myelination culture to decouple axonal influence from direct OL effect in myelination.

1.4.2.b Micropillars for Myelination

Although electrospun fibers represent a major advance in the myelination culture, it is not suitable for high-throughput screening of therapeutics. Fibers have to be electrospun and secured manually, which is relatively time-consuming⁶. Moreover, the electrospinning process always produces polymeric fibers of a small range of fiber diameter. As such, the photolithography process was employed to reproducibly fabricate micropillars that could be imaged automatically in a high throughput manner⁴⁹. OPCs could be differentiated and OLs could produce concentric membranes around the micropillars. Correspondingly, the size of the ring of myelin basic proteins around the micropillar serves as an indicator of myelination. The effectiveness of micropillar assay is demonstrated by the screening of 1000 bioactive molecules to pinpoint the cluster of antimuscarinic compounds that promote OL differentiation and remyelination (illustrated below)⁴⁹.

Adapted, credit to aforementioned authors

1.4.2.c Microsphere for Myelination

The use of microsphere in myelination culture is unique. While electrospun fibers mimic the architecture and size scale of axons, microspheres are the equivalence of microfibers for the neuronal cell bodies⁵⁰. OLs do not myelinate neuronal cell bodies physiologically. However, myelination of 20 μm microbeads shows that spherical architectural is biophysically permissive to OL myelination. As such, biochemical cues must be present on the neuronal cell bodies to inhibit its myelination⁵⁰. Factors could be surface functionalized on the microsphere for the screening of inhibitory effect on myelination that is relevant to the neuronal cell bodies.

Altogether, in vitro non-axonal myelination culture provides an intriguing platform to further our understanding of the mechanism of myelination and to perform drugs/genes screening. It provides an opportunity to uncover OL-specific effect which is decoupled from the neuronal influence.

1.4.3 Mechanotransduction in Myelination

On top of biochemical signaling, biophysical cues play an equally crucial role in defining cellular processes⁶¹. Mechanical forces such as substrate stiffness, matrix degradability and transient nonlinear mechanics are known to influence cellular behavior and stem cell lineage selection. Further evidence in more recent years also supports the notion that OPCs are also mechanosensitive, as the geometric and spatial constrain of the microenvironment could promote OPC differentiation⁴⁸. We review the effect of mechanical properties and architecture on cellular fate, mechanotransduction pathways and current state of mechanobiology understanding in OL lineage.

1.4.3.a Mechanical Cues that Influence Cellular Behavior

Substrate stiffness is the most commonly studied mechanical cues for its effect in cellular morphology, spreading, adhesion, migration, proliferation, differentiation and apoptosis ⁶². Typical techniques to explore substrate stiffness effect involve the use of polyacrylamide (PAA) gel and polydimethylsiloxane. A wide range of stiffness could be attained by these 2-dimensional (2D) materials through a varying degree of crosslinking with good control ⁶³. Adherent cells on these materials could generate a different degree of force to match the stiffness of the substrate through cytoskeletal machinery to exhibit differences in cellular behavior ^{64,65}. For instance, Wang et al cultured NIH3T3 cells on PAA gels with varying elasticity. Softer substrates resulted in lower rate of DNA synthesis and higher apoptosis coupled with a lower spreading area ⁶⁶. Interestingly, oncogenically transformed cells lose the ability to respond to substrate rigidity, giving a possible explanation for unregulated growth ⁶⁶. Similarly, fibroblast exhibited stress-fiber formation and more spreading on stiff PAA gels, but neutrophils do not exhibit behavioral changes to substrate stiffness ⁶⁴. The importance of substrate stiffness to cellular migration was further manifested through the use of 3D matrigel environment ⁶⁷. A prostate cancer cell line was encapsulated in the Matrigel substrate with uniform matrix ligand and cell integrin receptor levels. Maximal cell movement was observed in matrices with lower stiffness in this 3D environment, pointing to the importance of the balance between counteracting tractile and adhesion forces ⁶⁷. Last but not least, human dermal fibroblast was shown to increase its proliferation in stiff collagen matrices ⁶⁸.

More interestingly, mechanical stiffness could bias stem cells lineage selection ^{69,70}, providing an alternative avenue to alter stem cell fate. Mesenchymal Stem cells (MSCs) favors osteogenic differentiation when cultured on collagen substrates of high stiffness, and favors neuronal differentiation when cultured on matrices with stiffness close to the brain ⁶⁹. These MSCs exhibit changes in cellular morphology, genetic profile and cytoskeleton to indicate lineage specification when cultured on hydrogel of varying stiffness ⁶⁹. This mechanosensitivity is shared by neural stem cells, where low substrate stiffness favoring neuronal differentiation and high substrate stiffness favoring OL differentiation ⁷⁰. This was investigated through the use of a photopolymerizable methacrylamide chitosan hydrogel, a 2D substrate that could mimic the elastic modulus of the native CNS environment. Thus, substrate mechanical stiffness is an important physical parameter that influences cell behavior and cell fate.

Advancement of the field of mechanobiology from 2D to 3D culture enables the research on the effect of matrix degradability on cellular response. While cells on 2D hydrogel spread well and polarized on a stiff substrate, encapsulating mechanosensitive cells in 3D hydrogels revealed a polar opposite trend as these cells would remain round in stiff 3D substrates. Thus, it was hypothesized that the matrix degradability is another mechanical cues to influence cellular behavior ⁷¹. This is supported by observations that MSC favor osteogenesis in an environment with high cell-mediated matrix degradation and adipogenesis in an environment with restricted cell-mediated matrix degradation ⁷². Using hyaluronic acid hydrogels, the differentiation of MSC was found to be directed by degradation-mediated

cellular traction. Thus, matrix degradability could shares the same capacity as mechanical stiffness in influencing cell fate and serve as another possible tool in stem cell engineering.

In more recent years, the effect of transient nonlinear mechanics on cells is gaining traction ⁶². In particular, stress-relaxation kinetics or viscoelasticity is of huge interest after Mooney et al demonstrated how rapid stress relaxation enables fibroblast to spread and proliferate more rapidly and enable osteogenic differentiation of MSC ⁷³. The changes in stress-relaxation kinetics were attained with ionic crosslinking of varying the molecular weight of alginate with PEG spacer, and the stress relaxation-induced differentiation is also proved to be mediated through extracellular matrix (ECM) ligands ⁷³. This study of stress-relaxation on cellular behavior was later extended to myoblast where stress-relaxation promotes myoblast proliferation ⁷⁴. Similarly, alginate hydrogels were engineered to exhibit differential stress-relaxation kinetics through ionic crosslinking and PEG-doping. Altogether, the innovative hydrogel opens more avenues for the research on the additional physical regulator on cell behavior and cell fate. However, much is not known about the mechanotransduction of stress-relation process. The key regulatory element of mechanical signal Yes-associated Proteins (YAP) is not by itself controlling the differentiation of MSC ⁷³. Thus, it is crucial to look into established transduction pathways for physical and geometrical cues.

1.4.3.b Mechanotransduction Pathways

Mechanosensitive cells sensing the physical cues in their extracellular microenvironment have to transduce these cues into biochemical signals, which destined to the intranuclear space to influence transcriptional activities and gene expression. The recent boom in the mechanotransduction field has revealed some mechanistic pathways on how these physical signals could be transmitted across the cytoskeletal network to influence proceedings in the nucleus.

Mechanotransduction starts from the cellular adhesion to ECM ligands through integrin receptors. This interaction activates focal adhesion kinase, which serves as the integrin-actin linker by enhancing talin recruitment ⁷⁵. Phosphorylation of focal adhesion kinase is crucial to cellular adhesion and subsequent recruitment of other signaling molecules such as SRC tyrosine kinase. In OL development, SRC family of Fyn and Lyn kinases are known to be an important regulator for downstream signaling pathways that regulates OPC proliferation and differentiation. In particular, Fyn associated with ECM-bind $\alpha 6\beta 1$ integrin to trigger OPC differentiation. On the other hand, Lyn regulate OPC proliferation through integrin-specific response to ECM stimulus ⁷⁶. Adhesion complexes and cytoskeletal components assembled by FAK phosphorylation trigger downstream mechanoresponsive signaling, which typically involves mitogen-activated protein kinase and the Rho family of GTPase. Both signals are crucial to the regulation of OPC differentiation ^{76,77}. As a member of Rho family of GTPase, RhoA phosphorylates downstream effector protein Rho-associated protein kinase and play an important role in the regulation of cytoskeleton and actin dynamics. Altogether, mechanical signals are transduced into the intracellular environment through integrin receptors and cytoskeleton (see below).

Transduction of mechanical signal continues from the cytoskeleton to nucleus through the linker of the nucleoskeleton and cytoskeleton complex (LINC). LINC is associated with both inner and outer domain of nuclear envelop with SUN domain protein functioning as molecular chain links. The complex connects actin cytoskeleton to nuclear lamins in the nucleus via nesprin protein by having specific molecular orientation between SUN and KASH domain of nesprin ⁷⁸. Mechanical forces could trigger the entry of transcriptional regulators into the nucleus through the assembly of LINC and opening of nuclear pores ⁷⁹. The known transcriptional regulators include YAP, transcriptional co-activator with PDZ-binding motif (TAZ), megakaryocytic acute leukaemia (AMKL) and β -catenin ⁸⁰.

The role of YAP/TAZ in mechanotransduction is examined intensively in recent years due to their prominent role in responding to a variety of mechanical cues such as stiffness and geometrical constraints ⁸¹. YAP/TAZ has a predominant cytoplasmic expression on soft substrates but translocates into the nucleus upon increasing stiffness. Similarly, YAP/TAZ nuclear translocation is also observed with increasing MSC spreading ⁸². The nuclear localization of YAP/TAZ is maintained by Rho and actin cytoskeleton but not dependent on the ratio of monomeric/filamentous actin. Crucially, MSN osteogenic differentiation induced by stiff substrates is inhibited by depletion of YAP/TAZ, pointing to a significant role of YAP/TAZ in the mechanotransduction pathway ⁸². In OL, YAP was also shown to function as mechanotransducer in a variety of cellular events including OL maturation. Specifically, forced expression of nuclear YAP significantly increased the number of MBP+ cells, which is the crucial myelinating stage of OL ⁸³. In the peripheral nervous system, YAP/TAZ controls the

myelination of schwann cells, through regulating radial sorting and expression of laminin receptors. Moreover, YAP/TAZ regulates the expression of lipid synthetic enzymes, potentially pointing to a prominent role in controlling lipid synthesis for myelination ⁸⁴.

A less well-known transcriptional regulator activated by mechanical cues is MAL. Rho induces actin polymerization and depolymerization to activate MAL, which is a coactivator of transcription factor serum response factor to control gene expression ⁸⁵. Similar to YAP/TAZ, MAL translocate into the nucleus from cytoplasm upon mechanical signals, which is dependent on alternations in actin dynamics. Specifically, stabilization of F-actin induced MAL nuclear accumulation and activation of SRF for gene transcription ⁸⁵. In the context of myelination, actin dynamic is crucial for the growth of OL myelin sheaths. Compaction of myelin is accompanied by concurrent F-actin disassembly, which is mediated by myelin basic proteins ⁸⁶. Moreover, actin turnover, or the assembly of filamentous F-actin and subsequently disassembly into monomeric G-actin, drives the growth of myelin at the leading edges ⁸⁷. Thus, mechanical cues could control OL myelination through MAL-regulated pathways.

Chapter 2: Microfiber Drug/Gene Delivery Platform for the Study of Oligodendrocyte Myelination

Summary

Our ability to rescue functional deficits after demyelinating diseases or spinal cord injuries is limited by our lack of understanding of the complex remyelination process, which is crucial to functional recovery. In this study, we developed an electrospun suspended poly(ϵ -caprolactone) microfiber platform to enable the screening of therapeutics for remyelination. As a proof of concept, this platform employed scaffold-mediated non-viral delivery of a microRNA (miR) cocktail to promote oligodendrocyte precursor cells (OPCs) differentiation and myelination. We observed enhanced OPCs differentiation when the cells were transfected with miR-219 and miR-338 on the microfiber substrates. Moreover, miRs promoted the formation of MBP+ tubular extensions around the suspended fibers, which was indicative of myelination, instead of flat myelin membranes on 2D substrates. In addition, OPCs that were transfected with the cocktail of miRs formed significantly longer and larger amounts of MBP+ extensions. Taken together, these results demonstrate the efficacy of this functional screening platform for understanding myelination (Figure 2.1).

Figure 2.1: Graphical Summary of Chapter 2. We engineered a suspended microfiber platform for in vitro myelination. Microfibers could be surface modified with polydopamine to deliver promyelinogenic molecules to enhance myelination. The effect of surface modification on myelination was also examined.

2.1 Introduction

Oligodendrocytes (OLs), an important class of glia, are the myelinating cells of the central nervous system (CNS). Myelination, the process by which OLs form multiple multilamellar sheaths around axons, allows rapid sensory-motor coordination in vertebrates.

As such, pathological loss of myelin, or demyelination, impairs function due to disrupted action potential propagation as seen in Multiple Sclerosis (MS) and spinal cord injuries (SCI).

Remyelination restores myelin sheaths to demyelinated axons to reinstate salutatory conduction by following a sequence of events in a precise and temporally controlled fashion¹⁷. Following oligodendrocyte precursor cells (OPCs) activation and recruitment, OPCs differentiate into OLs and ensheath axons (i.e. nascent myelin sheaths) before compacting into multilamellar myelin sheaths (i.e. mature myelin wraps)^{17,88-90}. However, these final stages of remyelination often fail to transpire in MS and SCI¹⁷. The functional loss associated with failure in remyelination has prompted the search for better therapeutics. Yet, the development of therapeutic strategies for remyelination has been largely hindered by our lack of understanding of the intricate differentiation and myelination processes. Hence, *in vitro* platforms that allow us both to study these processes and at the same time enable functional screening of promising pro-myelinogenic therapeutics are highly valuable⁴⁹.

Among the various stages that are involved in OPC differentiation, maturation and myelination, the first two steps of differentiation and maturation of OPCs are often analyzed by fairly straightforward immunohistochemical evaluations of specific OL markers such as CNPase, Myelin Basic Protein (MBP) and others^{33,39,58,91}. However, the main functionality of an OL is determined at the final stage of myelination, where radial and longitudinal growths of myelin wraps occur⁹². Unfortunately, this final stage of myelination remains highly challenging to model in an *in vitro* culture. Yet it is crucial to achieve robust aforementioned

growth of myelin wraps to enable the systematic examination of OL functionality and screening of therapeutics that can modulate remyelination.

By mimicking the size scale and architecture of axons, electrospun fibers have been utilized to replace neurons in conventional neuron-OL co-cultures for studies on myelination. This strategy helps to remove influences of axonal signalling and reveals the intrinsic myelination capabilities of OL in response to physical signals ^{6,51}. While platforms comprising of dense electrospun fiber mesh provide axon-mimicking physical cues to support OL myelination, it is difficult to do direct imaging and quantification of the myelin ^{58,91}. On the contrary, the sparse fiber platforms that were engineered previously do not provide suspended fibers to prevent OPCs from sensing the stiff underlying supporting substrate ⁵. Moreover, the role of biochemical signalling is also crucial. Yet, such a biofunctional axon-like substrate remains unavailable for probing the effects of potential biochemical signals on myelination.

Here, we introduce a simple and versatile method to biofunctionalise microfiber scaffolds to allow controlled and sustained delivery of promyelogenic biomolecules to OPCs. This platform is comprised of suspended-axon-mimicking fibers, which supports OL myelination. As compared to conventional electrospun membranes, this platform also allows straightforward visualization of myelination. We show that using the versatile mussel-inspired bioadhesive, polydopamine (PD), coating, these axon-like fibers could be easily functionalized with drugs to serve as a reservoir for sustained drug/gene delivery. Correspondingly, such

scaffolds not only serve as potential drug/gene screening platforms for identifying important promyelinating therapeutics but can also be easily translated into direct implantable devices for future *in vivo* regeneration and remyelination purposes ⁶⁰.

As a proof-of-concept, we delivered a cocktail of microRNA (miR) mimics using this platform to control, visualize and quantify OL differentiation and myelination. In particular, miR-219 and miR-338 are potent post-transcriptional regulators that are involved in OPC development ^{93,94}. We previously showed that these miRs act synergistically to down-regulate the expression of inhibitors of OPCs differentiation and promoted OPCs differentiation and maturation ^{58,91}. More recently, further evidence also suggests that the two miRs act in tandem to promote myelination ³⁴. Thus, we hypothesized that PD fiber-mediated delivery of miR-219 and miR-338 will promote OPC differentiation and myelination, which can be evaluated and quantified using our suspended biofunctional, axon-mimicking microfiber platform. We also examined the effect of our surface modifications on oligodendrocyte myelination.

2.2 Materials and Methods

2.2.1 Materials

Polycaprolactone (PCL, Mw: 200,000) was purchased from PolySciTech. Polycaprolactone (PCL, Mw: 45,000), 2,2,2-trifluoroethanol (TFE), dopamine hydrochloride, Dnase 1 type IV, Poly-D-Lysine (PDL), N-Acetyl-L-Cysteine (NAC), D-biotin, ITS, Putrescine, L-Thyroxine (T4), Tri-iodothyronine (T3), Progesterone, Bovine Serum Albumin (BSA), L-cysteine,

Triton X-100 were purchased from Sigma-Aldrich. Scramble negative control miR, miR-219, miR-338-3p, miR-338-5p mimics, Alexa-Fluor 488 goat anti-rat, Alexa-Fluor 633 goat anti-rabbit antibodies, minimum essential media (MEM), DMEM High Glucose, Penicillin/Streptomycin (Pen/Strep) were purchased from Life Technologies. NS21 supplement and Neuro Medium were purchased from Miltenyi Biotech. TransIT-TKO was purchased from MirusBio. Rat anti-Myelin Basic Protein (MBP, aa82-87) was purchased from Bio-Rad. Rabbit anti-Oligodendrocyte Transcription Factor 2 (Olig2, AB9610) was purchased from Merck. Papain suspension was purchased from Worthington.

2.2.2 Fabrication and characterization of suspended microfiber substrate

Figure 2.3 illustrates the engineering of the suspended microfiber platform. PCL pellets (MW: 45,000) were melted on a hot plate in a mould and allowed to cool and solidify into a block of PCL. Following that, the block was embedded into optimal cutting temperature medium and cryosectioned into 20 μm films, which were then dried in a 37 $^{\circ}\text{C}$ oven. Two dried pieces of PCL films were then placed at two opposing ends of a 15-mm diameter glass coverslip at a distance of 0.5 cm apart and melted briefly on a hotplate at about 50 $^{\circ}\text{C}$. These glass coverslips were then pasted onto a rotating wheel with carbon tape. For electrospinning of microfibers, PCL (MW: 200,000) was dissolved in TFE to make a 7 wt% solution. Thereafter, the homogeneous solution was loaded into a syringe and dispensed at a fixed rate of 2 ml/h by a syringe pump (New Era pump system Inc., USA) through a 22G needle. Positive 4 kV and -4 kV voltages (Gamma High Voltage, USA) were then applied to the needle and rotating collector (730 rpm) respectively. The distance between the needle and the collector was kept

at 11.5 cm. After the fibers were collected, PCL films were melted briefly again to allow the fibers to stick to the PCL films. Since the melted films may cause fibers to slack and sink to the glass coverslips, to check if the fibers remained taut and suspended after the process, we repeated the melting and collection processes 3 times. Thereafter, the dried suspended microfiber scaffolds were secured by applying a layer of silicon glue around the circumference of each coverslip.

For 3-dimensional (3D) imaging of myelin, di(thiophene-2-yl)-diketopyrrolopyrrole conjugated PCL (PCL-DPP-PCL) fluorescent fibers were prepared according to previously reported protocol ⁹⁵. Briefly, to ensure similar fiber diameter as plain PCL fibers, PCL was mixed with 1 wt% of PCL-DPP-PCL in TFE to make a 12.5 wt% solution. Thereafter, the solution was dispensed at a flow rate of 1.5 ml/h through a 21G needle for electrospinning. The collector was located 12 cm away and rotated at 1500 rpm. Positive 12 kV and negative 4 kV voltages were applied on the needle and collector respectively.

2.2.3 Scanning Electron Microscopy

The morphology of the microfibers was evaluated using scanning electron microscopy (SEM) (JEOL, JSM-6390LA, Japan). Briefly, PCL substrates were attached onto the SEM support by carbon tapes and sputter-coated with platinum for 60 s at 20 mA. Thereafter, the samples were imaged under an accelerating voltage of 10 kV.

2.2.4 Preparation of miR-loaded Fibers

The microfiber substrates were sterilised and wetted with 70% ethanol for 15 min. The substrates were then incubated in 1 ml of 0.5 mg/ml dopamine hydrochloride solution for 4 h on an orbital shaker to form the polydopamine (PD) coating. After 3 washes with DI water, the fibers were further coated with 2 ml of 5 µg/ml PDL overnight and washed 3 times with DI water. For miR adsorption, 2 µg of miR was complexed at 1:1 v/v ratio with the transfection reagent, TKO. The amount of miR and its ratio with TKO have been optimised for high gene silencing and low cellular toxicity based on our previous works^{58,91}. Specifically, 3 µl of 50 µM miR was complexed with 3 µl of TKO for 15 min in 100 µl of DMEM. The complex was then added onto the PDL coated fibers and incubated at 37 °C for an hour. All substrates were divided into two groups: TKO with scrambled Negative Control miR (NEG miR control); and TKO with equal masses of miR-219, miR-338-3p and miR-338-5p (miR-219/miR-338). Substrates that were not used for the delivery of miRs were coated with 5 µg/ml of PDL overnight and washed 3 times with DI water before cell seeding. Bolus transfection for different diameter fibers was performed with Lipofectamine RNAimax according to manufacturer protocol after 7 days in culture. 10 nM of miRs was used with equal masses of miR-219 / miR-338.

