DESIGN AND DEVELOPMENT OF TWO NOVEL 3D CONDUCTIVE THREAD SCAFFOLDS FOR NERVE TISSUE ENGINEERING

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Nooshin Sadeghi Taheri
Masters of Engineering (By research)
Swinburne University of Technology

School of Applied Sciences
College of Science Engineering and Health
RMIT University

February 2017
Design and development of two novel 3D conductive thread scaffolds for nerve tissue engineering

Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Nooshin Sadeghi Taheri

28 Feb. 17
“THE MOST COMPLETE GIFT OF GOD IS
A LIFE BASED ON KNOWLEDGE”

IMAM ALI
DEDICATION

In the name of God, the most gracious, the most merciful

In honor of

My loving mother

Who taught me that it is never too late to pursue your true passion

In memory of

My beloved father who always encouraged to strive for excellence in education and have faith

To my husband Dr Shahin Khoddam for his support, encouragement and faith in me

And to my beautiful children who always supported me with their love
ACKNOWLEDGEMENT

I would like to express my gratitude to Dr Peggy Chan, for giving me the opportunity to do research in tissue engineering field that has become my passion. For her supportive and friendly supervision, faith and guidance throughout this research project. I have learned enormously from you and I am always grateful to you for your support.

My special gratitude should be directed to my second supervisor Dr Andrew Hung for his great support, understanding and being positive.

I would also like to extend my gratitude to Professor Wei Shen for his valuable knowledge, great inspiration and excellent advice during my candidature.

I also acknowledge the collaboration of Associate Professor Tan Thatt Yang Timothy, with his valuable knowledge on first part of the research.

My sincere appreciation is also extended to Professor Kourosh Kalantar Zadeh for the collaboration and assistance with his priceless knowledge, intelligence and enthusiasm. He was an inspiration to have as a great mentor and advisor.

My special thanks go to my colleagues and friends, especially Kai Wei Chan, Dr Layla Mehdi Alhasan; the general/support staff at RMIT School of Applied Science (especially Nadia Zakhartchouk and Lisa Diaz) for their support and friendship during my research.

I would like to thank Australian Federal Government and RMIT University for presenting me with the Australian Postgraduate Awards to support me throughout my studies.

Last but not least I am grateful to my family for all their love and support always.
# TABLE OF CONTENTS

Design and Development of two novel 3D conductive thread scaffolds for nerve tissue engineering ......................................................... 1
Dedication .......................................................................................................................................................................................................................... iii
Acknowledgement .......................................................................................................................................................................................... iv
Table of Contents ......................................................................................................................................................................................... v
List of Figures .......................................................................................................................................................................................... xi
List of Tables .......................................................................................................................................................................................... xiv
Publications ............................................................................................................................................................................................. xv
Abstract .................................................................................................................................................................................................................. xvi

## CHAPTER ONE

Introduction and Literature Review .......................................................................................................................................................... 20

1.1. Overview ........................................................................................................................................................................................................................................... 20

1.2. Significance and innovation ................................................................................................................................................................................................. 21

1.3. Outline of the thesis ................................................................................................................................................................................................................................. 22

1.4. Literature review ........................................................................................................................................................................................................................................... 25

1.4.1. Tissue engineering ................................................................................................................................................................................................................................. 25

1.4.1.1. Peripheral nerve injury ................................................................................................................................................................................................................................. 27

1.4.2. Biomaterials for nerve tissue engineering ......................................................................................................................................................................................... 29

1.4.2.1. Conducting polymers (CPs) ................................................................................................................................................................................................................................. 29

1.4.2.2. Carbon Nanotubes CNTs ................................................................................................................................................................................................................................. 30

1.4.2.3. Graphene ................................................................................................................................................................................................................................. 30

1.4.2.3.1. Graphene Oxide synthesis ................................................................................................................................................................................................................................. 31

1.4.2.3.2. Graphene Oxide reduction methods ................................................................................................................................................................................................................................. 32

1.4.2.4. Biocompatibility of reduced graphene oxide ................................................................................................................................................................................................................................. 33

1.4.3. Thread ................................................................................................................................................................................................................................. 35

1.4.3.1. Conductive Thread ................................................................................................................................................................................................................................. 38

1.4.3.2. Silk applications in tissue engineering repair ................................................................................................................................................................................................................................. 39

1.4.3.3. Electrospun nanofibers ................................................................................................................................................................................................................................. 40

1.4.4. Molybdenum disulphide ................................................................................................................................................................................................................................. 41

1.4.4.1. Applications of MoS2 ................................................................................................................................................................................................................................. 42

1.4.4.2. Molybdenum disulphide in tissue engineering applications ................................................................................................................................................................................................................................. 43
1.4.4.3. Molybdenum disulphide lithium ion intercalation ........................................ 43
1.4.4.4. Biological applications of Molybdenum disulphide ............................... 44
1.4.5. Cell adhesion .............................................................................................. 45
1.4.6. Electrical stimulation of neural cells seeded on nerve scaffolds .............. 46
1.4.6.1. Electrical activity recording ................................................................. 49

CHAPTER TWO .................................................................................................... 51
Materials and Methods ...................................................................................... 51
2.1. Introduction ................................................................................................... 51
2.1.1. Synthesis of graphene oxide .................................................................... 51
2.1.2. Preparation of GO solution ...................................................................... 52
2.1.3. Cotton thread preparation ......................................................................... 53
2.1.4. Cationic Polyacrylamide preparation ......................................................... 53
2.1.5. Coating GO onto the CPAM treated threads ............................................. 53
2.1.6. Graphene Oxide reduction ......................................................................... 54
2.1.6.1. Metal iodide treatment with FeI2 ........................................................... 54
2.1.6.2. Electrochemical reduction of graphene oxide ......................................... 54
2.1.7. Scanning electron microscopy –SEM ........................................................ 55
2.1.8. Electrical conductivity of the thread samples ............................................. 55
2.1.9. X-ray photoelectron spectroscopy-XPS .................................................... 57
2.1.10. Biocompatibility of rGO-thread ............................................................... 57
2.1.10.1. PC12 cell culture .................................................................................. 57
2.1.10.2. AlamarBlue® Assay ......................................................................... 58
2.2. Molybdenum disulfide .................................................................................. 58
2.2.1. MoS2 nanoflakes preparation .................................................................... 58
2.2.2. MoS2 coating onto the cotton thread ......................................................... 59
2.2.3. Lithium Ion Intercalation of MoS2-Thread ............................................... 59
2.2.4. Electrical conductivity of rMoS2-thread ................................................... 60
2.2.5. X-ray photoelectron spectroscopy of MoS2 thread .................................. 60
2