2.2.5 OPCs Isolation and Myelination Culture

The isolation of primary OPCs from rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. The isolation and culture of primary OPCs and the myelination culture were carried out according to two previously reported protocols^{51,96}. Briefly, P0-P2 neonatal rat cortices that were cleaned from

meninges were enzymatically digested with 1.2 U Papain and 40 µg/ml DNase at 37 °C for 1 h. Thereafter, the enzymatic activity was stopped with 8 ml of DMEM with 10% Fetal Bovine Serum (FBS) and the dissociated tissues were triturated with a 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 OPCs were separated from the mixed glia culture by shaking on an orbital shaker at 200 rpm for 1 h at 37 °C to remove the loosely attached microglia. This was followed by a further 16-17 h of shaking. OPCs were then further purified by differential adhesion on untreated petri dishes for 25 min. Through this isolation method, the purity of the OPCs attained was 95.3 ± 1.6 % (Figure 2.2). Purified OPCs were then seeded at a density of 35,000 onto each suspended fiber substrate, and cultured for 7 days in myelin medium, which consisted of DMEM:Neurobasal (50:50), NS21, 1% Pen/Strep, Glutamax, 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).

2.2.6 Immunofluorescent staining

After 7 days, the cultures were fixed with 4% paraformaldehyde (PFA). The fixed myelin-fiber cultures were then permeabilised with 0.1% Triton-X for 15 min at room temperature. Next, the samples were incubated with primary antibodies overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature. The primary antibodies used were: rat anti-MBP (1:200) and rabbit anti-Olig2 (1:1000). The secondary antibodies used were: Alexa-Fluor 488 goat anti-rat and Alexa Fluor 633 goat anti-rabbit (both at 1:1000). The nuclei were counterstained with DAPI. The samples were then mounted onto 60 mm coverglass with Fluoromount-G and imaged under a Zeiss LSM 800 confocal microscope.

To calculate the percentage of MBP⁺ cells with respect to DAPI (MBP⁺/DAPI), 10 non-overlapping regions of interest (ROI) were chosen randomly for each substrate at 20x magnification. The percentages of MBP⁺/DAPI were then calculated by (number of MBP⁺ cells/number of DAPI) x 100% with an average of more than 140 DAPI⁺ cells counted for each group per experiment. The percentages of MBP⁺ cells with respect to OLIG2 (MBP⁺/OLIG2⁺) were computed by counting on an average of more than 110 OLIG2⁺ cells from each group and using the formula: (number of MBP⁺ cells/number of OLIG2⁺ cells) x 100%. The experiment was repeated 3 times, resulting in the analysis of a total of more than 420 DAPI⁺ and 330 OLIG2⁺ cells per sample group.

To calculate the percentage of MBP⁺ cells with myelin-like extensions/wrappings on fibers only, MBP⁺ structures were thoroughly examined by looking at the entire z-layer (step size 0.75 μm) to observe any membrane formation on fibers and/or coverglass at 20x magnification. MBP⁺ cells that formed membranes on fibers but not on cover glass were qualified as “MBP⁺ cells with only myelin-like extensions on fibers”. The percentage of MBP⁺ cells with myelin-like extensions on fibers only was computed by (number of MBP⁺ cells with only myelin-like extension on fibers only/total number of MBP⁺ cells) x 100%. On average more than 50 MBP⁺ cells were evaluated from at least 10 ROI per substrate for each group. The experiment was repeated 3 times.

A myelin-like extension was defined as a continuous tube of MBP staining that stretched along a fiber. To measure the length of myelin-like extensions on fibers, MBP signals from single DAPI were traced along individual fibers at 40 x magnification. The measured lengths were then plotted in logarithmic scale as “log(nascent sheath length)”. Gaussian distribution was then used to fit the distribution of log(nascent sheath length) for average length comparison ⁵¹. On average 30 myelin-like wrappings were measured per experiment, resulting in a total of 196 nascent sheaths being quantified in all 3 experimental repeats. To compute for the number of nascent myelin sheaths per cell, 35 MBP⁺ cells in the miR-219/miR-338 group and 30 MBP⁺ cells in the negative miR (NEG miR) group from 3 independent experiments were included in the analysis. All measurements were done using the Fiji version of ImageJ ⁹⁷.

2.2.7 Evaluating Surface Charges with Anton Paar Surpass

Electrospun fibrous mesh was prepared according to protocol established in our previous work ⁵⁸. Briefly, poly-caprolactone (PCL) fibers were electrospun onto a piece of aluminium foil. Fibrous mesh was wetted with 70% ethanol and washed with water before cutting into 1 cm x 2 cm dimension and coated with 0.5 mg/ml of Dopamine for 4 h. Any further PDL coating was done at 5 µg/ml for overnight at 37 C. Surface potential measurements were performed with Anton Paar SurPASS 3 equipped with pH and conductivity electrodes and autotitrator according to manufacturer’s protocol. Briefly, a pair of coated fibrous mesh was mounted onto the adjustable gap cell with a Red industrial-grade tape. pH and conductivity electrodes were calibrated with standard solutions before sample measurements. For each pair of sample, the gap between the mesh was adjusted to in

between 100-120 μm . 0.001 M KCl was used as the electrolyte for surface potential measurements.

2.2.8 Statistical Analysis

All values were presented as mean $\pm$ standard error of mean (S.E.M). Student's t-test was used to compare between NEG miR control and the miR-219/miR-338 group. P-values of less than 0.05 were considered to be statistically significant.

2.3 Results

2.3.1 Characterizations of Suspended PCL Microfiber Substrates

Figure 2.3 illustrates the engineered suspended-microfiber substrate. The average diameter of the microfibers was $2.0 \pm 0.3 \mu\text{m}$, a diameter that is known to be permissive for myelination (Figure 2.3B) ^{5,51}. Figure 2.4 shows that the fibers remained taut and suspended even after 3 layers of PCL films were repeatedly melted and 3 layers of PCL fibers were deposited after each melting.

Figure 2.3C shows that OPCs differentiated into MBP⁺ OLs and appeared to ensheath the microfiber substrates in the absence of miRs. Multiple MBP⁺ extensions that were indicative of nascent myelination were formed by OLs, as shown by the bright green MBP signals. These myelin-like extensions ran along the electrospun fibers, as shown in Figure 2.5. PCL-DPP-PCL fibers fluoresce brightly at 568 nm wavelength upon excitation while green MBP

signals ran in conjunction. Figure 2.5B shows a confocal 3D reconstruction view of an OL on the red PCL-DPP-PCL fibers. Tubes of MBP signals were seen around red fibers, confirming MBP⁺ ensheathment of fibers by the OL. In the absence of growth factors, some OPCs underwent apoptosis after 7 days in culture as indicated by the abnormally small and bright spots of DAPI staining (Figure 2.3C). Concurrently, Olig2 expression was not detectable in these cells. Nonetheless, the results suggest that this platform could support the formation of myelin-like extensions on the suspended microfibers.

Figure 2.2: Confocal Images show more than 95% pure OPCs following isolation. Scale bar represents 10 μ m.

Figure 2.3: Electrospun microfiber platform for OL functional drug testing. (A) The workflow of suspended microfiber substrate. (B) SEM image showed that electrospun microfiber has a diameter of $2.0 \pm 0.3 \mu\text{m}$ (100 fibers were measured). (C) OPCs differentiated into OL and extended myelin-like MBP⁺ structures along the suspended fibers as shown by lines of green MBP signals.

Figure 2.4: SEM images of the cross-section of microfiber substrate. With repeated melting of PCL film and repeated fiber deposition, suspended fibers remained taut in layers. (A) Low magnification view shows fibers suspended in layers above glass coverslip. (B) Higher magnification view shows taut fibers.

Figure 2.5: MBP⁺ wrapping on fluorescent PCL-DPP-PCL fibers. (A) Confocal image shows MBP signals ran along red coloured fibers. (B) 3D-reconstruction image shows MBP⁺ tubular structures surrounding red fluorescent PCL-DPP-PCL fibers. (C) 95.3 ± 1.6 % of the cells expressed OPCs NG2 marker 1-day post seeding.

2.3.2 Scaffold-mediated miR-219 / miR-338 Delivery Promoted OPC Differentiation in the Absence of Mitogen

OPCs remained viable and differentiated into MBP⁺ OLs on PD-PDL coated substrates after transfection with miRs. Figure 2.6A shows that purified OPCs differentiated into OLIG2

and MBP expressing OLs on the miR-adsorbed platforms. In particular, a higher percentage of MBP⁺ OLs was seen with miR-219 and miR-338 treatment. Thus, miR cocktail promoted OPC differentiation in a pro-differentiation, mitogen-free environment. Specifically, a 1.42-fold and 1.40-fold increase in the percentage of MBP⁺ cells were observed when normalized against DAPI and OLIG2 respectively, when the OPCs were transfected with 2 μ g of miR cocktail ($p < 0.05$, Figure 2.6B and Figure 2.6C). The results show that miR-219 and miR-338 acted in synergy with the myelination medium and further promoted the differentiation and maturation of OPCs.

Figure 2.6: Scaffold-mediated delivery of miR-219/miR-338 promoted OPCs differentiation in the absence of mitogen. (A) Representative confocal images show that cocktail of miRs

promoted differentiation of OPCs into MBP⁺ OL. (B) Quantitative data shows miR-219 and miR-338 treatment resulted in more MBP⁺ cells per DAPI. (C) Quantitative data shows miR-219 and miR-338 treatment resulted in more MBP⁺ cells per OLIG2⁺ cell. # indicates $p < 0.05$; two-tailed t-test, with an average of more than 140 DAPI and 110 OLIG2⁺ cells quantified from each experimental group per experiment (n=3).

2.3.3 Scaffold-mediated miR-219 / miR-338 Delivery Promoted the formation of myelin-like extensions on fiber

In our platform, PCL fibers were suspended at a distance of about 20 μm above the glass coverslip surface using PCL films. The low fiber density allowed OPCs to pass through the gaps between electrospun fibers and adhere to the coverslip while maintaining contact with the suspended fibers. As OPCs were in contact with both 2D and 3D substrates, they were able to form both flat membranes on the cover-glass and myelin processes that tracked along or partially covered the fibers as they differentiated into OLs (Figure 2.7A).

As shown in Figure 2.7B, OLs preferred to form MBP⁺ extensions or nascent myelin sheaths along the axon-mimicking fibers when they were treated with the cocktail of miRs. Specifically, miR-219 / miR-338 treatment induced 2.9-fold more MBP⁺ cells forming only myelin-like wrappings on fibers ($p < 0.01$, Figure 2.7C). On the other hand, OLs that were subjected to Neg miR treatment (Control group) were inclined towards forming both MBP⁺ extensions or nascent myelin sheaths on fibers and myelin membranes on coverglass, or myelin membranes only (Figure 2.7A). Thus, miR-219 / miR-338 enhanced the interaction of

OPCs with 3D axon-like substrates, suggesting that biomolecules could alter cellular response towards physical cues.

*Figure 2.7: Scaffold-mediated delivery of miR-219/miR-338 promoted the formation of MBP⁺ myelin-like extensions on fiber instead of membrane on a glass coverslip. (A) Confocal images showing the formation of both myelin-like extensions on fiber and myelin membrane on coverslip by the same cell. (B) Representative confocal images show miR-219/miR-338 promoted the formation of myelin-like wrappings around fibers. (C) Quantitative data shows miR-219 and miR-338 treatment allowed a higher percentage of MBP⁺ cells to form myelin-like extensions only. * indicate $p < 0.01$; two-tailed t-test, with at least 50 MBP⁺ cells from each group per experiment ($n = 3$).*

2.3.4 Scaffold-mediated miR-219 / miR-338 Delivery Promoted Longer Fiber

Ensheatment

Substrate-mediated delivery of miRs increased the length of fiber ensheathment by OLs on the substrates (Figure 2.8). In the presence of miR-219 and miR-338, OLs formed significantly longer nascent myelin sheaths around the suspended fibers (Figure 2.8A). Quantification results as illustrated in Figure 2.8B shows that the distribution of the length of fiber ensheathment shifted towards a larger value when the cells were treated with the miR cocktail. Specifically, 1.94-fold longer nascent myelin sheaths were formed by the OLs when they were treated with the cocktail of miR-219 / miR-338 (Figure 2.8C).

*Figure 2.8: Scaffold-mediated delivery of miR-219/miR-338 increased myelin-like extension length of OL. (A) Representative confocal images show miR-219 and miR-338 treatment resulted in longer myelin-like extension length on suspended fiber platform. (B) A frequency distribution shows a shift of extension length distribution towards higher values when OPCs were treated with miR-219 and miR-338. (C) Log transformation of lengths shows the distribution of length of myelin-like extensions, with a significant difference between the mean of miR-219/miR-338 and NEG miR treated group. * indicate $p < 0.01$; two-tailed t-test, 196 extensions measured from 3 independent experiments.*

2.3.5 Scaffold-mediated miR-219 / miR-338 Delivery Promoted the Formation of More Myelin-like Extensions per Cell

OLs formed multiple myelin-like extensions along multiple fibers on the substrates. This attribute of OLs was altered by substrate-mediated delivery of miRs. In particular, miR-219 and miR-338-treated OLs had a higher number of myelin-like extensions being formed by each cell (Figure 2.9A). Specifically, miR-219 and miR-338-treated group had on average of 8.69 myelin extensions per OL as compared to 4.77 in the NEG miR treated group ($p < 0.01$, Figure 2.9B). Thus, both sheath number and length that were formed by individual OLs were enhanced on this substrate by substrate-mediated non-viral delivery of miR-219 / miR-338.

We further extended the application of non-viral miR delivery platform to enhance OL myelination. As shown in Figures 2.10A to C, miR-219/miR-338 treatment significantly enhanced OPC differentiation and maturation when cells were cultured in myelin medium after 14 days, resulting in larger numbers of 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 proliferative 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 2.10A and D). 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 2.10E) 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 we did not detect the formation of complete sheaths from either sample at earlier time points (Day 7), 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 2.10: Scaffold-mediated delivery of miR-219/miR-338 promoted long-term 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. Anova with Tukey Post-Hoc test; at least 100 cells quantified per sample group in each experiment repeats (n = 3 independent experiments) *p<0.01. (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 a higher average number of compact myelin sheath per OL. Each dot represents one cell **p<0.001; two-tailed t-test, 40 cells in miR-219/miR-338 group and 35 cells in NEG miR group, from 3 independent experiments.*

2.3.7 Synergistic Effect of miR-219 / miR-338 and Fiber Diameter on Myelination

We investigated the synergistic effect of topographical cues and chemical cues on myelination. Figure 2.11 shows 3 distinct types of PCL fiber engineered through electrospinning, one of which has fiber diameter below the biophysical requirement for OL myelination (Figure 2.11A, $0.460 \pm 0.095 \mu\text{m}$). We observed that OL myelination occurs when fiber diameter is above a biophysical threshold (Figure 2.12A) in agreement with earlier work⁶. Similarly, we observed an increase in the length of myelin sheaths with increasing fiber diameter (Figure 2.12 NEG miR Control Medium vs Large fiber, miR-219 / miR-338 Medium vs Large fiber)⁵¹. Moreover, miR-219 / miR-338 act in synergy with fiber diameter to enhance OL myelination when the biophysical requirement of myelination is satisfied, with an increasing average length of myelin sheaths formed by miR-219 / miR-338 transfected cells in Medium and Large fiber (Figure 2.12C).

Figure 2.11: Electrospun fiber of varying diameter. (A, B, C) Representative SEM images show a distinct electrospun fiber diameter. (D) Distribution of electrospun fiber diameter with significant differences between groups. A: $0.460 \pm 0.095 \mu\text{m}$, B: $2.231 \pm 0.382 \mu\text{m}$, C: $3.219 \pm 0.294 \mu\text{m}$. ** $p < 0.001$; ANOVA, at least 95 fibers measured in all groups.

*Figure 2.12: Myelination response to bolus miR transfection on fiber of varying diameter (A) Representative confocal images show miR-219/miR-338 promote OL myelination when the biophysical requirement is met. (B) Quantitative data shows miR-219 and miR-338 treatment resulted in rightward shift of ensheathment length distribution (C) Log transformation of lengths shows the distribution of length of myelin sheaths. ** $p < 0.001$; # $p < 0.05$ ANOVA, at least 33 sheaths were measured from each group.*

2.3.8 Effect of surface chemistry/charges on Myelination

We investigated the effect of transfection agent adsorption, dopamine and PDL coating on myelination irrespective of the presence of miR. Uncoated electrospun PCL fibers carry negative potential. PDL coating as expected, create slight positive charges on the fiber (Figure 2.13a). When coated with dopamine, all fibers displayed negative charges with negligible differences between coating time. Interestingly, negative charges created with Dopamine is of lower magnitude than uncoated PCL fiber or PDL-coated PCL fibers (Figure 2.13a).

Fibers coated with dopamine was left at room temperature for 4 or 7 days before zeta potential measurement. Dopamine coating has decreasing zeta potential following longer time exposed to air (Figure 2.13b). Dopamine-coated PCL fibers were further coated with PDL. Longer dopamine coating results in more positive surface potential after further PDL coating of the same amount (Figure 2.13c). Finally, TKO coating on dopamine coated fibers created highly positive charge surface (Figure 2.13d). Oligodendrocyte were culture in myelin media

for 14 days on fibers modified with different materials. Dopamine coated, negatively charged surfaces impede oligodendrocyte myelination. However, highly charged surfaces created by TKO coating restores the myelin permissiveness of the fiber substrate (Figure 2.13e).

Figure 2.13: Surface chemistry and charges influence oligodendrocyte myelination response.

*(A) Zeta potential data of unmodified, PDL-coated, dopamine-coated PCL fibers. PDL coating creates slightly more positive charges in comparison with uncoated fiber. Dopamine coating induced negative potentials regardless of coating time. (B) Dopamine-coated fibers left at room temperature has more negative potential as time progresses. (C) Longer durations of dopamine coating resulted in more positive potential. (D) Transfection reagents on PCL fibers resulted in highly positive potential. (E) Surface chemistry of PCL fiber influences the degree of in-vitro myelination. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, One-way ANOVA with Tukey Post-hoc.*

2.4 Discussions

OLs are known to not require dynamic axonal signalling to initiate myelination ⁹⁸. Hence, microfibers, micropillars and microbeads have been engineered to uncouple the axon-OL crosstalk and provide biophysical cues for myelination studies ^{6,49,50}. While bolus supplementation of biomolecules could provide the necessary biochemical signals for cells on the aforementioned substrates, integrating biomolecules into tissue-engineered scaffolds could better recapitulate the *in vivo* microenvironment, which is known to impact OLs behaviour ⁴⁸. Thus, we developed a surface-functionalized platform to evaluate myelination using mussel-inspired PD coating.

OL differentiation and myelination are two different stages of OPC development with distinctive mechanisms ⁹⁹. In our previous work, our fiber mesh-mediated miR delivery platform promoted the differentiation and maturation of NG2⁺ OPCs into RIP⁺ and MBP⁺ cells

with high viability ^{58,91}. However, robust myelination *in vitro* requires optimisation of culture conditions and scaffold design for optimal biochemical and physical signaling. Moreover, the new platform should enable simple visualization, imaging and quantification of the extent of myelination. Thus, we engineered a low fiber density, axon-mimicking drug delivery model, specifically for these purposes. A low fiber density platform allows straightforward confocal imaging and quantification of myelin. With this, we were able to demonstrate physiologically-relevant myelin membrane extension by amalgamating physical cues from the low-density microfibers and biochemical signalling from the miR-219 / miR-338 cocktail (Figure 2.3, 2.8, 2.9).

PD-coated suspended microfiber substrates enabled effective surface adsorption of miRs without altering the topography of the electrospun fibers. These miRs could then be released in a sustained fashion and provide surface-mediated signalling, as shown in our previous studies ^{56,58,91,100}. Conventional siRNA or miRNA studies employ bolus transfection, where cells are exposed to RNAs transiently. However, sustained delivery of RNA complexes can have significantly better gene silencing efficiency than bolus transfection, which can be translated to more efficient OPC differentiation and maturation ⁹¹. Furthermore, surface-adsorbed RNAs that were not released could be directly internalised by OPCs ⁵⁶. This mimics axon-OL biochemical signalling and further asserts the physiological relevance of our platform. All in all, we showed that substrate-mediated miR delivery enhanced OPC differentiation and membrane extension on our platform (Figure 2.6-2.9). Moreover, we have previously employed similar electrospun substrates for the delivery of proteins and low molecular

weight drugs^{57,101,102}. Thus, this microfiber substrate could be a versatile functional drug/gene screening platform for OLs.

In our previous study, we employed a similar platform to knock down REST in mesenchymal stem cells (MSCs) using siRNA to enhance neuronal differentiation¹⁰⁰. While we observed pro-neuronal differentiation effect of siREST when MSCs were subjected to conditions that were non-specific and suboptimal for such differentiation, the advantage of siREST was not seen when optimal neuronal differentiation condition was employed¹⁰⁰. The results suggested that the gene silencing of REST was effective only in non-specific differentiation conditions. In contrast, miR-219 and miR-338 appeared to act in synergy with pro-myelinogenic culture conditions. miR-219 and miR-338 have been elucidated to be the key miRs that are involved in OL maturation. These two miRs suppress critical inhibitory genes of OPC differentiation and myelination (PDGFR α , Sox6, FoxJ3, ZFP238) or inhibitory signalling pathways (NOTCH, LINGO)^{33,34}. Specifically, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are well known mitogenic factors. Their removal from culture medium results in the rapid differentiation of OPCs into OLs *in vitro*. All previous *in vitro* experiments that analyzed the efficacy of miR-219 and miR-338 on directing OL differentiation utilized PDGF and FGF supplemented culture media^{33,58,91}. Under such proliferative conditions, miR-219 / miR-338 promoted OPC differentiation into MBP⁺ OLs. In contrast, we employed myelin-promoting medium in this study, which comprised of a defined medium with no OPC mitogenic factors. Correspondingly, we showed for the first time that miR-219 and miR-338 could significantly promote OPC differentiation into mature MBP⁺ OLs,

even in such mitogen-free, pro-differentiation environment (Figure 2.6). This suggests that miR-219 and miR-338 are potent factors in controlling OPC cell fate.

OPCs cultured on 2-dimensional (2D) glass coverslips or culture dishes form flat myelin membrane sheets on the glass or plastic surface as they differentiate and mature into OLs ⁸⁶. On the other hand, OLs cultured on suspended fibers formed only myelin-like wrappings around the fibers ⁵¹. In our platform, we observed an interesting phenomenon where scaffold-mediated delivery of miR-219 and miR-338 promoted the formation of only MBP⁺ extensions on fibers (Figure 2.7). The inclination to form structures that are indicative of myelin sheaths by wrapping around the fibers rather than spreading membranes on the glass surface could be a pro-myelination indicator. Future studies could elucidate the mechanistic differences between flat myelin membrane and myelin sheath formation by OLs under the influence of miR-219/miR-338. In addition, ultrastructural analyses using transmission electron microscopy could also provide useful insights into the effects of miR-219/miR-338 on the integrity and structure of the formed myelin.

Although *in vitro* CNS myelin sheath length is an intrinsic property of OL ⁵¹, this “myelinogenic potential” ¹⁰³ of OL can be influenced by the underlying substrates. Firstly, substrates can alter this potential by activating signalling molecules through a surface receptor. Specifically, coating fiber substrates with laminin to reconstruct physiological OL-ECM interactions promoted the formation of significantly more myelin sheaths per OL ⁵¹. In this work, we presented distinctive mechanism on how substrates could also be employed to

deliver molecules into OPCs/OLs and remodel their myelinogenic potential. Specifically, our substrate-mediated delivery of miR-219 and miR-338 changed how OLs spread their membranes on glass or fibers (Figure 2.7-2.9). The diverse mechanisms underlined the versatility of electrospun substrates in controlling myelination and reinforced the notion that our microfiber-substrate could serve as an effective functional testing platform for OL myelination.

Apart from serving as an *in vitro* platform for understanding myelination, electrospun fibers may also be designed into directly implantable substrates for *in vivo* nerve regeneration applications, such as in the case of spinal cord injury treatment ⁶⁰. In particular, the biomimicking topography of electrospun fibers provides contact guidance to nerve regeneration while the sustained delivery of drugs and genes from these scaffolds offers synergistic biochemical signaling to direct cell fate and tissue regrowth ⁶⁰. Thus, promising drugs or genes as assessed on these fiber platforms may be directly translated to *in vivo* testing. This avoids the need to redesign a new implantable platform that carries the promising drug/gene; hence speeding up the process of drug development.

PDL coating as expected, creates slight positive charges on the fiber, likely due to polar interactions with water. On the other hand, dopamine is known to undergo oxidative polymerization. Further oxidative polymerisation is a likely cause of more negative potential, which impedes *in-vitro* myelination. Nonetheless, longer dopamine coating likely allows more PDL to adhere, thereby creating more positive charges. Though this was not sufficient to

restore the myelin sheaths on PCL fibers. Highly charged surfaces created by TKO coating restores the myelin permissiveness of the fiber substrate, likely due to favourable electrochemical environment. This understanding of the interaction between surface charges and oligodendrocytes would be crucial to engineer fibrous substrates capable of robust *in-vitro* myelination.

2.5 Conclusion

In the present study, we developed a microfiber platform for *in vitro* myelination study and functional screening of drugs/genes. Using miR-219 and miR-338 as the model drugs, we showed that these miRs promoted OPC differentiation on our microfiber constructs. Moreover, we also showed that the miRs promoted the ensheathment of fibers instead of the formation of flat myelin membranes. OLs transfected non-virally with miRs also possessed longer nascent myelin sheaths and more MBP⁺ extensions per cell. This platform could be employed for further drug/gene testing and translation into *in vivo* remyelination scaffold design.

Chapter 3: Evaluating Potential of Mesoporous Silica Nanoparticles as RNA Transfection Reagent for Neural Cells

Summary

Chapter 2 highlights the efficacy of microRNA in promoting oligodendrocyte differentiation and myelination. The successful implementation of such regenerative strategy depends upon the availability of low toxicity, efficient and versatile delivery system. However, conventional transfection reagents are inflexible in its design, often could only be used to deliver a single type of payload. On the contrary, mesoporous silica nanoparticle (MSN) has been widely used due to its biocompatibility and customizable properties. We investigated the potential of substituting commercially available transfection reagent in our drug/gene delivery system with MSN. As a proof of concept, we delivered cyclophilin B siRNA (siPPIB) using polyethylenimine-conjugated MSN (MSN-PEI) for gene silencing neural cells, which are known to be difficult to transfect. We show that siRNA-MSN-PEI complexes are efficiently up-taken by neurons, astrocytes, neural progenitors and oligodendrocytes precursor cells (OPCs). Bolus delivery of siRNA-MSN-PEI complexes could deliver similar gene silencing efficacy as conventional transfection reagents.

3.1 Introduction

Central Nervous System (CNS) regeneration following traumatic injuries or diseases has been subpar. Regeneration is often hindered by the limited regenerative capacity of the CNS neurons and glia cells in the native inhibitory microenvironment. One strategy to promote regeneration is to enhance the regenerative capacity of CNS cells by delivering pro-regenerative molecules directly to neurons, neural progenitor cells (NPCs) and OLs ^{91,104,105}.

However, activated astrocytes glia scar forms a formidable barrier for CNS neurons to regeneration. Regenerative therapy could be accompanied by digestion of glia scar to promote robust nerve regenerations. Thus, effective delivery of drug/gene to all neural and glia cells could be key to better CNS tissue regrowth.

RNA interference (RNAi) mechanisms play a crucial role during CNS development and diseases. Specifically, microRNA (miR) expression has been well characterized in the rodent central nervous system during development ¹⁰⁶, indicating the importance of RNAi in this phase of growth. Moreover, dysregulation of miR has also been linked to disorders, further illustrating the significance of RNAi in the CNS ¹⁰⁷. Given the crucial role of RNAi, significant advances have been made to develop RNAi therapies for CNS ¹⁰⁸. Despite signs of progress in RNAi therapies in recent years, challenges still remain on how to effectively delivery small-interfering RNA (siRNA) and miR to neural cells ¹⁰⁹.

Delivery of RNAi molecules has conventionally been achieved with viral vectors, electroporation, cationic liposomes or polymers ¹⁰⁹. Viral vectors could achieve stable gene transfections and long term expression of RNA. However, concerns on immunogenicity and mutagenesis remain. Electroporation has low transfection efficiency. High efficiencies are often achieved at the expense of low cellular viability ¹⁰⁹. Similarly, commercially available cationic liposomes or polymers exhibit a strong correlation between high efficiency and high toxicity ¹¹⁰. Thus, there is an urgent need to develop a highly efficient, non-toxic and highly versatile transfection vehicle.

Mesoporous silica nanoparticles (MSNs) have emerged as a prime candidate for drug/gene delivery due to its biocompatibility, high efficiency and high tenability. As such, MSNs have been widely adopted in many tissue engineering applications ¹¹¹⁻¹¹³. The highly porous structure of MSN enables high drug loading due to large surface area and the presence of active groups on the surfaces support versatile functionalization to allow controlled drug/gene delivery ¹¹⁴. In particular, positively charged groups such as polyethyleneimine (PEI) could be conjugated on MSN surfaces for effective RNA delivery and uptake ¹¹⁵. However, applications of MSN-mediated RNA interference in the CNS remain limited ¹¹³.

In this study, we investigated the efficacy of MSN in delivering RNA to neural and glia cells for RNAi. Specifically, using cyclophilin B siRNA (siPPIB) as the model RNA, we examined the feasibility of using PEI-modified MSN to deliver siRNA to all major neural cell lineages. We characterized the siRNA/MSN-PEI complexes to be used in transfection and examined the efficiency of scaffold-mediated delivery of these complexes. Using suspended microfiber platform developed in chapter 2, which mimics the architecture of the natural extracellular matrix and allows easy imaging and quantification of neural cells, we study the cellular uptake of siRNA/MSN-PEI complexes by neurons, neural progenitor cells (NPC), OPCs and astrocytes. We also study the gene silencing efficacy of siRNA/MSN-PEI complexes in both bolus delivery and scaffold-mediated delivery. We hypothesize that MSN-PEI could be an alternative to conventional transfection reagents for RNAi in neural tissue engineering applications.

3.2 Materials and Methods

3.2.1 Materials

Polycaprolactone (PCL, Mw: 200,000) was purchased from PolySciTech. Polycaprolactone (PCL, Mw: 45,000), 2,2,2-trifluoroethanol (TFE), dopamine hydrochloride, Dnase 1 type IV, Poly-D-Lysine (PDL), N-Acetyl-L-Cysteine (NAC), D-biotin, ITS, Putrescine, L-Thyroxine (T4), Tri-iodothyronine (T3), Progesterone, Bovine Serum Albumin (BSA), L-cysteine, Triton X-100, triethanolamine (TEA), branched PEI, cetyltrimethylammonium p-toluenesulfonate (CTAT), poly-L-ornithine were purchased from Sigma-Aldrich. Scramble negative control siRNA, siPPIB (s10908), siREST (s11932), secondary antibodies, minimum essential media (MEM), DMEM High Glucose, Penicillin/Streptomycin (Pen/Strep), B21, N2, lipofectamine RNAimax and natural mouse laminin were purchased from Life Technologies. NS21 supplement and Neuro Medium were purchased from Miltenyi Biotech. Rabbit anti-Oligodendrocyte Transcription Factor 2 (Olig2, AB9610) Chicken anti-Glial Fibrillary Acidic Protein (GFAP, AB5541), Mouse anti-Nestin (Nestin, MAB5326) were purchased from Merck. Mouse anti-Tubulin β III (Tuj1, MAB5564) was purchased from Biolegend. Papain suspension was purchased from Worthington. Cy5-labeled double-stranded oligonucleotides (cy5-RNA) was purchased from IDT. Tetraethoxysilane (TEOS), bis[3-(triethoxysilyl)propyl]tetrasulfide (BTES), and 1,4-dithio-DL-threitol (DTT, 98%) were obtained from Alfa Aesar. SingleShot SYBR Green One-Step Kit was purchased from Bio-rad.

3.2.2 Fabrication and characterization of suspended microfiber substrate

Chapter 2 illustrates the engineering of the suspended microfiber platform. Briefly, 20 μ m PCL films were then placed on a 15-mm diameter glass coverslip and melted briefly on a

hotplate at about 50 °C. These glass coverslips were then pasted onto a rotating wheel with carbon tape. For electrospinning of microfibers, PCL (MW: 200,000) was dissolved in TFE to make a 7 wt% solution. Thereafter, the homogeneous solution was loaded into a syringe and dispensed at a fixed rate of 2 ml/h by a syringe pump (New Era pump system Inc., USA) through a 22G needle. Positive 4 kV and -4 kV voltages (Gamma High Voltage, USA) were then applied to the needle and rotating collector (730 rpm) respectively. The distance between the needle and the collector was kept at 11.5 cm. After the fibers were collected, PCL films were melted briefly again to allow the fibers to stick to the PCL films. Thereafter, the dried suspended microfiber scaffolds were secured by applying a layer of silicon glue around the circumference of each coverslip.

3.2.3 Synthesis of PEI-modified mesoporous silica nanoparticles (MSN-PEI)

MSN-PEI was synthesized according to a previously reported method¹¹³. Briefly, CTAT and TEA were dissolved in water and stirred at 80 °C for 2 h. Following which, a mixture of BTES and TEOS was added dropwise and reacted at 80 °C for 4 h. Subsequently, the solution was cooled to room temperature while stirring. The product was collected by centrifugation, washed with ethanol and water three times and dried under vacuum. Then, sample was extracted thrice with a solution of HCl (37%) and ethanol by refluxing at 80 °C for 12 h to efficiently remove the CTAT template. Finally, the nanoparticles were washed with water, and dried under vacuum.

The obtained MSNs were dispersed in toluene and sonicated for 90 min. After that, 3-GPS was added, and the mixture was stirred for 2 h at room temperature. Then, the reaction solution was refluxed at 80 °C for 24 h. The product was separated by centrifugation and washed with ethanol and water before lyophilisation to obtain Epoxysilane-functionalized MSN powder. Epoxysilane functionalized MSN was then suspended in pure water by sonication before adding PEI solution at room temperature for 24 h. PEI-modified MSNs were then centrifuged and wash with water.

3.2.4 Preparation of MSN-PEI Complexes and Reverse Transfection Fibers

Stock solution of MSN-PEI was sonicated for 15 min prior use. Sonicated stock solution was further diluted in RNase-free water and sonicated for further 15 min. Cy5-RNA and siRNA were reconstituted to a stock solution of 100 μ M. MSN-PEI and RNA were mixed at various mass ratios (1:5, 1:10, 1:20, 1:40, 1:80) for 15 min complexation. 0.2 μ g of RNA and 1.0 μ g of RNA were used for bolus transfection and scaffold-mediated transfection respectively. As a control, 1 μ g of RNA was complexed at manufacturer recommended ratio with the transfection reagent lipofectamine in 100 μ l of DMEM.

For scaffold-mediated transfection, the microfiber substrates were sterilised and wetted with 70% ethanol for 15 min. The substrates were then incubated in 1 ml of 0.5 mg/ml dopamine hydrochloride solution for 4 h on an orbital shaker to form the polydopamine (PD) coating. After 3 washes with DI water, the fibers were further coated with 2 ml of 5 μ g/ml

PDL overnight and washed 3 times with DI water. The complexes were then added onto the PDL coated fibers and incubated at 37 °C for an hour.

3.2.5 Neuronal and Glia Cell Culture

The isolation of primary neurons, OPCs and astrocytes from rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. The isolation and culture of primary OPCs was carried out according to previously reported in chapter 2. Briefly, P0-P2 neonatal rat cortices that were cleaned from meninges were enzymatically digested with 1.2 U Papain and 40 µg/ml DNase at 37 °C for 1 h. Thereafter, the enzymatic activity was stopped with 8 ml of DMEM with 10% Fetal Bovine Serum (FBS) and the dissociated tissues were triturated with a 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 OPCs were separated from the mixed glia culture by shaking on an orbital shaker at 200 rpm for 1 h at 37 °C to remove the loosely attached microglia. This was followed by a further 16-17 h of shaking. OPCs were then further purified by differential adhesion on untreated petri dishes for 25 min. Purified OPCs were then seeded at a density of 50,000 onto each suspended fiber substrate, and cultured in myelin medium, which consisted of DMEM:Neurobasal (50:50), NS21, 1% Pen/Strep, Glutamax, 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).

For primary rat cortical neurons, rat cortices were isolated and digested as above. Thereafter, the dissociated tissues were triturated carefully with a 1 ml pipette. Purified cortical neurons were then seeded at a density of 50,000 onto each suspended fiber substrate and cultured in DMEM:Neurobasal (50:50), 2% B21, 10% FBS, 1% Pen/Strep and Glutamax. For primary rat astrocytes, OPCs culture flasks were further shake overnight to remove most of the attached OPCs. 0.1% trypsin was then used to remove the astrocyte layer for seeding⁹⁶. Rat astrocytes were cultured in DMEM, 10% FBS, 1% Pen/Strep.

Human NPCs were cultured as previously reported¹⁰⁴. Briefly, Human NPCs were derived from hiPSCs using a Neuro EB protocol. NPCs were cultured on poly-L-ornithine and laminin coated culture dishes in maintenance medium. The maintenance medium consisted of DMEM/F12 with 1% Glutamax, 1% N2 supplement, 2% B27 supplement, 1% pen/strep and 20 ng/ml FGF2. The cell culture medium was changed every 2 days and P7–P8 cells were used for seeding onto the suspended-fiber structure.

3.2.6 Immunofluorescent staining

For cellular uptake, after 24 h, the cultures were fixed with 4% paraformaldehyde (PFA). The fixed fiber cultures were then permeabilised with 0.1% Triton-X for 15 min at room temperature. Next, the samples were incubated with primary antibodies overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature. The primary antibodies used were: rabbit anti-Olig2 to label OPCs, mouse anti-Tuj1 to label cortical neurons, chicken anti-GFAP to label astrocytes and mouse anti-Nestin to label NPC. The respective antibodies used

were at 1:1000. To quantify cellular uptake of cy5-RNA, 488-phalloidin was used to mark out the cellular shape to identify neural cells that have successfully intake cy5-RNA. The nuclei were counterstained with DAPI. The samples were then mounted onto 60 mm coverglass with Fluoromount-G and imaged under a Zeiss LSM 800 confocal microscope.

3.2.7 *In vitro* Gene Silencing and qPCR

To evaluate gene silencing after siRNA/MSN-PEI delivery, RNAs from the cell cultures were extracted after 3 days to detect significant changes in gene expressions. RNA isolation and qPCR were performed with SingleShot SYBR Green One Step Kit from Bio-rad according to manufacturer protocol. Briefly, medium was removed from cells on suspended fiber scaffold and washed with DPBS. Following which, 50 μ l of cell lysis buffer mix (48 μ l lysis buffer, 1 μ l Proteinase K and DNase solutions) was added to each culture on suspended fiber and incubated for 10 mins. Cell lysate was collected and put into thermal cycler for 5 min at 37 °C and 5 min at 75 °C. Meanwhile, a mixture of SYBR Green reaction mix, reverse transcriptase and water were added to individual PCR wells accordingly. Cell lysate was then added to the qPCR reaction mix and put into StepOne qPCR machine for transcription reaction, polymerase activate & DNA denaturation and annealing/extension reactions with melt curve analysis. Table 3.2.1 shows the primer sequences used for qPCR reactions. Primers were verified to have similar amplication efficiency. Thus, $\Delta\Delta C_t$ method was used to evaluate changes in gene expression.

Target	Sequence (5' to 3')
hPPIB Forward	TGTGGTGTGGCAAAGTTC
hPPIB Reverse	TTTATCCCGGCTGTCTGTCT
h18S RNA Forward	AACGAGACTCTGGCATGCTAACT
h18S RNA Reverse	CGCCACTTGTCCCTCTAAGAA

hREST Forward	TGGAAAATGCAACTATTTTTCAGA
hREST Reverse	GAAC TTGAGTAAGGACAAAGTTCACA
rPPIB Forward	TCGGAGCGCAATATGAAGGT
rPPIB Reverse	CTTCTTCTTATCGTTGGCCACG
rB-actin Forward	ACGGTCAGGTCATCACTATCG
rB-actin Reverse	TGCCACAGGATTCCATACCCAG

Table 3.1: Sequences of primer used for qPCR

3.3 Results

3.3.1 Characterizations of MSN-PEI complexes

Figure 3.1 shows the amount of RNA that could be complexed with a fixed weight of MSN-PEI. Ethidium bromide acts as a RNA intercalating agent to visualise RNA location after gel electrophoresis. Free RNA moves down the channel rapidly due to its small size and form a bright band at the bottom end of the gel. Much heavier and larger MSN-PEI remained in the well of the gel together with the amount of RNA that has complexed to it. Bright RNA bands observed at low RNA/MSN-PEI w/w ratio indicating insufficient amount of MSN-PEI to fully complex the excess RNA, allowing some free RNA to escape down the channel. RNA/MSN-PEI weight ratios above 1:20 no longer produce RNA bands at the bottom of the gel. Thus, a minimum weight ratio of 1:20 is required to fully adsorb all RNA to MSN-PEI.

Table 3.2 shows the particles size and zeta potential data of the RNA/MSN-PEI complexes above the minimum weight ratio. Presence of RNA on MSN-PEI surfaces increased the overall size of complexes initially. However, increasing RNA:MSN-PEI ratio was correlated with decreasing size of complexes. Higher amount of RNA also decreased the zeta potential of MSN-PEI complexes.

Figure 3.1: Gel electrophoresis data of RNA/MSN-PEI complexes. Negative control microRNA was used as a model RNA for complexation study. A lower amount of free-RNA escaped down the gel with an increasing mass of MSN-PEI. At least 20 times the weight of MSN was required to fully adsorbed all RNA.

	MSN only	1:20 w/w	1:40 w/w	1:80 w/w
Particles Diameter (nm)	188.18	202.26	190.02	164.85
Zeta Potential (mV)	0.700	-0.242	-1.16	-1.4162

Table 3.2: Particle sizes and zeta potential data of RNA/MSN-PEI Complexes from Dynamic Light Scattering. Presence of RNA increases the overall complexes size initially. Higher RNA to MSN-PEI ratio decreased the overall particle size thereafter. RNA adsorbed on MSN-PEI also decreased the zeta potential of complexes.

3.3.2 Cellular Uptake of MSN-PEI Complexes

Cy5-RNA, or Cy5-tagged oligonucleotides (cy5-ODN) was employed to investigate the efficacy of neuronal and glia cellular uptake of these complexes. Cortical neurons, OPCs/OLs, NPCs and astrocytes were verified to be viable after cy5-ODN transfections with Tuj1, Olig2, Nestin and GFAP respectively (Figure 3.2). To better visualise the cellular shape of OPCs, NPCs and astrocytes, phalloidin was used to label their actin cytoskeleton.

To investigate the transfection efficiency of MSN-PEI complexes on all four neuronal and glia cells, we delivered cy5-ODN to the cells at 1:40 and 1:80 weight ratios to ensure all free RNAs were adsorbed onto MSN-PEI particles. The complexes were dropped directly into the culture for bolus transfection or loaded onto suspended fiber substrates prior to cell seeding for scaffold-mediated transfection. Generally, a higher mass ratio of MSN-PEI enabled more efficient transfections as observed by the cy5 signal within the cells in the 3-dimensional confocal view (Figure 3.3). Uptake of complexes varied widely among the four cell types. Post-mitotic, matured neurons and astrocytes have poor uptake of RNA/MSN-PEI complexes while other mitotic, precursor cells have a higher percentage of cells with cy5 signal inside its cytoplasm (Figure 3.3B).

Figure 3.2: Neuronal and glia lineage cell culture viability following MSN-PEI transfection. Neuronal and glia cells remain viable on suspended fiber substrates and expressed its marker (GFAP for Astrocytes, Nestin for NPCs, Olig2 for OPCs and Tuj1 for Neurons). Since Tuj1 labels the tubulin of neurons, no cytoskeleton stain with phalloidin was necessary.

Figure 3.3: Cy5-ODN/MSN-PEI complexes uptake efficiency. (A) Representative confocal images show the uptake of complexes by post-mitotic and mitotic cells. Post-mitotic and matured astrocytes and neurons show limited uptake at low MSN-PEI ratio. Mitotic OPCs and NPCs show efficient uptake of complexes in most conditions. (B) Quantitative data show the percentage of cells with cy5-ODN/MSN-PEI complexes uptake. Post-mitotic cells have significantly lower uptake of complexes with neurons being the most difficult to transfect. Nonetheless, high MSN-PEI to RNA ratio still enables sufficiently high cy5-ODN uptake through bolus transfection. At least 30 cells were imaged at high confocal magnification in each group.

3.3.3 Gene Silencing by MSN-PEI Complexes

To investigate the gene silencing efficacy of MSN-PEI complexes, we targeted PPIB as the model gene with siRNA. Astrocytes were used as the model for matured CNS cells, while NPCs were used to as the model for young progenitors CNS cells. To achieve the highest transfection efficiency, the highest MSN-PEI mass ratio was used. Concurrently, scaffold-mediated delivery of complexes was performed in two ratios to examine the efficacy of

localized delivery. In agreement with cellular uptake data, matured CNS astrocytes have low gene silencing even with commercial lipofectamine RNAiMax. However, MSN-PEI showed similar gene silencing outcome to the commercial transfection reagent. On the other hand, neural progenitors showed at least 50% silencing of PPIB, with MSN-PEI also achieved a similar silencing outcome in comparison with commercial transfection reagent. However, scaffold-mediated delivery of MSN-PEI complexes showed limited gene silencing in all cell types (Figure 3.4A).

We ask if MSN-PEI could be employed to silent gene of low abundance. We compared the gene silencing outcome achieved by MSN-PEI complexes with previously established protocol in RE-1 silencing transcription factor (REST) silencing⁵⁶. Commercial transfection reagent achieved at least 50% silencing of REST, a level matched by bolus delivery of siREST/MSN-PEI complexes. However, scaffold-mediated delivery of siREST by MSN-PEI remained inefficient (Figure 3.4B).

Figure 3.4: siRNA/MSN-PEI complexes gene silencing efficacy in neural cells. (A) Quantitative data showing fold change in PPIB expression obtained through $\Delta\Delta C_t$ approach following transfection. Matured astrocytes show inefficient knockdown of PPIB regardless of transfection reagent used while NPCs show significant PPIB knockdown with both lipofectamine and MSN-PEI with bolus transfection. MSN-PEI show similar efficiency as lipofectamine in gene silencing outcome. ** $P < 0.01$, *** $P < 0.001$, One-way ANOVA. Error bar showing S.E.M. with 3 biological repeats for astrocytes and 2 biological repeats for NPC. (B) Quantitative data showing fold change in REST expression obtained through $\Delta\Delta C_t$ approach following transfection. Bolus delivery of siREST with MSN-PEI achieved the same level of gene silencing as commercial lipofectamine. Gene silencing through scaffold-mediated delivery of siREST with MSN-PEI remained poor. Error bar showing S.E.M. with 2 biological repeats.

3.4 Discussions

Transfection of neural cells is notoriously difficult due to its sensitivity to physical stress and other changes in the microenvironment ¹⁰⁹. The lack of an established method to deliver plasmid, siRNA and other drug/gene hinders studies of many aspects of neuronal cell biology ¹⁰⁹. More recently, this limitation has extended to neural tissue engineering, impeding our progress in finding therapeutics for nervous system injuries and disorders. Thus, finding an optimal transfection protocol for neural and glia cells could dramatically widen the range of tool in our disposal to address neurological injuries and diseases. Although commercially available transfection reagents have improved drastically over the past years, it has a key drawback in the lack of versatility. Hence, MSN emerged as a promising alternative. We experimented on the use of PEI-modified MSN in bringing RNA to major neuronal and glia cell lineages. As a proof-of-concept, we employed MSN-PEI with model gene PPIB or fluorescent-tagged RNA to study the efficacy of gene silencing and uptake in these cells.

Transfection reagents carry net-positive charges to enable the easy crossing of negatively charged lipid bilayer for efficient transfections. In the context of MSN, positively-charged groups were conjugated onto MSN for complexation with negatively charged RNA ¹¹⁵. However, high positive charges are known to induce cytotoxicity ¹¹⁶, especially to environmental-sensitive neural cells. Thus, MSN-PEI in our study carries low positive charges, sufficient to complex with RNA for gene delivery and minimised cell death. Interestingly, an increasing ratio of MSN-PEI to RNA decreases the overall size of the complex (Table 3.2). This is likely due to a lower amount of RNA adsorbed onto each MSN-PEI particle.

We investigated the efficacy of siRNA/MSN-PEI complexes through cellular uptake and gene silencing in all major neuronal and glia lineage cells. These cells could be classified into precursor cells (NPCs and OPCs) and mature cells (astrocytes and neurons). The developmental stages of a cell could heavily influence transfection efficiency. For instance, the transfection efficiency of post-mitotic and differentiated cells like neurons is often lower than cells undergoing active mitosis ¹⁰⁹. This phenomenon was also observed in MSN-PEI-mediated transfection. In comparison with NPCs and OPCs, astrocytes and neurons have a significantly lower percentage of cells with RNA uptake (Figure 3.3). The lower uptake was translated to lower efficacy of gene silencing (Figure 3.4). Thus, the results highlight the challenge of delivering effective gene therapy in CNS.

The conventional approach of delivering drug/gene involved bolus delivery *in vitro* and systemic delivery *in vivo*. However, such delivery methods are suboptimal, especially when sustained and long-term drug/gene delivery is vital ¹¹³. Thus, scaffold-mediated, localized delivery of drug/gene is an attractive alternative. We have previously detailed effective reverse transfections of siRNA and microRNA with commercial transfection reagents in many neuronal cell types ^{56,58,91}. However, the efficacy of reverse transfection with engineered nanoparticles on neural cells is not known. Surprisingly, while cy5-RNA cellular uptake and bolus delivery gene silencing efficiency remains high, siRNA gene silencing with reverse transfection is poor (Figure 3.2 and 3.3). The results point to possible stability issue with engineered nanoparticles. Interactions between MSN-PEI and scaffolds during the particle loading and release process have to be further examined in future work.

3.5 Conclusion

In the present study, we investigated the efficacy of using engineered MSN-PEI to deliver RNA to major neuronal and glia cells. RNA could be effectively adsorbed onto MSN-PEI to form a stable complex and be delivered to neurons, astrocytes, NPCs and OPCs. We found that bolus delivery of MSN-PEI to be as effective as commercial transfection reagents in achieving high gene silencing efficacy in neuronal and glia cells. Thus, an easily modifiable and versatile MSN could be a potential drug/gene delivering agents to be used in the CNS.

Chapter 4: Effect of Structural Stiffness of Oligodendrocyte Myelination

Summary

Oligodendrocyte (OL) myelination, a critical process where OLs produce multilamellar wraps around axons, is necessary for the efficient and rapid transmission of action potentials in the central nervous system (CNS). However, the effect of microenvironment mechanical stiffness on myelination is poorly understood. Here, we report an artificial axon platform with tunable stiffness that decouples the microenvironmental stiffness from the influence of axonal calibre and topography. We verified that this system replicates the established trend of OL differentiation on two-dimensional hydrogel surfaces. Moreover, we show for the first time, that compliant microenvironment enhances OL myelination, independently and distinctively from OL differentiation. We identify Yes-associated protein (YAP), a critical nuclear relay of mechanical signals, to be required to influence OL myelination through mechanotransduction. We also identified actin skeleton changes during myelination on tunable stiffness scaffolds. This work pioneered an *in vitro* platform for the understanding of the biophysical effect on OL myelination, potentially shedding new lights on the relationship between CNS tissue mechanical dysregulation and demyelinating diseases.

4.1 Introduction

Extracellular matrix (ECM) biophysical forces heavily influence embryonic development ¹¹⁷, diseases ¹¹⁸, stem cell fate and lineage specification ^{69,119,120}. These processes are termed mechanotransduction, where cells sense and respond to mechanical stimuli of the microenvironment by converting physical cues into biochemical signals to regulate cellular expression. Pathological dysregulation of the mechanical environment is

associated with cancer, diseases and traumatic injuries ^{118,121}. Thus, novel platforms that enable the understanding of the interaction between cells and mechanical microenvironment are highly valued ^{72,122}.

OLs, the neuroglia responsible for CNS myelination, are mechanosensitive. OPCs are capable of sensing the physical environment and respond accordingly to stimuli, which influence its cellular outcome ⁴⁸. It is, therefore, crucial to investigate how pathological changes in mechanical microenvironment in the CNS could influence OPCs behavior. As such, two-dimensional, tunable stiffness hydrogel surfaces were employed to establish the relationship between mechanical stiffness and OL viability, differentiation, maturation and membrane spreading ¹²³. However, the functionality of an OL is in myelination, which is characterized by the concentric wrapping around axons and is distinctively regulated from other OL processes ¹⁷. Despite the clinical relevance in the relationship between CNS tissue mechanical stiffness and myelination ¹²¹, the lack of a myelination permissive substrate with tunable mechanical stiffness handicapped the effort to investigate such association. Given the significant amount of work in enhancing remyelination for therapeutic purposes ¹⁰, it is crucial to understand how OL microenvironment mechanical stiffness could modulate myelination. Thus, there is a need for myelination permissive substrate with tunable mechanical stiffness to investigate this elusive relationship.

In vitro OLs could myelinate non-axonal substrates. Among which, electrospun microfibers are commonly used as artificial axons due to its similarity in shape and topography

to live axons ^{6,7,51}. Chapter 2 has also detailed robust myelination that closely mimics in vivo physiological myelin could be attained with electrospun fibers. This platform effectively decoupled the crosstalk between axons and OLs to investigate the direct effect of chemical or physical cues on OL myelination. Moreover, electrospun fibers also mimic the natural structure of the ECM microenvironment, providing topographical cues to influence OPC differentiation and maturation ⁵⁸. Thus, a tunable stiffness platform based on electrospun fibers would be ideal for investigating the effect of mechanical properties on OL myelination.

In this work, we engineered a tunable structural stiffness platform with electrospun poly-caprolactone (PCL) fibers as artificial axons to study the effect of structural stiffness on myelination. We controlled the structural stiffness of artificial axons through the variable length of supporting structure. Our platform effectively decoupled the effect of structural stiffness from other factors that influence OL myelination, including fiber caliber, topography and fiber density. We hypothesized that structural stiffness of the underlying substrate is necessary and sufficient to influence OL myelination.

4.2 Materials and Methods

4.2.1 Materials

Polycaprolactone (PCL, Mw: 200,000) was purchased from PolySciTech. Polycaprolactone (PCL, Mw: 45,000), 2,2,2-trifluoroethanol (TFE), Dnase 1 type IV, Poly-D-Lysine (PDL), N-Acetyl-L-Cysteine (NAC), D-biotin, ITS, Putrescine, L-Thyroxine (T4), Tri-iodothyronine (T3), Progesterone, Bovine Serum Albumin (BSA), L-cysteine, Triton X-100, and

components of NS21 were purchased from Sigma-Aldrich. Alexa-Fluor 488 goat anti-rat, Alexa-Fluor 633 goat anti-rabbit antibodies, minimum essential media (MEM), DMEM High Glucose, Penicillin/Streptomycin (Pen/Strep) were purchased from Life Technologies. NS21 supplement and Neuro Medium were purchased from Miltenyi Biotech. Rat anti-Myelin Basic Protein (MBP, aa82-87) was purchased from Bio-Rad. Rabbit anti-Oligodendrocyte Transcription Factor 2 (Olig2, AB9610) was purchased from Merck. Papain suspension was purchased from Worthington.

4.2.2 Fabrication of tunable stiffness platform

Figure 4.1 illustrates the engineering of the tunable stiffness microfiber platform. PCL pellets (MW: 45,000) were pressed into 50 μm PCL film with a heat press and cut into thin frame with customized cutter (Outer area: 11.5 cm x 1.5 cm, Inner area: 9.5 cm x 1 cm). The thin PCL film was mounted onto electrically conductive wheel for electrospinning to collect electrospun PCL fibers.

PCL pellets were also pressed into 0.5 mm thick film and cut into frames of different dimensions with customized cutter. PCL frames were used to secure fibers from the same sheet of thin film with silicon glue. Inverting the scaffold creates a 0.5 mm suspended fiber structure for OPCs seeding.

4.2.3 Scanning Electron Microscopy

The morphology of the microfibers was evaluated using scanning electron microscopy (SEM) (JEOL, JSM-6390LA, Japan). Briefly, PCL substrates were attached onto the SEM support by carbon tapes and sputter-coated with platinum for 60 s at 20 mA. Thereafter, the samples were imaged under an accelerating voltage of 10 kV.

4.2.4 Atomic Force Microscopy

The substrate bending experiments were performed using a Dimension Icon AFM (Bruker). Force curves were obtained using the peakforce quantitative nanomechanical (QNM) fluid mode of the AFM. The probe consisted of a 25 μm diameter polystyrene bead attached to a silicon nitride cantilever with a spring constant of 0.406 N/m (Novascan Technologies, Inc., Ames, IA). The suspended microfiber scaffold is fixed on both ends and immersed in PBS with 0.1% BSA added. A maximum force of 10 nN and loading rate of 8 $\mu\text{m/s}$ were applied to ensure the microfiber scaffold undergoes elastic bending. At least 8 force curves across the midspan of the suspended microfiber scaffold were obtained. The slope of the force-distance curve which corresponds to the stiffness (N/m) was extracted using the Nanoscope analysis software v1.9.

4.2.5 OPCs Isolation and Myelination Culture

The isolation of primary OPCs from rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. The isolation and culture of primary OPCs and the myelination culture were described in chapter 2. Briefly, P0-P2 neonatal rat cortices that were cleaned from meninges were enzymatically digested

with 1.2 U Papain and 40 µg/ml DNase at 37 °C for 1 h. Thereafter, the enzymatic activity was stopped with 8 ml of DMEM with 10% Fetal Bovine Serum (FBS) and the dissociated tissues were triturated with a 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 OPCs were separated from the mixed glia culture by shaking on an orbital shaker at 200 rpm for 1 h at 37 °C to remove the loosely attached microglia. This was followed by a further 16-17 h of shaking. OPCs were then further purified by differential adhesion on untreated petri dishes for 25 min. Purified OPCs were then seeded at a density of 35,000 onto each suspended fiber substrate, and cultured for 7 days in myelin medium, which consisted of DMEM:Neurobasal (50:50), NS21, 1% Pen/Strep, Glutamax, 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).

4.2.6 Immunofluorescent staining

After 3 or 14 days, the cultures were fixed with 4% paraformaldehyde (PFA). The fixed myelin-fiber cultures were then permeabilised with 0.1% Triton-X for 15 min at room temperature. Next, the samples were incubated with primary antibodies overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature. The primary antibodies used were: rat anti-MBP (1:200) and rabbit anti-Olig2 (1:1000). The secondary antibodies used were: Alexa-Fluor 488 goat anti-rat and Alexa Fluor 633 goat anti-rabbit (both at 1:1000). For F-actin and G-actin visualisation, samples were incubated with Alexa Fluor 633 Phalloidin and Alexa Fluor 594 Deoxyribonuclease I respectively for an hour in room temperature following triton treatment. The nuclei were counterstained with DAPI. The samples were then mounted

onto 60 mm coverglass with Fluoromount-G and imaged under a Zeiss LSM 800 confocal microscope. Myelin sheaths were examined by scanning through 40x, 0.45 μm z-stack images acquired by confocal microscopy. Each sheath is defined as continuous tube of MBP signals along the microfiber.

4.2.7 Statistical Analysis

All values were presented as mean $\pm$ standard deviation (S.D). Student's t-test was used to compare between 2 independent samples. Levene's test of homogeneity was used to check for homogeneity of variance when comparing 3 or more sample groups. One-way ANOVA with Tukey post-hoc test was used for samples that passed the homogeneity test. Otherwise, non-parametric Kruskal-Wallis pair-wise comparison was made. P-values of less than 0.05 were considered to be statistically significant.

4.3 Results

4.3.1 Engineering of Tunable Stiffness Microfiber Platform

Electrospun fibres mimic the size scale and architecture of the natural extracellular matrix. In the context of the CNS, electrospun fibres also mimic axons and can be used as effective in vitro artificial axon models to decouple the biochemical vs. physical effects of axons on OL myelination⁵¹. However, due to the lack of effective platform designs, it remains unknown how the mechanical properties of the microenvironment/axons affect OL myelination seen in disease state (Figure 4.1A). We utilize classical structural mechanics concepts to establish a suspended artificial axon platform that allows the systematic testing

of axon structural stiffness effects on OL myelination (Figure 4.1B). Similar to the concepts underlying the use of micropillars for traction force microscopy, the stiffness of each suspended artificial axon may be approximated by assuming each axon as a long and slender cylindrical beam that is simply supported over 2 structural supports. Hence, by continuum beam theory, the displacement, d , of a cylindrical supported beam that is subjected to a force at the midpoint of the beam, F , is given by:

$$F = \frac{48EI}{l^3} \times d = \frac{12 E \pi r^4}{l^3} \times d \quad \Rightarrow \text{stiffness}, k = \frac{F}{d} = \frac{12 E \pi r^4}{l^3}$$

Where E = Young's modulus of material from which artificial axon is made; r = radius of artificial axon; and l = length of suspended artificial axon. The stiffness of the suspended artificial axon at the midpoint, k , would be proportional to r^4/l^3 . Therefore, by choosing the appropriate radius of the artificial axon (r) and varying the length of the suspended artificial axon (l) and vice versa, this platform can provide independent control of structural stiffness, axon topography and axon diameter.

Figure 4.1: Fabrication of artificial axons with tunable structural stiffness to mimic environmental signals experienced by oligodendrocytes. (A) Axon structural stiffness (i.e. ability of axons to deflect due to stiffness of tissues and extracellular microenvironment that surrounds axons) affects oligodendrocyte myelination. (B) Schematic showing principle of tunable axon structural stiffness. By assuming each artificial axon as a simple supported beam, structural stiffness along the midline of the supported axon is related to the ratio of axon radius, r , and length of support, l .

Figure 4.2 shows the engineering of our tunable stiffness platform. As the fibrous structure would come from the same sheet of PCL film, axonal calibre and the topography of

underlying substrate are consistent across all PCL frames inserted into PCL film regardless of its dimensions (Figure 4.3). We electrospun two distinct types of fibers onto the PCL film and inserted 3 PCL frames with different length onto each type of fiber (Figure 4.2A,D). Regardless of the length of the PCL frame and types of electrospun fiber, the fiber diameter of the microfiber remains constant (Figure 4.2B,E). To ensure no changes in substrate topography that could influence cellular behavior, we measure the average deviation of the individual fibers away from the average angle as a proxy for topography. Unsurprisingly, we find no significant differences in fiber alignment regardless of the length of PCL frame (Figure 4.2C,F). Thus, this platform elegantly decouples the influence of other biophysical cues from structural stiffness to investigate cellular behavior.

Figure 4.2: Engineering of variable stiffness platform. 50 μm thick, 8 cm long PCL film was mounted onto the collector wheel to collect the electrospun fibers. 0.5 mm thick PCL frames of various sizes were cut out by customized punchers and used to secure the fibers on PCL films with silicon glue. Individual scaffolds were cut and utilized for downstream experiments.

Figure 4.3: Fibers from the same source have consistent diameter and topography in different PCL frame dimension. (A) Representative SEM images showing $1\ \mu\text{m}$ diameter fibers in 3 PCL frames length have similar fiber diameter and alignment. Scale bar represents $50\ \mu\text{m}$ (B) Quantitative data showing consistent fiber diameter in structures of different dimension in 1

μm fiber source. (C) Quantitative data showing consistent fiber alignment in structures of different dimension in 1 μm fiber source. (D) Representative SEM images showing 3 μm diameter fibers reservoir in 3 PCL frames length have similar fiber diameter and alignment. Scale bar represents 50 μm (E) Quantitative data showing consistent fiber diameter in structures of different dimension in 3 μm fiber source. (F) Quantitative data showing consistent fiber alignment in structures of different dimension in 3 μm fiber source.

4.3.2 Effect of Structural Stiffness on OPC Differentiation

To investigate the effect of changes in structural stiffness on OPC differentiation with our platform, we electrospun 1 μm diameter fibers onto PCL film and secured the same film with 3 lengths of PCL frames (1 cm, 2 cm and 3 cm). By bending the fibrous structure along the midline with a suitable force, we confirmed that longer length of PCL frame produces a more compliant structure as predicted by the aforementioned equation (Figure 4.4A). We electrospun 1 and 2 μm diameter fibers with 80,000 M_n or 200,000 M_n PCL onto PCL film and secured the fiber with the same combination of PCL frames. We confirmed that regardless of the types of fiber, the longer PCL frames continue to produce more compliant structure (Figure 4.4A). Following which, we seeded OPCs on these three variable stiffness structures. In agreement with results on two-dimensional tunable stiffness hydrogels, higher structural stiffness substrate produced by the shortest length of PCL frame resulted in the highest percentage of MBP⁺ cells after 3 days (Figure 4.4B). This suggests that higher structural stiffness of the suspended fiber promoted OPC differentiation. Thus, our engineered platform effectively altered structural stiffness according to the length of the PCL frame without affecting fiber diameter, density and topography, to influence OPC differentiation.

*Figure 4.4: Changes in structural stiffness of artificial axons microenvironment by altering PCL frame dimension. Higher structural stiffness promoted OPC differentiation. (A) Scanning Electron Microscopy images of fiber of 4 artificial axon source, structural stiffness and fiber density data with Atomic Force Microscopy. Scale bar represents 50 μm . Quantitative AFM data shows a decreasing trend in structural stiffness of artificial axons with increasing PCL frame dimension regardless of artificial fiber source. Density of artificial axons were kept constant. *** $P < 0.001$, ** $P < 0.01$. Kruskal-Wallis (B) Representative confocal images show the number of MBP+ cells on structures with the same artificial axon source but different PCL frame length. Scale bar represents 50 μm . Quantitative data show increasing structural stiffness of microenvironment with shorter PCL frame dimension increased the percentage of MBP+ cells. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ One-way ANOVA with Tukey post-hoc.*

4.3.3 Effect of Structural Stiffness on OPC Myelination

We next investigated the elusive relationship between structural stiffness and OL myelination. Using myelination permissive 1 μm electrospun fiber secured by three different lengths of PCL frames, we cultured primary rat OPCs for 14 days on the tunable stiffness structure (Figure 4.5 & 4.6). Surprisingly, while short PCL frame with high structural stiffness promoted OPC differentiation, OL myelination was impeded in high stiffness environment. OL formed fewer myelin sheaths per cell and shorter myelin sheath length on a stiffer substrate with fibers secured by shorter PCL frame (Figure 4.5A-D). Thus, in contrast to the effect of structural stiffness on differentiation, high structural stiffness impeded OL myelination on our tunable stiffness platform.

Figure 4.5: High scaffold structural stiffness impedes OPC myelination. (A) Representative confocal images showing less complete myelin sheaths and shorter sheath length with stiffer artificial axons. Scale bar represents 20 μm . (B) Quantitative data and representative images show lower average myelin sheath number per OL with increasing scaffold stiffness. ***P<0.001, One-way ANOVA, with an average of more than 90 cells pooled from 9 animals quantified per sample group. (C-D) Quantitative data showing the distribution of myelin sheath length in 3 sample groups. High artificial axon structural stiffness resulted in shorter average myelin sheath length. **P<0.001, One-way ANOVA, with a total of 494 sheaths quantified from cells pooled from 9 animals.

To confirm the observed phenomena, we look at OL at the edges of 3 cm PCL frame secured fiber. Structural stiffness is higher at the edges as it was where fibers were secured with glue, and thus less bendable. AFM verified this prediction (Figure 4.6A). Structural stiffness was at least 3 times higher at the edges and decreased as we indent nearer to the midline. We compared OL myelination at two edges and the midline of the structure (Figure 4.6). OL along the midline formed more myelin sheath per cell and longer myelin sheath, where the structure is the most compliant. Hence, these results suggest while favourable to OPC differentiation, high structural stiffness inhibits OL myelination.

Figure 4.6: High structural stiffness impedes OPC myelination within the same cell population on the same substrate. (A) Atomic force microscopy data showing V-shaped trend in variation of artificial axon structural stiffness across the platform. *** $p < 0.001$ One-way ANOVA, with

*an average of 15 randomly selected points per sample length in 3 distinct sets of scaffolds. (B) Confocal images showing more complete myelin sheaths and greater sheath length on artificial axons, where structural stiffness was lowest, i.e. along the midline of 3.0 cm PCL frame structure. Scale bar represents 20 μ m. (C-E) Quantitative data showing lower myelin sheath number per oligodendrocyte and shorter average myelin sheath length at structurally stiff ends vs structurally soft midpoint of artificial axon platform. *** $p < 0.001$, Kruskal-Wallis. A total of 487 sheaths quantified from cells pooled from 9 animals.*

To demonstrate that our platform could effectively decouple structural stiffness and axon calibre, we compared OL myelination on structures made by 80,000 Mn PCL, secured by 3.0 cm PCL frame. Two different calibre (1 μ m and 2 μ m) artificial axons in these structures have similar stiffness as measured by AFM and similar density (Figure 4.7B, D). Unsurprisingly, we found longer length of myelin sheaths in structure with higher fiber calibre (Figure 4.7A,E). As such, our platform decoupled two key determinants of OL myelination, to show that both high structural stiffness and low axonal calibre hinder myelination independently.

Figure 4.7: High artificial axons calibre promotes OL myelination independently from structural stiffness. (A) Representative confocal images show the enhanced myelination on structures with the same structural stiffness but higher fiber calibre. Scale bar represents 20 μm . (B) Quantitative AFM data showing the ability to modulate axon calibre and suspended length L to obtain platforms with similar axon structural stiffness. Samples indicated in blue

were used for understanding axon calibre effect on myelination, independent of axon stiffness effects. $**p < 0.01$, $***p < 0.001$, One-way ANOVA with Tukey post-hoc. Quantitative data shows significantly higher artificial axon diameter formed with the same PCL polymer. (C-D) Quantitative results showing larger axon calibre and similar fiber density of artificial axons. $***P < 0.001$, independent t-test. (E-F) Higher axonal calibre promotes formation of significantly longer myelin sheaths and slightly more sheaths per cells with similar axon structural stiffness, $***p < 0.001$, Independent T-test.

4.3.4 Effect of Structural Stiffness on YAP Signaling

Mechanical forces influence cellular behavior through mechanotransduction pathways. In particular, YAP/TAZ is an essential transcriptional regulator, which responds to a variety of mechanical cues such as stiffness to influence cellular outcome including OPC differentiation and peripheral myelination. We asked if our artificial axon platform could control YAP translocation to modulate CNS myelination (Figure 4.8A-C). We observed significant translocation of YAP away from OL nuclei in compliant substrates regardless of the time point studied (Figure 4.8C). This suggests that mechanical stiffness from artificial axons platform could be transduced into OL to influence the degree of myelination through YAP signaling pathway.

Figure 4.8: YAP relay mechanotransduction signals in myelinating oligodendrocytes. (A) Confocal images show structurally stiff axons promoted YAP nuclear translocation throughout oligodendrocyte differentiation (D3-D7) and myelination (D10). Scale bar represents 20 μ m. (B) Quantitative data show consistent enhanced YAP nuclear translocation on structurally stiff axons throughout OPC differentiation and myelination. * $p < 0.05$, one-way ANOVA with Tukey post-hoc test. (C) Schematic showing enhanced YAP nuclear translocation on structurally stiff axons.

4.3.5 Effect of Structural Stiffness on Actin Turnover

Actin turnover is vital for the growth of CNS myelin sheath. Crucially, actin is also a key component of cellular mechanosensing mechanism. We investigated the effect of structural

stiffness on actin assembly and disassembly process. Actin assembly drives the extension of the leading edges of myelin, while disassembly enabled compaction to form compact myelin sheaths⁸⁷. OL cultured on the compliant substrate extends the myelin along artificial axons at early time point with high F-actin content, which later disassembled into monomeric units to produce compact myelin sheaths. However, f-actin in OL on stiffness substrates failed to disassemble at a similar time point (Figure 4.9). Mechanical stiffness-induced dysregulation of actin turnover could, therefore, prevent myelin compaction, thereby resulted in shorter and fewer myelin sheaths.

*Figure 4.9: Myosin II relay mechanotransduction signals in myelinating oligodendrocytes. (A) Confocal images showing decreased F-actin expression by differentiated oligodendrocytes cultured on structurally compliant artificial axons. (B) Quantification showing a significant reduction in F/G actin ratio by oligodendrocytes cultured on structurally compliant artificial axons. ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Tukey post-hoc test. (C) Schematic showing enhanced F-actin expression on structurally stiff axons.*

4.4 Discussion

OL's unique myelination and remyelination process in the CNS are not fully understood¹⁰. Myelination is a crucial part of CNS development and remyelination following demyelination is important to achieve functional recovery. Thus, efforts have been made to improve our understanding of these two vital processes. In vitro axon-free platforms have significantly enhanced our ability to study these processes by decoupling cellular crosstalks from myelination^{6,7,51}. However, the effect of biophysical cues, specifically structural stiffness, on myelination is not known. Myelination is a remarkable process which OL produces myelin membrane of an exceptionally huge area around a single fiber¹²⁴. Due to the distinctive nature of the process, no tunable stiffness platform was developed to investigate the crucial link between stiffness and myelination. We presented our solution in this chapter. By clever engineering, we showed that our platform could effectively control structural stiffness of scaffold without changing fiber calibre, density and orientation. Thus, we have decoupled structural stiffness from other biophysical cues that could influence myelination.

Biophysical forces in the surrounding ECM microenvironment are known to affect the cellular outcome. Substrates stiffness is among the first such physical forces to be discovered, which was proven in many instances to affect the rate of cellular fate, migration, etc ^{67,119}. More recently, a novel platform was developed to prove that viscoelasticity and stress-relaxation characteristics could also regulate cellular activities ¹²⁵. It exemplified how new platforms could advance our understanding of how physical processes affect cellular development and functions. Herein, we addressed the difficulty of decoupling structural stiffness of a substrate from other biophysical cues in the important context of OL myelination. *In vitro* myelination requires artificial axons. To alter the structural stiffness experienced by OL, we developed a novel platform, which decouples structural stiffness from parameters that influence myelination (E.g. substrate diameter) (Figure 4.1). In the simplest form, the stiffness or bending of the structure could solely be dependent on the distance between two supports. As such, we suspended PCL artificial axons with various distances between supports to create a tunable stiffness platform. Stiffer or compliant substrates could be achieved simply by decreasing or increasing the distance between supports respectively. Thus, we were able to keep other determinants of myelination constant while controlling the structure stiffness.

The functionality of an OL lies in myelination to promote efficient saltatory conduction of electrical signal through an axon. Myelination is a complex and precisely orchestrated process that is regulated differently from other OL processes such as proliferation and differentiation ¹⁰. Two-dimensional tunable stiffness hydrogel could investigate the effect of structural stiffness on the early processes of OPCs including OPC viability, proliferation and differentiation ¹²⁶. However, such platforms are unable to investigate the effect of stiffness

of myelin as myelination is a 3D phenomenon. In our tunable stiffness microfiber platform, we observed that higher structural stiffness promotes OPC differentiation. This was consistent in 2 other work with hydrogel platform (Figure 4.3) ^{70,126}. 3D-printed artificial axon platform was also developed to investigate stiffness effect on myelination. Lower printed axon stiffness appeared to increase the percentage of wrapped artificial axons by oligodendrocytes ¹²⁷. However, this platform was unable to replicate the robust myelination found *in vivo*. With our unique tunable stiffness platform, we were able to observe robust myelination response to structural stiffness. Contrary to stiffness effect on OPC differentiation, we found that low structural stiffness promoted OL myelination (Figure 4.4, Figure 4.5). It could explain how active axonal grow was coincide with active remyelination ¹²⁸, as the biophysical environment required for both processes is fundamentally similar.

Our results on how stiffer microenvironment promoted OPC differentiation and compliant substrate promoted myelination could have implications on ageing and diseases. Advancement in magnetic resonance (MR) elastography enabled mapping of brain stiffness. When used in studying the effect of ageing on brain stiffness, ageing was found to be associated with decreasing brain stiffness ¹²⁹. Crucially, ageing is also associated with decreasing remyelination efficiency due to impairment in OPC differentiation ¹³⁰. Thus, decreasing in brain stiffness with age could explain the lack of OPC differentiation needed for remyelination in older multiple sclerosis patients. In multiple sclerosis, cortical remyelination is well known to be significantly more extensive than white matter ¹³¹. The higher remyelination capacity was attributed to the lack of astrogliosis in the cortex, which allowed OPC differentiation for remyelination ¹³¹. However, it is possible that while lack of astrogliosis

promoted OPC differentiation, the significantly softer cortices allowed efficient subsequent remyelination¹³². Animal models that could decouple the two intimate OPC processes *in vivo* would be crucial in explaining this possibility.

Transduction of external mechanical signal into nuclear requires two key cellular components, cytoskeleton and linker of the nucleoskeleton and cytoskeleton complex (LINC). As such, we examined changes in OL cytoskeleton and transcriptional regulator of LINC (YAP) in our tunable stiffness platform (Figure 4.7). Actin turnover plays a significant role in the extension of myelin^{86,87}. We hypothesized that this crucial process was disrupted in the stiffer environment and therefore explain the short myelin sheaths formed by OL (Figure 4.4, Figure 4.5). Indeed, we observed significantly higher F-actin ratio in OL on high stiffness substrates (Figure 4.8), suggesting dysregulation of actin turnover during myelination. Moreover, the role of YAP/TAZ in mechanotransduction is in focus due to their prominent role in responding to a variety of mechanical cues such as stiffness and geometrical constraints⁸¹. YAP/TAZ was found to have a predominant cytoplasmic expression on soft substrates but translocate into the nucleus upon increasing stiffness⁸². We observed such YAP translocation on our tunable stiffness scaffold by means of two scaffolds with distinct structural stiffness along the midline (Figure 4.5). With differences in myelination likewise observed between edges and the midline of the same scaffold (Figure 4.6), we postulate that similar YAP translocation could also be seen in this setting. Together with alterations in actin ratio, these data support our view that variable structural stiffness of our platform was transduced to the OL, which influence the degree of myelin extensions. This platform identified changes in YAP expression and actin turnover as the mechanotransduction pathways associated with structural stiffness.

However, future work could dive into the detailed mechanism by investigating how inhibition of these two pathways could influence the pro-myelination effect of compliant substrates.

4.5 Conclusion

In the present study, we engineered a tunable stiffness microfiber platform to investigate the effect of structural stiffness on OL myelination. Our platform effectively decoupled structural stiffness from other biophysical cues to allow simple study of structural stiffness effect on OL. We found that our tunable stiffness platform has a similar effect to conventional tunable stiffness hydrogel in OPC differentiation. We also showed that, for the first time, lower structural stiffness promotes OL myelination. We identified YAP, a crucial transcriptional regulator of mechanotransduction and rate actin turnover as a possible explanation to the differences in myelination observed in tunable stiffness scaffolds. This study showed that our tunable stiffness microfiber platform could be a crucial tool to develop a deeper understanding of the complex OL myelination process.

Chapter 5: Effect of Intrinsic Stiffness of Oligodendrocyte Myelination

Summary

High extracellular matrix stiffness prevents deflection of embedded axons, contributing to high structural stiffness. Chapter 4 highlighted that with clever engineering, we could decouple this structural stiffness from other parameters to investigate the effect of structural stiffness on OL myelination. However, axonal intrinsic stiffness, another important aspect of mechanical stiffness that could influence OL myelination, was not addressed. Axonal intrinsic stiffness measures its resistance to deformation, which could be affected by changes in axon cytoskeleton during aging and neural diseases. In this chapter, we employed a unique crystallinity property of poly-lactic acid (PLA) to mimic changes in axonal cytoskeleton, which influence the intrinsic stiffness of axons. We show that intrinsic stiffness of artificial axons could be controlled by the ratio of PLA isomers. When built into suspended artificial axons, we continued to observe impairment in OL myelination with high structural stiffness regardless of intrinsic stiffness of artificial axons. Similarly, we observed that high artificial axon intrinsic stiffness impairs OL myelination.

5.1 Introduction

Neuronal axons, like most eukaryotic cells, have actin filaments, microtubules and intermediate filaments as the three main types of cytoskeletal polymer. Collectively, they resist deformation in response to external forces, arranging intracellular compartments, drive changes in cell shape and cellular migration¹³³. Crucially, these cytoskeletal elements influence the mechanical properties of the axons¹³⁴, potentially affecting cellular processes that require close physical contact between different cell types, such as oligodendrocytes (OL)

myelination. OL myelination of axons is a complex process that is heavily influenced by biophysical cues ⁶. However, how changes in the mechanical properties of individual axons affect OL myelination is not known.

Poly-lactic acid (PLA) is a well-characterized synthetic polymer that is widely employed in tissue engineering due to its biocompatibility and biodegradability ¹³⁵. More recently, electrospun PLA fiber was successfully used as artificial axons for robust *in vitro* myelination ⁵¹, which shows the potential of PLA fiber as a platform to investigate the effect of mechanical properties on OL myelination. PLA is a chiral polypeptide with two enantiomers, P-D-LA and P-L-LA. The crystallinity of PLA structure is dependent on the stereochemical makeup of the two lactic acid enantiomers, which in turn affects the mechanical properties ¹³⁶. By controlling the ratio of enantiomers in PLA, we could engineer a tunable stiffness single fiber with the same surface chemistry, fiber diameter, etc.

In Chapter 4, we investigated the effect of structural stiffness on OL myelination. Using electrospun PCL fibers, we mimic the embedment of axons in different ECM stiffness to investigate the effect of structural stiffness on OL. We found that while highly stiff structure promoted OPCs differentiation, high structure stiffness impaired myelination. This echoed the lack of remyelination in stiff white matter and robust remyelination in softer gray matter during multiple sclerosis ^{35,132}. However, the ability of an axon to be deflected, as measured by axonal structural stiffness, may not fully account for the effect of stiffness on myelination. Aging and neural diseases change axon cytoskeleton and viscosity ¹³⁷. Since myelination

involved concentric wrapping on single axon, ease of axonal deformation, as measured by axonal intrinsic stiffness, could heavily influence the myelin wrapping process.

In this chapter, we investigated the effect of axon intrinsic stiffness on OL myelination. We engineered two types of artificial axons with a different ratio of PLA enantiomers to create fibers with different crystallinity. An equal mixture of PLLA and PDLA (PLA-50) created more amorphous PLA microfiber while optically pure PLLA (PLA-94, 94% PLA with 6% PDLA) created semi-crystalline PLA microfiber. Higher crystallinity contributed to higher intrinsic stiffness of the artificial axons¹³⁶. Our platform effectively decoupled the effect of intrinsic stiffness from surface chemistry, fiber calibre and structural stiffness to study the effect of single axon mechanical properties on OL myelination. We hypothesized that individual axonal mechanical property could influence the myelin wrapping process, similar to mechanical properties of the entire structure.

5.2 Materials and Methods

5.2.1 Materials

Polycaprolactone (PCL, Mw: 200,000) was purchased from PolySciTech. Polycaprolactone (PCL, Mw: 45,000), 2,2,2-trifluoroethanol (TFE), Dnase 1 type IV, Poly-D-Lysine (PDL), N-Acetyl-L-Cysteine (NAC), D-biotin, ITS, Putrescine, L-Thyroxine (T4), Tri-iodothyronine (T3), Progesterone, Bovine Serum Albumin (BSA), L-cysteine, Triton X-100, Benzyl alcohol, tin(II)2-ethyl-hexanoate (Sn(Oct)₂, 95%), dichloromethane, diethyl ether, tetrahydrofuran (THF) and components of NS21 were purchased from Sigma-Aldrich. Alexa-

Fluor 488 goat anti-rat, Alexa-Fluor 633 goat anti-rabbit antibodies, minimum essential media (MEM), DMEM High Glucose, Penicillin/Streptomycin (Pen/Strep) were purchased from Life Technologies. NS21 supplement and Neuro Medium were purchased from Miltenyi Biotech. Rat anti-Myelin Basic Protein (MBP, aa82-87) was purchased from Bio-Rad. Rabbit anti-Oligodendrocyte Transcription Factor 2 (Olig2, AB9610) was purchased from Merck. Papain suspension was purchased from Worthington. D,L-lactide and L,L-lactide were purchased from Purac.

5.2.2 Synthesis of Poly-lactic Acid

PLA-94 and PLA-50 were synthesized through ring opening polymerization as reported previously^{138,139}. Briefly, predetermined amounts of dry D,L-lactide, L,L-lactide, benzyl alcohol and tin(II) 2-ethylhexanoate (0.1 molar % with respect to lactic acid units) were introduced into a dry flask. After five argon/vacuum alternations to remove oxygen, the flasks were sealed and placed 5 days under rotation at 130°C to allow polymerization. Then, the polymers were solubilized in dichloromethane and precipitated in cold diethyl ether. Finally, the product was dried under reduced pressure.

The number average molecular weight (M_n) and the polydispersity ($\mathcal{D}$) of the polymers were determined by size exclusion chromatography (SEC) using a Shimadzu LC-200AD system equipped with two PLgel Miwed-C 5 μ m columns (300 mm) column and a RID-20A detector. In short, polymers were solubilized in THF (1mg/ml) and then filtered before 100 μ l

was injected into the system. Measurement were performed at 30°C at a flow rate of 1 ml/min. The molecular weights were expressed according to a polystyrene calibration.

^1H NMR spectra were recorded at room temperature using an AMX300 Bruker spectrometer at 300 MHz. Chemical shifts were referenced to the peak of deuterated chloroform.

^1H NMR: (300 MHz; CDCl_3): δ (ppm) = 5.1 (q, 1H, CO-CH(CH₃)-O), 1.5 (m, 3H, CO-CH(CH₃)-O).

5.2.3 X-ray Diffraction and Differential Scanning Calorimetry

PLA degree of crystallinity analysis was performed with x-ray diffraction (XRD) Bruker D2 Phaser according to manufacturer protocol. PLA samples were loaded onto a clean sample holder and scanned with a range of 5.0 to 80.0 degree at 0.02 degree step width. Background was removed with Bruker analysis software and raw data was exported into excel spreadsheet. Absolute intensity at every point were then plotted in Graphpad Prism to visualised the location of peaks and relative peak intensity of PLA-50 and PLA-94.

Differential scanning calorimetry (DSC) measurements were carried out under nitrogen on a Perkin Elmer Instrument DSC 6000 Thermal Analyzer. Samples were subjected to a thermal analysis first by heating to 300°C (10 °C.min⁻¹), followed by cooling (10 °C.min⁻¹ from 300 °C to 0 °C) and then a second heating to 300°C (10 °C.min⁻¹). Glass transition temperature (T_g), melting temperature (T_m) and melting enthalpy (ΔH_m) were measured on the first heating ramp.

5.2.4 Fabrication of tunable stiffness platform with PLA

Chapter 4 detailed the engineering of the tunable stiffness microfiber platform. Briefly, PCL pellets were pressed into 50 μm PCL film with a heat press and cut into thin frame with customized cutter. The thin PCL film was mounted onto electrically conductive wheel for electrospinning to collect electrospun PLA-50 or PLA-94 fibers. PCL pellets were also pressed into 0.5 mm thick film and cut into frames of different dimensions with customized cutter. PCL frames were used to secure fibers from the same sheet of thin film with silicone glue. Inverting the scaffold creates a 0.5 mm suspended fiber structure for OPCs seeding.

5.2.5 Scanning Electron Microscopy

The morphology of the microfibers was evaluated using scanning electron microscopy (SEM) (JEOL, JSM-6390LA, Japan). Briefly, PLA fibrous substrates were attached onto the SEM support by carbon tapes and sputter-coated with platinum for 60 s at 20 mA. Thereafter, the samples were imaged under an accelerating voltage of 10 kV. For fiber diameter measurement, at least 100 fibers from each group were imaged and measured.

5.2.6 Atomic Force Microscopy Bending and Indentation

The substrate bending experiments were described in chapter 4. Briefly, force curves were obtained with a 0.406 N/m silicon nitride cantilever probe attached with a 25 μm diameter polystyrene bead (Novascan Technologies, Inc., Ames, IA). A maximum force of 10 nN and loading rate of 8 $\mu\text{m/s}$ were applied to ensure the microfiber scaffold undergoes

elastic bending. At least 8 force curves across the midspan of the suspended microfiber scaffold were obtained. The slope of the force-distance curve which corresponds to the stiffness (N/m) was extracted and analyzed.

Single fiber indentations were performed with the relative DMT model of contact mechanics using a Dimension Icon AFM (Bruker) in peakforce quantitative nanomechanical mapping mode. The probe is antimony doped silicon with a spring constant of 200 N/m and 400 nm tip radius (Tap525 Novascan Technologies, Inc., Ames, IA). A reference polystyrene sample (2.7 GPa) was used to obtain the tip end radius for a given indentation depth. Electrospun fibers resting on glass were indented to match the deformation depth using peak force setpoint. The modulus of fiber was extracted from the force-deformation field data using Nanoscope analysis software v1.9.

5.2.7 Nanotensile

Nanotensile evaluation of single eletrospun fiber was performed according to our previously reported protocol ¹⁴⁰. Briefly, electrospun PLA fibers were collected onto an aluminium frame mounted onto the electrospinning setup. A similar cardboard as what was reported previously ¹⁴⁰ was used to collect single fiber and secured with carbon tape. The sample collection was verified under a dissection microscope. The gauge length of the sample was set at 1.0 cm.

The sample was mounted onto MTS Nanomechanical Testing System. Two sides of the cardboard were cut cleanly to ensure no fibril remains. The fiber sample was pulled at a strain rate of 0.005 s^{-1} until fracture. Data from specimens that pass the ASTM standard testing criteria were collected. The slope of the stress-strain curves were analysed to obtain data on Young's Modulus.

5.2.8 OPCs Isolation and Myelination Culture

The isolation of primary OPCs from rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. The isolation and culture of primary OPCs and the myelination culture were described in chapter 2 and 4. Briefly, P0-P2 neonatal rat cortices that were cleaned from meninges were enzymatically digested with 1.2 U Papain and 40 $\mu\text{g/ml}$ DNase at 37 °C for 1 h. Thereafter, the enzymatic activity was stopped with 8 ml of DMEM with 10% Fetal Bovine Serum (FBS) and the dissociated tissues were triturated with a 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 OPCs were separated from the mixed glia culture by shaking on an orbital shaker at 200 rpm for 1 h at 37 °C to remove the loosely attached microglia. This was followed by a further 16-17 h of shaking. OPCs were then further purified by differential adhesion on untreated petri dishes for 25 min. Purified OPCs were then seeded at a density of 35,000 onto each suspended fiber substrate, and cultured for 7 days in myelin medium, which consisted of DMEM:Neurobasal (50:50), NS21, 1% Pen/Strep, Glutamax, ITS, 10 ng/ml biotin, 5 $\mu\text{g/ml}$ NAC and SATO (100 $\mu\text{g/ml}$ BSA, 60 ng/ml Progesterone, 16 $\mu\text{g/ml}$ Putrescine, 400 ng/ml T3 and T4).

5.2.9 Immunofluorescent staining

After 3 or 10 days, the cultures were fixed with 4% paraformaldehyde (PFA). The fixed myelin-fiber cultures were then permeabilised with 0.1% Triton-X for 15 min at room temperature. Next, the samples were incubated with primary antibodies overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature. The primary antibodies used were: rat anti-MBP (1:200) and rabbit anti-Olig2 (1:1000). The secondary antibodies used were: Alexa-Fluor 488 goat anti-rat and Alexa Fluor 633 goat anti-rabbit (both at 1:1000). The nuclei were counterstained with DAPI. The samples were then mounted onto 60 mm coverglass with Fluoromount-G and imaged under a Zeiss LSM 800 confocal microscope. Myelin sheaths were examined by scanning through 40x, 0.45 μ m z-stack images acquired by confocal microscopy. Each sheath is defined as continuous tube of MBP signals along the microfiber.

5.2.10 Statistical Analysis

All values were presented as mean $\pm$ standard deviation (S.D). Student's t-test was used to compare between 2 independent samples. Levene's test of homogeneity was used to check for homogeneity of variance when comparing 3 or more sample groups. One-way ANOVA with Tukey post-hoc test was used for samples that passed the homogeneity test. Otherwise, non-parametric Kruskal-Wallis pair-wise comparison was made. P-values of less than 0.05 were considered to be statistically significant.

5.3 Results

5.3.1 Characterization of PLA Fibrous Structure

To study the effect of axonal intrinsic stiffness on myelination, we modelled changes in the axonal cytoskeleton (Figure 5.1A). We could increase axon intrinsic stiffness independently from other biophysical parameters by increasing the degree of crystallinity of artificial axons (Figure 5.1B). This could be achieved by increasing the ratio of PLLA to PDLA enantiomer (PLA₉₄ > PLA₅₀). Enantiomers of PLA have the same chemical structure and thus, similar surface chemistry, but should differ in certain physical properties due to the stereocomplex arrangement (Figure 5.2). We verified that our synthesized PLA-50 and PLA-94 have the same proton ratio of 3:1 in NMR as predicted by its structure (Figure 5.2A). Moreover, we verified with both XRD and DSC that the stereocomplex of PLA-50 contributed to changes in the crystallinity of PLA. Specifically, XRD showed a sharp and high peak intensity for semi-crystalline, optically pure PLA-94, which was absent in PLA-50 (Figure 5.2C). Likewise, DSC analysis of PLA-94 showed crystallization peak at a higher temperature, which was absent in PLA-50. While both types of PLA showed glass transition temperature at 52-56°C, enthalpy of PLA-50 at this amorphous region is significantly higher than PLA-94 (Figure 5.2B), suggesting the presence of large amorphous region in its structure.

Figure 5.1: Fabrication of artificial axons with tunable intrinsic stiffness to mimic environmental signals experienced by oligodendrocytes. (A) Axon intrinsic stiffness (i.e. how easily an axon can be compressed due to changes in axon viscosity and/or cytoskeletal changes) affects oligodendrocyte myelination. (B) Schematic showing principle of tunable axon intrinsic stiffness by altering the degree of crystallinity of artificial axons.

A

NMR

PLA-50

PLA-94

B**DSC****PLA-50** **PLA-94**

Figure 5.2: Physical characterization of PLA artificial axons. (A) ^1H NMR data shows the predicted PLA protons with a ratio of 1:3 in both PLA-50 and PLA-94 (B) Differential Scanning Calorimetry data comparing the effect of heat on the melting characteristics of PLA-50 and PLA-94. PLA-50 shows single peak correlated to an amorphous structure while PLA-94 shows an additional peak representing crystalline structure. Higher heat capacity of PLA-50 suggests a large proportion of amorphous structure. (C) X-ray diffraction data shows a more intense peak for PLA-94 fiber, indicating a more crystalline structure than PLA-50 fiber.

L/D motif distribution of PLA did not hinder the electrospinning of the polymer as both PLA-50 and optically pure PLA-94 produced similar artificial axons upon electrospinning as seen under SEM (Figure 5.3A). We verified that both artificial axons have similar fiber calibre (Figure 5.3B). Furthermore, when built into a suspended structure as described in chapter 4, both PLA artificial axons have similar fiber density (Figure 5.3C). We used AFM to investigate the stiffness of the structure built by suspended PLA fibers. With the same length of PCL frames, the structural stiffness along the midline of the structure was similar across the two types of PLA (Figure 5.3D). Nonetheless, increasing length of PCL frames decreased structural

stiffness of the substrate as expected from three-point bending theory (Figure 5.3D). Thus, our platform decoupled the effect of axonal calibre, axonal density and structural stiffness from changes crystallinity of PLA fibers.

We next verified if changes in crystallinity of PLA fibers would influence the mechanical properties of single artificial axon. By indenting single microfiber on glass with AFM, we observed significantly higher Young's Modulus in microfibers electrospun with semi-crystalline PLA-94 (1.4-fold higher modulus in PLA-94, Figure 5.3E). Direct modulus measurement from the stress-strain relationship with nanotensile system produced similar results (Figure 5.3F). Thus, electrospinning of optically pure PLA-94 created semi-crystalline PLA artificial axons, which was associated with higher intrinsic stiffness.

Figure 5.3: A higher degree of crystallinity increases PLA artificial axons intrinsic stiffness. (A) Scanning electron microscopy images show similar polymeric fiber formed from electrospinning with PLA-50 and PLA-94 polymer. Scale bar represents 50 μm . (B) Quantitative data shows both electrospun fibers from both polymers has similar fiber diameter. (C) Quantitative data shows similar fiber density in suspended structure engineered by the two PLA polymer. (D) Atomic force microscopy indentation of the fibrous structure shows similar structural stiffness between structures of the same suspended length. Increasing suspension length decreases structural stiffness. ** $P < 0.01$, *** $P < 0.001$, One-way ANOVA. (E) Atomic force microscopy indentation of single fiber confirms PLA-94 has higher Young's modulus and

*thus, stiffer fiber. *** $P < 0.001$, Independent T-test. (F) Single fiber nanotensile test shows PLA-94 electrospun fiber has higher Young's modulus, indicating a stiffer fiber in comparison to PLA-50 fiber. ** $P < 0.01$, Independent T-test.*

5.3.2 Effect of Intrinsic Stiffness on OPC Differentiation

Artificial axons engineered with PLA-50 and PLA-94 were built into suspended fiber format as described in chapter 4 to investigate the effect of mechanical properties on OPC cellular outcome. We evaluated the differentiation and maturation of OPC on the different PLA artificial axons after 3 days of culture. Increased structural stiffness promoted OPC differentiation into MBP⁺ cells regardless of the types of PLA artificial axons, as substrates made with shorter frames have a higher percentage of matured OL (Figure 5.3). Crucially, increasing intrinsic stiffness of individual artificial fibers also promoted OPC differentiation, especially with stiffer structures. PLA-94 artificial axons secured by 1 cm PCL frames have 1.8-fold higher percentage of MBP⁺ cells than PLA-50 artificial axons secured by the same PCL frames. This effect is slightly less pronounced with more compliant 2 cm frame (1.7-fold higher in PLA-94) and negligible in most compliant 3 cm frame (Figure 5.3). Since the structural stiffness, fiber calibre, topography, etc were similar, the data suggest intrinsic stiffness of individual artificial axons promoted OPC differentiation and maturation.

Figure 5.4: High structural and intrinsic stiffness of PLA promote OPC differentiation. (A) Representative confocal images show the number of MBP⁺ cells on structures with the same artificial axon source of PLA-50 but different PLA frame length. Scale bar represents 50 μ m. Quantitative data show increasing structural stiffness of microenvironment with shorter PCL frame dimension increased the percentage of MBP⁺ cells. (B) Representative confocal images show the number of MBP⁺ cells on structures with the same artificial axon source of PLA-94 but different PLA frame length. Scale bar represents 50 μ m. Quantitative data show increasing structural stiffness of microenvironment with shorter PCL frame dimension increased the percentage of MBP⁺ cells. **P<0.01, *P<0.05 One-way ANOVA with Tukey post-hoc.

5.3.3 Effect of Intrinsic Stiffness on OPC Myelination

Complete myelin sheaths were observed by 10 days of OPC culture on PLA artificial axons. We first investigated the effect of structural stiffness on myelination with different PLA

fibers. Rigid structures with shorter PCL frames hindered OL myelination regardless of the types of PLA artificial axons (Figure 5.5). Structures secured with 2 cm PCL frames saw 1.9-fold and 2.1-fold decrease in the number of complete myelin sheaths formed per OL in PLA-50 and PLA-94 artificial axons respectively (Figure 5.5B,E). This was supplemented with a shift of sheath length distributions to the left for both PLA fibers (Figure 5.5C,F).

Figure 5.5: High PLA artificial axons structural stiffness impedes oligodendrocyte myelination.

*(A) Representative confocal images show the extent of oligodendrocyte myelination on PLA-50 artificial axons. Scale bar represents 20 μ m. (B) Quantitative data shows shorter suspension and thus, higher structural stiffness impedes the formation of myelin sheath per cell on PLA-50 fibers. *** $P < 0.001$, T-test. (C) Quantitative data shows shorter suspension length and higher structural stiffness also decrease the length of myelin sheath formation on PLA-50 fibers. (D) Representative confocal images show the extent of oligodendrocyte myelination on PLA-94 artificial axons. Scale bar represents 20 μ m. (E) Quantitative data shows longer suspension and thus, lower structural stiffness impedes the formation of myelin sheath per cell on PLA-94 fibers. *** $P < 0.001$, T-test. (F) Quantitative data shows shorter suspension length and higher structural stiffness also decrease the length of myelin sheath formation on PLA-94 fibers.*

To examine the effect of intrinsic stiffness on myelination, we compared the complete myelin sheaths formed by PLA-50 and PLA-94 artificial axons in the same structure (Figure 5.6). More rigid artificial axons PLA-94 saw a 2.1-fold decrease in the number of complete myelin sheaths formed per OL in 2 cm frames when compared to more compliant PLA-50 (Figure 5.6B). Similarly, PLA-94 fibers saw 1.8-fold lower number of complete sheaths in 3 cm frames substrates when compared to PLA-50 artificial axons (Figure 5.6E). The data was also supported by a shift of sheath length distributions to the left for the more rigid PLA-94 fibers (Figure 5.6C,F). Thus, our data suggest that higher intrinsic stiffness hindered OL myelination.

Figure 5.6: High PLA artificial axons intrinsic stiffness impedes oligodendrocyte myelination.

*(A) Representative confocal images show the extent of oligodendrocyte myelination on 2 cm structure PLA artificial axons. Scale bar represents 20 μ m. (B) Quantitative data shows more crystalline and thus, higher intrinsic stiffness PLA-94 fiber decreases the number of myelin sheath per cell. *** $P < 0.001$, T-test. (C) Quantitative data shows high crystallinity and higher intrinsic stiffness resulted in the formation of shorter myelin sheath on PLA-94 fibers. (D) Representative confocal images show the extent of oligodendrocyte myelination on 3 cm structure PLA artificial axons. Scale bar represents 20 μ m. (E) Quantitative data shows more crystalline and thus, higher intrinsic stiffness PLA-94 fiber decreases the number of myelin sheath per cell on more compliance structure. *** $P < 0.001$, T-test. (F) Quantitative data shows high crystallinity and higher intrinsic stiffness resulted in the formation of shorter myelin sheath on PLA-94 fibers on more compliance structure.*

5.4 Discussion

Remyelination capacity of an OL varied widely across regions of CNS and seemed to be inversely correlated with ageing. For instance, remyelination is often robust in the gray matter but limited in the white matter ¹³¹. Similarly, remyelination capability of OL decreases with ageing in the CNS ¹³⁰. Thus, understanding how age or disease factors affect OL differentiation and myelination could help develop new therapy to target demyelination. Biochemical signalling pathway has been the core focus of OL and myelin neurobiology. As such, significant advances have been made in the past decade on cell extrinsic and intrinsic factors that are essential for remyelination ¹⁰. However, development in our understanding of biophysical cues on OL myelination remained relatively stagnant, largely due to the lack of

platform as illustrated in chapter 4. Thus, we aim to contribute our engineering knowledge into the development of artificial axons platforms, to improve our understanding of the effect of biophysical signalling in OL myelination. Building upon our tunable structural stiffness platform in chapter 4, which effectively decouple the effect of biophysical cues such as axonal calibre, we engineered tunable intrinsic stiffness platform to investigate the effect of intrinsic stiffness of individual axons on OL myelination. We found that similar to the effect of structural stiffness, high intrinsic fiber stiffness promoted OPC differentiation into OL but hinders OL myelination.

OL in the intrinsic CNS matrix microenvironment experience a complex milieu of biophysical signals. Firstly, it is well-known that biophysical cues from axonal calibre control if its permissive to OL myelination ^{6,51}. Second, geometric and spatial restriction around OPC promotes its differentiation and maturation ⁴⁸. Third, myelin supply is correlated with axonal demand. Ectopic myelination is associated with lower demand for myelination ¹⁴¹. Fourth, higher structural stiffness promotes OPC differentiation but hinders myelination as shown in chapter 4. As such, similar to the development of tunable structural stiffness platform, the key conundrum remains in decoupling intrinsic stiffness from other determinants of OL myelination. In this work, we developed a platform that decoupled axonal calibre, cellular & axonal density and structural stiffness from intrinsic stiffness of artificial axons to investigate how changes in stiffness of individual single fiber would influence OL myelination. The conventional way of altering the intrinsic stiffness of a fiber is to simply use another material. However, materials surface chemistry places a role in regulating cellular responses ¹⁴². Alternatively, a core-shell fiber with the same shell could theoretically alter the stiffness of

individual fiber without changing the surfaces in contact with cells. However, controlling the fiber calibre, an important myelination determinant, in such platform is tricky. Utilizing unique chemical properties of PLA, we engineered an amorphous and a semi-crystalline PLA artificial axon with different compositions of PLA isomers. Changes in crystallinity enabled us to control the intrinsic stiffness of individual fibers. When built into suspended-fiber, we showed that all other physical parameters that influence OL differentiation and myelination were kept consistent. Thus, our platform enabled us to study the effect of intrinsic stiffness on OL myelination, which was otherwise not possible due to the presence of many confounding factors.

OL myelination has long been associated with aspects of the axonal cytoskeleton. Myelin is involved in the regulation of the axonal cytoskeleton, transport and node of ranvier assembly. Demyelination disrupts axonal cytoskeleton, which could result in subsequent neurodegeneration¹⁴³. Herein, we attempted to model changes in the axonal cytoskeleton in artificial axons, which facilitate studies of biophysical effect, to investigate intrinsic stiffness effect on myelination. Specifically, through varying the crystallinity of PLA artificial axons, we investigated how OL myelination would respond to such alteration in the underlying substrate. We found that higher crystallinity of artificial axons increased intrinsic stiffness and hindered the formation of complete myelin sheaths by an OL. This could have implications in neurodegeneration as dysregulation of axonal cytoskeleton could induce axon demyelination. Loss of myelin could further exacerbate disruptions in the axonal cytoskeleton due to the loss of structural and metabolic support. We presented a model of mimicking axonal cytoskeleton with fiber crystallinity to show the intimate relationship between axons and myelin. Future

work could model the pathological changes in the axonal cytoskeleton and investigate the effect on OL myelination.

Mechanical stiffness of microenvironment could influence OL cellular outcome. As explained in chapter 4, it was reported that high structural stiffness promoted OPC differentiation into OL ¹²⁶. We verified this relationship with our tunable structural stiffness scaffold as illustrated in the previous chapter. Furthermore, we showed that high structural stiffness of OL microenvironment perturb myelination. Thus, this work set out to uncover more biophysical effect on OL myelination. During OL myelination, OL processes are in close contact with axons throughout the concentric wrapping process ¹²⁴. We hypothesized that the mechanical properties of individual axons would impact the way OLs spread its membrane. Interestingly, we observed the same inverse relationship between OL differentiation and myelination with changes in intrinsic stiffness. Together with chapter 4, our data showed an important relationship between key physical forces acting on OL during myelination.

5.5 Conclusion

In the present study, we engineered a tunable intrinsic stiffness microfiber platform to investigate the effect of artificial axons intrinsic stiffness on OL myelination. Our platform effectively decoupled intrinsic stiffness from other biophysical cues to allow simple study of intrinsic stiffness effect on OL. We showed that intrinsic stiffness of artificial axons could be modulated by controlling the degree of crystallinity through mixing different ratio of enantiomers. High crystallinity from optically pure PLA-94 artificial axons has significantly

higher intrinsic stiffness as measured by both AFM and nanotensile machine. Similar to the effect of structural stiffness, higher intrinsic stiffness of artificial axons supported OPC differentiation into MBP⁺ OL. For the first time, we revealed that the intrinsic stiffness of artificial axons is could alter the degree of OL myelination. Higher intrinsic stiffness of individual artificial axons resulted in lower length and number of myelin sheaths formed by OL. Our data suggest that high axonal stiffness could promote OPC differentiation but perturb OL myelination.

Chapter 6: Conclusion and Future Work

6.1 Summary

CNS myelination is a complex and crucial process for efficient conduction of action potentials, which enables rapid sensory and motor functions. In this thesis, we explored the use of electrospun microfiber platforms to better understand the OL myelination. We hypothesized that suspended artificial axons could enable 1) robust multilamellar myelination by OLs; 2) engineering of tunable stiffness substrate. With the microfiber platform, we answer difficult questions on the effect of mechanical stiffness on OLs myelination. Improve understanding of the unique CNS myelination process would allow new therapeutics to be discovered for many neurological injuries and diseases.

We first set out to establish a microfiber platform for OL myelination. OL could myelinate non-living axons, artificial axons and other engineered platforms. However, robust myelination that closely mimics the robust concentric wrapping process seen in the presence of living axons was not possible. Amalgamating appropriate physical and chemical cues, we showed that our engineered artificial axons could closely mimic myelination of live axons *in-vitro*, as seen by the robust MBP⁺ signals along microfiber. We also showed that this platform could be a drug/gene screening platform to explore how small molecule drugs, proteins or RNAs could influence OL myelination. This platform would facilitate a rapid screening of potential therapeutics for demyelinating injuries and diseases.

We explored the alternatives of bringing crucial drugs/genes to sensitive neuronal cells through engineered nanoparticles. For our screening platform to be effective, drugs/genes have to be efficiently delivered or transfected into OLs or other neuronal cells. However, conventional transfection reagents have limited efficacy and lack the versatility to be modified for specific purposes. We herein showed that bolus MSN delivery could bring desired RNAs into both neuronal and glia cells with similar efficacy than commercial transfection reagents. Thus, engineered particles could be employed in conjunction with our microfiber platform as a versatile pre-animal model screening platform for the CNS.

We next answered the important question of the effect of mechanical microenvironment on OL myelination with modified microfiber platform. The lack of a tunable stiffness, myelin-permissive platform has impeded our ability to understand how changes in mechanical stiffness affect the ability of an OL to myelinate axons. Through engineering of suspended microfiber, we presented a platform that could decouple structural stiffness from parameters that are known to influence OL myelination. With this platform, we found that high structural stiffness promoted OPCs differentiation into MBP⁺ OL. Crucially, we revealed that high structural stiffness hindered OL myelination and this involves YAP signalling and actin turnover dysregulation. These findings could explain the failure of remyelination in the stiff white matter and active remyelination in the soft cortex following demyelinating disorders.

Last but not least, we postulated that cytoskeleton-induced changes in intrinsic stiffness of individual axons could have a huge impact on OL myelination. Thus, we mimic such changes in axon by controlling the crystallinity of PLA to engineer a tunable intrinsic stiffness platform for OL myelination. Similar to the structural stiffness platform, we decouple intrinsic stiffness from parameters that are known to influence OL myelination. We revealed that high intrinsic stiffness of artificial axons, like a pathological environment following injuries and diseases, inhibited OL myelination. The effect on OL myelination was not due to lack of MBP⁺ OL as we confirmed that high stiffness microenvironment did not prevent the differentiation of OPCs.

In summary, this dissertation demonstrated the functionality of an electrospun microfiber platform to study OL myelination. The platform decouples biochemical and biophysical factors that affect OLs differentiation and myelination to allow study of the multifaceted myelination processes. We presented on how our microfiber myelination platform could accelerate the process of drug development by providing more rapid and accurate *in vitro* screening. More significantly, we introduced novel methods to engineer tunable structural and intrinsic stiffness platforms for OL myelination. Our platforms show dysregulation in mechanical microenvironment could inhibit myelination. Moving forward, our microfiber platform could hold immense potential in helping us to enhance remyelination and recovery.

6.2 Future Directions

This dissertation highlighted the importance of engineering platforms in enhancing our understanding of the CNS processes and more in myelin research. However, there continue to be room for future research to translate our results into clinical applications. Currently, our microfiber platform for drug/gene testing could screen for single candidate at a time. Despite its physiological relevance and easy visualization of myelin, it lacks the ability to perform high throughput screening similar to the micropillar platform⁴⁹. High throughput screening is necessary to filter a large number of possible drug candidates, to sieve out the most promising therapeutics for clinical trial¹⁴⁴. Thus, advanced manufacturing method such as 3D-printing could potentially be an alternative to electrospinning in making microfibers¹²⁷. 3D-printing of microfiber in conventional microwells could enable high throughput production and screening of microfiber substrates, thereby enhancing the efficiency of screening. However, resolution of 3D-printing is a major hurdle to overcome before this technique could be used to engineer artificial axons of relevant sizes¹⁴⁵.

Our platform provides a simple system to decouple the crosstalk between OL and axons. Such a system is incredibly useful in enhancing our understanding of myelination. However, therapeutic drugs are likely to affect the CNS as a whole. This system would therefore unable to reveal potential side-effects or interactions between drugs and other crucial components of the CNS. For instance, OLs are closely associated with other CNS cells such as microglia¹⁴⁶. Interactions between OPCs and microglia are now thought to be crucial for normal myelinogenesis and maintenance¹⁴⁶. Thus, our platform could be modified with the addition of specific CNS modules to investigate the effect of their interactions on myelination. Simple OPC-microglia co-culture on microfiber substrate could enable

investigation of potential interactions. Alternatively, conditioned media culture could also be possible to investigate potential biochemical signaling effect on two different CNS cells. For such system to be successful, careful characterization of microglia will be needed. Microglia is known to have a range of possible polarization stage, which could have distinct impact on OL myelination ³⁹. *In-vitro* selection of polarized microglia is currently difficult and time-consuming. Unfortunately, efficient method of obtaining CNS cells does not currently enable such careful selection to be done ⁹⁶.

The ability to efficiently deliver materials to neuronal cells is another crucial aspect of translating our understanding of OL biology into clinical application. As such, we illustrated a better approach to deliver RNA into neural cells with MSN. Despite similar gene knockdown efficacy achieved with our engineered particles in comparison with conventional transfection reagents, localized and targeted drug/gene delivery with MSN remains suboptimal. Yet it is vital to attain localized or cell-specific delivery for better therapeutic outcome ^{56,91,147}. Thus, MSN release kinetics and characteristic from the reservoir have to be further studied for better therapeutic effect ¹¹³. Alternatively, other forms of cell-specific delivery method could be explored for MSN. For instance, cell-specific delivery by MSN is widely studied in oncology ¹⁴⁸. Similarly, MSN could be surface-modified with ligands specific to neuronal cells to improve the drug delivery efficacy. Lessons could be drawn from strategy to specifically target the peripheral nervous system, where novel liposomes modified with neural cell membrane properties are engineered to enhance internalization of particles by neuronal and glia cells ¹⁴⁹. This could be achieved with a combination of phospholipid and cholesterol to mimic compositions of neural cell membranes ¹⁵⁰. For such purpose, lipid-modified MSN could serve

as a platform to improve neural cell-specific delivery of pro-regenerative drugs or molecule to the CNS ¹⁵¹.

Platforms developed in this dissertation has enabled us to study crucial mechanotransduction pathways for OL myelination. In this work, we hypothesized that alterations of mechanical microenvironment, which could be due to injuries and disorders, would influence the degree of myelination. Naturally, the next question to ask is if we could arrest important cellular signaling pathways contributed to the inhibition of myelination we observed in stiff substrates. For instance, our tunable stiffness platform showed that YAP signaling is involved in the crucial process to OL myelination. YAP signaling is a part of the Hippo pathway, which consists of components known to influence cellular growth and differentiation. Compounds such as Verteporfin have been identified to target a specific part of Hippo pathway including YAP signaling for therapeutics ¹⁵². Thus, it would be interesting to amalgamate tunable stiffness platform with drug/gene screening platform to investigate the effect of these compounds on OL myelination. Upon identification of suitable drug candidates, these drugs could be administered to animal models of multiple sclerosis, Alzheimer's disease, traumatic injuries or ageing. Dysregulation in tissue mechanical microenvironment and lack of remyelination are hallmarks of these conditions ^{121,129,153}. Therapeutic compounds could promote remyelination by blocking signaling pathways responsible for remyelination failure following these conditions thereby providing novel treatment methods for CNS injuries and disorders.

Chapter 7: References

- 1 Spinal cord injury facts and figures at a glance. *J Spinal Cord Med* **35**, 197-198, doi:10.1179/1079026812Z.00000000063 (2012).
- 2 Thuret, S., Moon, L. D. & Gage, F. H. Therapeutic interventions after spinal cord injury. *Nat Rev Neurosci* **7**, 628-643, doi:10.1038/nrn1955 (2006).
- 3 Schmidt, C. E. & Leach, J. B. Neural tissue engineering: strategies for repair and regeneration. *Annu Rev Biomed Eng* **5**, 293-347, doi:10.1146/annurev.bioeng.5.011303.120731 (2003).
- 4 Richardson, P. M., McGuinness, U. M. & Aguayo, A. J. AXONS FROM CNS NEURONS REGENERATE INTO PNS GRAFTS. *Nature* **284**, 264-265, doi:10.1038/284264a0 (1980).
- 5 Lee, S., Chong, S. Y. C., Tuck, S. J., Corey, J. M. & Chan, J. R. A rapid and reproducible assay for modeling myelination by oligodendrocytes using engineered nanofibers. *Nature Protocols* **8**, 771-782, doi:10.1038/nprot.2013.039 (2013).
- 6 Lee, S. *et al.* A culture system to study oligodendrocyte myelination processes using engineered nanofibers. *Nat Methods* **9**, 917-922, doi:10.1038/nmeth.2105 (2012).
- 7 Ong, W. *et al.* Microfiber drug/gene delivery platform for study of myelination. *Acta Biomater* **75**, 152-160, doi:10.1016/j.actbio.2018.06.011 (2018).
- 8 Baker, B. M. & Chen, C. S. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *J Cell Sci* **125**, 3015-3024, doi:10.1242/jcs.079509 (2012).
- 9 Nashmi, R. & Fehlings, M. G. Mechanisms of axonal dysfunction after spinal cord injury: with an emphasis on the role of voltage-gated potassium channels. *Brain Research Reviews* **38**, 165-191, doi:10.1016/s0165-0173(01)00134-5 (2001).
- 10 Franklin, R. J. M. & Ffrench-Constant, C. Regenerating CNS myelin - from mechanisms to experimental medicines. *Nat Rev Neurosci* **18**, 753-769, doi:10.1038/nrn.2017.136 (2017).
- 11 Powers, B. E. *et al.* Remyelination reporter reveals prolonged refinement of spontaneously regenerated myelin. *Proceedings of the National Academy of Sciences* **110**, 4075-4080, doi:10.1073/pnas.1210293110 (2013).
- 12 Hesp, Z. C., Goldstein, E. Z., Miranda, C. J., Kaspar, B. K. & McTigue, D. M. Chronic oligodendrogenesis and remyelination after spinal cord injury in mice and rats. *J Neurosci* **35**, 1274-1290, doi:10.1523/JNEUROSCI.2568-14.2015 (2015).
- 13 Duncan, G. J. *et al.* Locomotor recovery following contusive spinal cord injury does not require oligodendrocyte remyelination. *Nat Commun* **9**, 3066, doi:10.1038/s41467-018-05473-1 (2018).
- 14 Assinck, P. *et al.* Myelinogenic Plasticity of Oligodendrocyte Precursor Cells following Spinal Cord Contusion Injury. *J Neurosci* **37**, 8635-8654, doi:10.1523/JNEUROSCI.2409-16.2017 (2017).
- 15 Watanabe, M., Hadzic, T. & Nishiyama, A. Transient upregulation of Nkx2.2 expression in oligodendrocyte lineage cells during remyelination. *Glia* **46**, 311-322, doi:10.1002/glia.20006 (2004).
- 16 Vana, A. C., Lucchinetti, C. F., Le, T. Q. & Armstrong, R. C. Myelin transcription factor 1 (Myt1) expression in demyelinated lesions of rodent and human CNS. *Glia* **55**, 687-697, doi:10.1002/glia.20492 (2007).
- 17 Franklin, R. J. & Ffrench-Constant, C. Remyelination in the CNS: from biology to therapy. *Nat Rev Neurosci* **9**, 839-855, doi:10.1038/nrn2480 (2008).

- 18 Mekhail, M., Almazan, G. & Tabrizian, M. Oligodendrocyte-protection and remyelination post-spinal cord injuries: a review. *Prog Neurobiol* **96**, 322-339, doi:10.1016/j.pneurobio.2012.01.008 (2012).
- 19 Zhang, Y. *et al.* Notch1 signaling plays a role in regulating precursor differentiation during CNS remyelination. *Proc Natl Acad Sci U S A* **106**, 19162-19167, doi:10.1073/pnas.0902834106 (2009).
- 20 Hammond, T. R. *et al.* Astrocyte-derived endothelin-1 inhibits remyelination through notch activation. *Neuron* **81**, 588-602, doi:10.1016/j.neuron.2013.11.015 (2014).
- 21 John, G. R. *et al.* Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. *Nat Med* **8**, 1115-1121, doi:10.1038/nm781 (2002).
- 22 Chen, J., Leong, S. Y. & Schachner, M. Differential expression of cell fate determinants in neurons and glial cells of adult mouse spinal cord after compression injury. *Eur J Neurosci* **22**, 1895-1906, doi:10.1111/j.1460-9568.2005.04348.x (2005).
- 23 Jurynczyk, M., Jurewicz, A., Bielecki, B., Raine, C. S. & Selmaj, K. Inhibition of Notch signaling enhances tissue repair in an animal model of multiple sclerosis. *J Neuroimmunol* **170**, 3-10, doi:10.1016/j.jneuroim.2005.10.013 (2005).
- 24 Mumm, J. S. & Kopan, R. Notch Signaling: From the Outside In. *Developmental Biology* **228**, 151-165, doi:http://dx.doi.org/10.1006/dbio.2000.9960 (2000).
- 25 Stidworthy, M. F. *et al.* Notch1 and Jagged1 are expressed after CNS demyelination, but are not a major rate-determining factor during remyelination. *Brain* **127**, 1928-1941, doi:10.1093/brain/awh217 (2004).
- 26 Mi, S., Pepinsky, R. B. & Cadavid, D. Blocking LINGO-1 as a therapy to promote CNS repair: from concept to the clinic. *CNS Drugs* **27**, 493-503, doi:10.1007/s40263-013-0068-8 (2013).
- 27 Ji, B. *et al.* LINGO-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury. *Mol Cell Neurosci* **33**, 311-320, doi:10.1016/j.mcn.2006.08.003 (2006).
- 28 Mi, S. *et al.* LINGO-1 negatively regulates myelination by oligodendrocytes. *Nat Neurosci* **8**, 745-751, doi:10.1038/nn1460 (2005).
- 29 Mi, S. *et al.* LINGO-1 antagonist promotes spinal cord remyelination and axonal integrity in MOG-induced experimental autoimmune encephalomyelitis. *Nat Med* **13**, 1228-1233, doi:10.1038/nm1664 (2007).
- 30 Mi, S. *et al.* Promotion of central nervous system remyelination by induced differentiation of oligodendrocyte precursor cells. *Ann Neurol* **65**, 304-315, doi:10.1002/ana.21581 (2009).
- 31 Shao, Z. *et al.* LINGO-1 Regulates Oligodendrocyte Differentiation through the Cytoplasmic Gelsolin Signaling Pathway. *J Neurosci* **37**, 3127-3137, doi:10.1523/JNEUROSCI.3722-16.2017 (2017).
- 32 Pasquinelli, A. E. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat Rev Genet* **13**, 271-282, doi:10.1038/nrg3162 (2012).
- 33 Zhao, X. *et al.* MicroRNA-mediated control of oligodendrocyte differentiation. *Neuron* **65**, 612-626, doi:10.1016/j.neuron.2010.02.018 (2010).
- 34 Wang, H. B. *et al.* miR-219 Cooperates with miR-338 in Myelination and Promotes Myelin Repair in the CNS. *Developmental Cell* **40**, 566-+, doi:10.1016/j.devcel.2017.03.001 (2017).

- 35 Katz, D. *et al.* CORRELATION BETWEEN MAGNETIC-RESONANCE-IMAGING FINDINGS AND LESION DEVELOPMENT IN CHRONIC, ACTIVE MULTIPLE-SCLEROSIS. *Annals of Neurology* **34**, 661-669, doi:10.1002/ana.410340507 (1993).
- 36 Peterson, J. W., Bo, L., Mork, S., Chang, A. & Trapp, B. D. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Annals of Neurology* **50**, 389-400, doi:10.1002/ana.1123 (2001).
- 37 Frischer, J. M. *et al.* The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* **132**, 1175-1189, doi:10.1093/brain/awp070 (2009).
- 38 Patel, J. R., McCandless, E. E., Dorsey, D. & Klein, R. S. CXCR4 promotes differentiation of oligodendrocyte progenitors and remyelination. *Proceedings of the National Academy of Sciences* **107**, 11062-11067 (2010).
- 39 Miron, V. E. *et al.* M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat Neurosci* **16**, 1211-1218, doi:10.1038/nn.3469 (2013).
- 40 Dombrowski, Y. *et al.* Regulatory T cells promote myelin regeneration in the central nervous system. *Nat Neurosci* **20**, 674-680, doi:10.1038/nn.4528 (2017).
- 41 Beck, K. D. *et al.* Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. *Brain* **133**, 433-447, doi:10.1093/brain/awp322 (2010).
- 42 Jarjour, A. A., Zhang, H., Bauer, N., Ffrench-Constant, C. & Williams, A. In vitro modeling of central nervous system myelination and remyelination. *Glia* **60**, 1-12, doi:10.1002/glia.21231 (2012).
- 43 Lubetzki, C. *et al.* EVEN IN CULTURE, OLIGODENDROCYTES MYELINATE SOLELY AXONS. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 6820-6824, doi:10.1073/pnas.90.14.6820 (1993).
- 44 Chan, J. R. *et al.* NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes. *Neuron* **43**, 183-191, doi:10.1016/j.neuron.2004.06.024 (2004).
- 45 Watkins, T. A., Emery, B., Mulinyawe, S. & Barres, B. A. Distinct Stages of Myelination Regulated by gamma-Secretase and Astrocytes in a Rapidly Myelinating CNS Coculture System. *Neuron* **60**, 555-569, doi:10.1016/j.neuron.2008.09.011 (2008).
- 46 Nave, K. A. & Trapp, B. D. Axon-glial signaling and the glial support of axon function. *Annual Review of Neuroscience* **31**, 535-561, doi:10.1146/annurev.neuro.30.051606.094309 (2008).
- 47 Colello, R. J. & Pott, U. Signals that initiate myelination in the developing mammalian nervous system. *Molecular Neurobiology* **15**, 83-100, doi:10.1007/bf02740617 (1997).
- 48 Rosenberg, S. S., Kelland, E. E., Tokar, E., De la Torre, A. R. & Chan, J. R. The geometric and spatial constraints of the microenvironment induce oligodendrocyte differentiation. *Proc Natl Acad Sci U S A* **105**, 14662-14667, doi:10.1073/pnas.0805640105 (2008).
- 49 Mei, F. *et al.* Micropillar arrays as a high-throughput screening platform for therapeutics in multiple sclerosis. *Nat Med* **20**, 954-960, doi:10.1038/nm.3618 (2014).
- 50 Redmond, S. A. *et al.* Somatodendritic Expression of JAM2 Inhibits Oligodendrocyte Myelination. *Neuron* **91**, 824-836, doi:10.1016/j.neuron.2016.07.021 (2016).
- 51 Bechler, M. E., Byrne, L. & Ffrench-Constant, C. CNS Myelin Sheath Lengths Are an Intrinsic Property of Oligodendrocytes. *Curr Biol* **25**, 2411-2416, doi:10.1016/j.cub.2015.07.056 (2015).

- 52 Cao, H., Liu, T. & Chew, S. Y. The application of nanofibrous scaffolds in neural tissue engineering. *Advanced Drug Delivery Reviews* **61**, 1055-1064, doi:https://doi.org/10.1016/j.addr.2009.07.009 (2009).
- 53 Lim, S. H., Liu, X. Y., Song, H. J., Yarema, K. J. & Mao, H. Q. The effect of nanofiber-guided cell alignment on the preferential differentiation of neural stem cells. *Biomaterials* **31**, 9031-9039, doi:10.1016/j.biomaterials.2010.08.021 (2010).
- 54 Christopherson, G. T., Song, H. & Mao, H. Q. The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation. *Biomaterials* **30**, 556-564, doi:10.1016/j.biomaterials.2008.10.004 (2009).
- 55 Carlberg, B., Axell, M. Z., Nannmark, U., Liu, J. & Kuhn, H. G. Electrospun polyurethane scaffolds for proliferation and neuronal differentiation of human embryonic stem cells. *Biomedical Materials* **4**, 7, doi:10.1088/1748-6041/4/4/045004 (2009).
- 56 Low, W. C. *et al.* Nanofibrous scaffold-mediated REST knockdown to enhance neuronal differentiation of stem cells. *Biomaterials* **34**, 3581-3590, doi:10.1016/j.biomaterials.2013.01.093 (2013).
- 57 Low, W. C., Rujitanaroj, P. O., Wang, F., Wang, J. & Chew, S. Y. Nanofiber-mediated release of retinoic acid and brain-derived neurotrophic factor for enhanced neuronal differentiation of neural progenitor cells. *Drug Delivery and Translational Research* **5**, 89-100, doi:10.1007/s13346-013-0131-5 (2015).
- 58 Diao, H. J., Low, W. C., Lu, Q. R. & Chew, S. Y. Topographical effects on fiber-mediated microRNA delivery to control oligodendroglial precursor cells development. *Biomaterials* **70**, 105-114, doi:10.1016/j.biomaterials.2015.08.029 (2015).
- 59 Milbreta, U. *et al.* Three-Dimensional Nanofiber Hybrid Scaffold Directs and Enhances Axonal Regeneration after Spinal Cord Injury. *ACS Biomaterials Science & Engineering* **2**, 1319-1329, doi:10.1021/acsbiomaterials.6b00248 (2016).
- 60 Nguyen, L. H. *et al.* Three-dimensional aligned nanofibers-hydrogel scaffold for controlled non-viral drug/gene delivery to direct axon regeneration in spinal cord injury treatment. *Sci Rep* **7**, 42212, doi:10.1038/srep42212 (2017).
- 61 Discher, D. E., Janmey, P. & Wang, Y. L. Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**, 1139-1143, doi:10.1126/science.1116995 (2005).
- 62 Li, L. Q., Eyckmans, J. & Chen, C. S. Designer biomaterials for mechanobiology. *Nature Materials* **16**, 1164-1168, doi:10.1038/nmat5049 (2017).
- 63 Lo, C. M., Wang, H. B., Dembo, M. & Wang, Y. L. Cell movement is guided by the rigidity of the substrate. *Biophysical Journal* **79**, 144-152, doi:10.1016/s0006-3495(00)76279-5 (2000).
- 64 Yeung, T. *et al.* Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton* **60**, 24-34, doi:10.1002/cm.20041 (2005).
- 65 Parsons, J. T., Horwitz, A. R. & Schwartz, M. A. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nature Reviews Molecular Cell Biology* **11**, 633-643, doi:10.1038/nrm2957 (2010).
- 66 Wang, H. B., Dembo, M. & Wang, Y. L. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. *American Journal of Physiology-Cell Physiology* **279**, C1345-C1350 (2000).
- 67 Zaman, M. H. *et al.* Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 10889-10894, doi:10.1073/pnas.0604460103 (2006).

- 68 Hadjipanayi, E., Mudera, V. & Brown, R. A. Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. *Journal of Tissue Engineering and Regenerative Medicine* **3**, 77-84, doi:10.1002/term.136 (2009).
- 69 Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677-689, doi:10.1016/j.cell.2006.06.044 (2006).
- 70 Leipzig, N. D. & Shoichet, M. S. The effect of substrate stiffness on adult neural stem cell behavior. *Biomaterials* **30**, 6867-6878, doi:10.1016/j.biomaterials.2009.09.002 (2009).
- 71 Pampaloni, F., Reynaud, E. G. & Stelzer, E. H. K. The third dimension bridges the gap between cell culture and live tissue. *Nature Reviews Molecular Cell Biology* **8**, 839-845, doi:10.1038/nrm2236 (2007).
- 72 Khetan, S. *et al.* Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. *Nat Mater* **12**, 458-465, doi:10.1038/nmat3586 (2013).
- 73 Chaudhuri, O. *et al.* Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat Mater* **15**, 326-334, doi:10.1038/nmat4489 (2016).
- 74 Bauer, A. *et al.* Hydrogel substrate stress-relaxation regulates the spreading and proliferation of mouse myoblasts. *Acta Biomater* **62**, 82-90, doi:10.1016/j.actbio.2017.08.041 (2017).
- 75 Lawson, C. *et al.* FAK promotes recruitment of talin to nascent adhesions to control cell motility. *J Cell Biol* **196**, 223-232, doi:10.1083/jcb.201108078 (2012).
- 76 Colognato, H., Ramachandrapa, S., Olsen, I. M. & ffrench-Constant, C. Integrins direct Src family kinases to regulate distinct phases of oligodendrocyte development. *J Cell Biol* **167**, 365-375, doi:10.1083/jcb.200404076 (2004).
- 77 Liang, X., Draghi, N. A. & Resh, M. D. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. *J Neurosci* **24**, 7140-7149, doi:10.1523/JNEUROSCI.5319-03.2004 (2004).
- 78 Crisp, M. *et al.* Coupling of the nucleus and cytoplasm: role of the LINC complex. *Journal of Cell Biology* **172**, 41-53, doi:10.1083/jcb.200509124 (2006).
- 79 Elosegui-Artola, A. *et al.* Force Triggers YAP Nuclear Entry by Regulating Transport across Nuclear Pores. *Cell* **171**, 1397-1410 e1314, doi:10.1016/j.cell.2017.10.008 (2017).
- 80 Vining, K. H. & Mooney, D. J. Mechanical forces direct stem cell behaviour in development and regeneration. *Nat Rev Mol Cell Biol* **18**, 728-742, doi:10.1038/nrm.2017.108 (2017).
- 81 Halder, G., Dupont, S. & Piccolo, S. Transduction of mechanical and cytoskeletal cues by YAP and TAZ. *Nat Rev Mol Cell Biol* **13**, 591-600, doi:10.1038/nrm3416 (2012).
- 82 Dupont, S. *et al.* Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179-183, doi:10.1038/nature10137 (2011).
- 83 Shimizu, T. *et al.* YAP functions as a mechanotransducer in oligodendrocyte morphogenesis and maturation. *Glia* **65**, 360-374, doi:10.1002/glia.23096 (2017).
- 84 Poitelon, Y. *et al.* YAP and TAZ control peripheral myelination and the expression of laminin receptors in Schwann cells. *Nat Neurosci* **19**, 879-887, doi:10.1038/nn.4316 (2016).
- 85 Miralles, F., Posern, G., Zaromytidou, A. I. & Treisman, R. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113**, 329-342, doi:10.1016/s0092-8674(03)00278-2 (2003).

- 86 Zuchero, J. B. *et al.* CNS myelin wrapping is driven by actin disassembly. *Dev Cell* **34**, 152-167, doi:10.1016/j.devcel.2015.06.011 (2015).
- 87 Nawaz, S. *et al.* Actin filament turnover drives leading edge growth during myelin sheath formation in the central nervous system. *Dev Cell* **34**, 139-151, doi:10.1016/j.devcel.2015.05.013 (2015).
- 88 Armstrong, R. C., Le, T. Q., Frost, E. E., Borke, R. C. & Vana, A. C. Absence of fibroblast growth factor 2 promotes oligodendroglial repopulation of demyelinated white matter. *The Journal of Neuroscience* **22**, 8574-8585 (2002).
- 89 Chari, D. M. & Blakemore, W. F. Efficient recolonisation of progenitor-depleted areas of the CNS by adult oligodendrocyte progenitor cells. *Glia* **37**, 307-313 (2002).
- 90 Franklin, R. J., Gilson, J. M. & Blakemore, W. F. Local recruitment of remyelinating cells in the repair of demyelination in the central nervous system. *Journal Of Neuroscience Research* **50**, 337-344 (1997).
- 91 Diao, H. J., Low, W. C., Milbreta, U., Lu, Q. R. & Chew, S. Y. Nanofiber-mediated microRNA delivery to enhance differentiation and maturation of oligodendroglial precursor cells. *J Control Release* **208**, 85-92, doi:10.1016/j.jconrel.2015.03.005 (2015).
- 92 Chang, K. J., Redmond, S. A. & Chan, J. R. Remodeling myelination: implications for mechanisms of neural plasticity. *Nat Neurosci* **19**, 190-197, doi:10.1038/nn.4200 (2016).
- 93 Emery, B. Transcriptional and post-transcriptional control of CNS myelination. *Current Opinion in Neurobiology* **20**, 601-607, doi:https://doi.org/10.1016/j.conb.2010.05.005 (2010).
- 94 He, X., Yu, Y., Awatramani, R. & Lu, Q. R. Unwrapping myelination by microRNAs. *Neuroscientist* **18**, 45-55, doi:10.1177/1073858410392382 (2012).
- 95 Diao, H. J., Wang, K., Long, H. Y., Wang, M. & Chew, S. Y. Highly Fluorescent and Photostable Polymeric Nanofibers as Scaffolds for Cell Interfacing and Long-Term Tracking. *Adv Healthc Mater* **5**, 529-533, doi:10.1002/adhm.201500693 (2016).
- 96 Chen, Y. *et al.* Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc* **2**, 1044-1051, doi:10.1038/nprot.2007.149 (2007).
- 97 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).
- 98 Rosenberg, S. S., Kelland, E. E., Tokar, E., De La Torre, A. R. & Chan, J. R. The geometric and spatial constraints of the microenvironment induce oligodendrocyte differentiation. *Proceedings of the National Academy of Sciences* **105**, 14662-14667 (2008).
- 99 Emery, B. Regulation of Oligodendrocyte Differentiation and Myelination. *Science* **330**, 779-782, doi:10.1126/science.1190927 (2010).
- 100 Low, W. C. *et al.* Mussel-Inspired Modification of Nanofibers for REST siRNA Delivery: Understanding the Effects of Gene-Silencing and Substrate Topography on Human Mesenchymal Stem Cell Neuronal Commitment. *Macromol Biosci* **15**, 1457-1468, doi:10.1002/mabi.201500101 (2015).
- 101 Handarmin *et al.* Nanofibrous scaffold with incorporated protein gradient for directing neurite outgrowth. *Drug Delivery and Translational Research* **1**, 147-160, doi:10.1007/s13346-011-0017-3 (2011).
- 102 Liu, T., Xu, J. Y., Chan, B. P. & Chew, S. Y. Sustained release of neurotrophin-3 and chondroitinase ABC from electrospun collagen nanofiber scaffold for spinal cord injury

- repair. *Journal of Biomedical Materials Research Part A* **100A**, 236-242, doi:10.1002/jbm.a.33271 (2012).
- 103 Chong, S. Y. C. *et al.* Neurite outgrowth inhibitor Nogo-A establishes spatial segregation and extent of oligodendrocyte myelination. *Proceedings of the National Academy of Sciences* **109**, 1299-1304 (2012).
- 104 Chooi, W. H. *et al.* Scaffold mediated gene knockdown for neuronal differentiation of human neural progenitor cells. *Biomater Sci* **6**, 3019-3029, doi:10.1039/c8bm01034j (2018).
- 105 Zhang, N. *et al.* Biomimicking Fiber Scaffold as an Effective In Vitro and In Vivo MicroRNA Screening Platform for Directing Tissue Regeneration. *Advanced Science*, 1800808, doi:10.1002/advs.201800808 (2019).
- 106 Bak, M. *et al.* MicroRNA expression in the adult mouse central nervous system. *Rna* **14**, 432-444, doi:10.1261/rna.783108 (2008).
- 107 Beveridge, N. J. & Cairns, M. J. MicroRNA dysregulation in schizophrenia. *Neurobiology of Disease* **46**, 263-271, doi:10.1016/j.nbd.2011.12.029 (2012).
- 108 Nguyen, L. H., Diao, H. J. & Chew, S. Y. MicroRNAs and their potential therapeutic applications in neural tissue engineering. *Advanced Drug Delivery Reviews* **88**, 53-66, doi:10.1016/j.addr.2015.05.007 (2015).
- 109 Karra, D. & Dahm, R. Transfection Techniques for Neuronal Cells. *Journal of Neuroscience* **30**, 6171-6177, doi:10.1523/jneurosci.0183-10.2010 (2010).
- 110 Breunig, M., Lungwitz, U., Liebl, R. & Goepferich, A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 14454-14459, doi:10.1073/pnas.0703882104 (2007).
- 111 Kim, T.-H. *et al.* Therapeutic foam scaffolds incorporating biopolymer-shelled mesoporous nanospheres with growth factors. *Acta Biomaterialia* **10**, 2612-2621, doi:10.1016/j.actbio.2014.02.005 (2014).
- 112 Kang, M. S., Kim, J. H., Singh, R. K., Jang, J. H. & Kim, H. W. Therapeutic-designed electrospun bone scaffolds: mesoporous bioactive nanocarriers in hollow fiber composites to sequentially deliver dual growth factors. *Acta Biomater* **16**, 103-116, doi:10.1016/j.actbio.2014.12.028 (2015).
- 113 Pinese, C. *et al.* Sustained delivery of siRNA/mesoporous silica nanoparticle complexes from nanofiber scaffolds for long-term gene silencing. *Acta Biomaterialia* **76**, 164-177, doi:10.1016/j.actbio.2018.05.054 (2018).
- 114 Slowing, II, Vivero-Escoto, J. L., Wu, C. W. & Lin, V. S. Y. Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers. *Advanced Drug Delivery Reviews* **60**, 1278-1288, doi:10.1016/j.addr.2008.03.012 (2008).
- 115 Xia, T. A. *et al.* Polyethyleneimine Coating Enhances the Cellular Uptake of Mesoporous Silica Nanoparticles and Allows Safe Delivery of siRNA and DNA Constructs. *Acs Nano* **3**, 3273-3286, doi:10.1021/nn900918w (2009).
- 116 Kedmi, R., Ben-Arie, N. & Peer, D. The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. *Biomaterials* **31**, 6867-6875, doi:10.1016/j.biomaterials.2010.05.027 (2010).
- 117 Mammoto, T. & Ingber, D. E. Mechanical control of tissue and organ development. *Development* **137**, 1407-1420, doi:10.1242/dev.024166 (2010).

- 118 Lampi, M. C. & Reinhart-King, C. A. Targeting extracellular matrix stiffness to attenuate disease: From molecular mechanisms to clinical trials. *Science Translational Medicine* **10**, 14, doi:10.1126/scitranslmed.aao0475 (2018).
- 119 Guilak, F. *et al.* Control of stem cell fate by physical interactions with the extracellular matrix. *Cell Stem Cell* **5**, 17-26, doi:10.1016/j.stem.2009.06.016 (2009).
- 120 Gilbert, P. M. *et al.* Substrate Elasticity Regulates Skeletal Muscle Stem Cell Self-Renewal in Culture. *Science* **329**, 1078-1081, doi:10.1126/science.1191035 (2010).
- 121 Moeendarbary, E. *et al.* The soft mechanical signature of glial scars in the central nervous system. *Nat Commun* **8**, 14787, doi:10.1038/ncomms14787 (2017).
- 122 Shao, Y. *et al.* Self-organized amniogenesis by human pluripotent stem cells in a biomimetic implantation-like niche. *Nat Mater* **16**, 419-425, doi:10.1038/nmat4829 (2017).
- 123 Lourenco, T. & Graos, M. Modulation of Oligodendrocyte Differentiation by Mechanotransduction. *Front Cell Neurosci* **10**, 277, doi:10.3389/fncel.2016.00277 (2016).
- 124 Snaidero, N. *et al.* Myelin membrane wrapping of CNS axons by PI(3,4,5)P3-dependent polarized growth at the inner tongue. *Cell* **156**, 277-290, doi:10.1016/j.cell.2013.11.044 (2014).
- 125 Chaudhuri, O. *et al.* Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nature Materials* **15**, 326-+, doi:10.1038/nmat4489 (2016).
- 126 Jagielska, A. *et al.* Mechanical Environment Modulates Biological Properties of Oligodendrocyte Progenitor Cells. *Stem Cells and Development* **21**, 2905-2914, doi:10.1089/scd.2012.0189 (2012).
- 127 Espinosa-Hoyos, D. *et al.* Engineered 3D-printed artificial axons. *Sci Rep* **8**, 478, doi:10.1038/s41598-017-18744-6 (2018).
- 128 Karttunen, M. J., Czopka, T., Goedhart, M., Early, J. J. & Lyons, D. A. Regeneration of myelin sheaths of normal length and thickness in the zebrafish CNS correlates with growth of axons in caliber. *PLoS One* **12**, e0178058, doi:10.1371/journal.pone.0178058 (2017).
- 129 Arani, A. *et al.* Measuring the effects of aging and sex on regional brain stiffness with MR elastography in healthy older adults. *Neuroimage* **111**, 59-64, doi:10.1016/j.neuroimage.2015.02.016 (2015).
- 130 Sim, F. J., Zhao, C., Penderis, J. & Franklin, R. J. M. The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. *Journal of Neuroscience* **22**, 2451-2459, doi:10.1523/jneurosci.22-07-02451.2002 (2002).
- 131 Chang, A. S. *et al.* Cortical Remyelination: A New Target for Repair Therapies in Multiple Sclerosis. *Annals of Neurology* **72**, 918-926, doi:10.1002/ana.23693 (2012).
- 132 Kruse, S. A. *et al.* Magnetic resonance elastography of the brain. *Neuroimage* **39**, 231-237, doi:10.1016/j.neuroimage.2007.08.030 (2008).
- 133 Fletcher, D. A. & Mullins, R. D. Cell mechanics and the cytoskeleton. *Nature* **463**, 485-492, doi:10.1038/nature08908 (2010).
- 134 Ouyang, H., Nauman, E. & Shi, R. Y. Contribution of cytoskeletal elements to the axonal mechanical properties. *Journal of Biological Engineering* **7**, 8, doi:10.1186/1754-1611-7-21 (2013).

- 135 Yang, F., Murugan, R., Wang, S. & Ramakrishna, S. Electrospinning of nano/micro scale poly(L-lactic acid) aligned fibers and their potential in neural tissue engineering. *Biomaterials* **26**, 2603-2610, doi:10.1016/j.biomaterials.2004.06.051 (2005).
- 136 Garlotta, D. A literature review of poly(lactic acid). *Journal of Polymers and the Environment* **9**, 63-84, doi:10.1023/a:1020200822435 (2001).
- 137 Lamoureux, P. L., O'Toole, M. R., Heidemann, S. R. & Miller, K. E. Slowing of axonal regeneration is correlated with increased axonal viscosity during aging. *BMC Neurosci.* **11**, 9, doi:10.1186/1471-2202-11-140 (2010).
- 138 Schwach, G., Coudane, J., Engel, R. & Vert, M. Ring opening polymerization of D,L-lactide in the presence of zinc metal and zinc lactate. *Polymer International* **46**, 177-182, doi:10.1002/(sici)1097-0126(199807)46:3<177::aid-pi937>3.0.co;2-s (1998).
- 139 Pinese, C. *et al.* Rolled knitted scaffolds based on PLA-pluronic copolymers for anterior cruciate ligament reinforcement: A step by step conception. *Journal of Biomedical Materials Research Part B-Applied Biomaterials* **105**, 735-743, doi:10.1002/jbm.b.33604 (2017).
- 140 Chew, S. Y., Hufnagel, T. C., Lim, C. T. & Leong, K. W. Mechanical properties of single electrospun drug-encapsulated nanofibres. *Nanotechnology* **17**, 3880-3891, doi:10.1088/0957-4484/17/15/045 (2006).
- 141 Almeida, R. G. *et al.* Myelination of Neuronal Cell Bodies when Myelin Supply Exceeds Axonal Demand. *Curr Biol* **28**, 1296-1305 e1295, doi:10.1016/j.cub.2018.02.068 (2018).
- 142 Boyan, B. D., Hummert, T. W., Dean, D. D. & Schwartz, Z. Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials* **17**, 137-146, doi:10.1016/0142-9612(96)85758-9 (1996).
- 143 Pan, S. & Chan, J. R. Regulation and dysregulation of axon infrastructure by myelinating glia. *Journal of Cell Biology* **216**, 3903-3916, doi:10.1083/jcb.201702150 (2017).
- 144 Green, A. J. *et al.* Clemastine fumarate as a remyelinating therapy for multiple sclerosis (ReBUILD): a randomised, controlled, double-blind, crossover trial. *Lancet* **390**, 2481-2489, doi:10.1016/s0140-6736(17)32346-2 (2017).
- 145 Gladman, A. S., Matsumoto, E. A., Nuzzo, R. G., Mahadevan, L. & Lewis, J. A. Biomimetic 4D printing. *Nat Mater* **15**, 413-418, doi:10.1038/nmat4544 (2016).
- 146 Hagemeyer, N. *et al.* Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. *Acta Neuropathologica* **134**, 441-458, doi:10.1007/s00401-017-1747-1 (2017).
- 147 Veiga, N. *et al.* Cell specific delivery of modified mRNA expressing therapeutic proteins to leukocytes. *Nat Commun* **9**, 4493, doi:10.1038/s41467-018-06936-1 (2018).
- 148 Rosenholm, J., Sahlgren, C. & Linden, M. Cancer-cell targeting and cell-specific delivery by mesoporous silica nanoparticles. *Journal of Materials Chemistry* **20**, 2707-2713, doi:10.1039/b920076b (2010).
- 149 Langert, K. A. & Brey, E. M. Strategies for Targeted Delivery to the Peripheral Nerve. *Front Neurosci* **12**, 887, doi:10.3389/fnins.2018.00887 (2018).
- 150 Lee, S., Ashizawa, A. T., Kim, K. S., Falk, D. J. & Notterpek, L. Liposomes to Target Peripheral Neurons and Schwann Cells. *Plos One* **8**, 8, doi:10.1371/journal.pone.0078724 (2013).
- 151 Meng, H. *et al.* Use of a Lipid-Coated Mesoporous Silica Nanoparticle Platform for Synergistic Gemcitabine and Paclitaxel Delivery to Human Pancreatic Cancer in Mice (vol 9, pg 3540, 2015). *Acs Nano* **10**, 6416-6416, doi:10.1021/acsnano.6b03110 (2016).

- 152 Johnson, R. & Halder, G. The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. *Nature Reviews Drug Discovery* **13**, 63-79, doi:10.1038/nrd4161 (2014).
- 153 Murphy, M. C. *et al.* Regional brain stiffness changes across the Alzheimer's disease spectrum. *Neuroimage-Clinical* **10**, 283-290, doi:10.1016/j.nicl.2015.12.007 (2016).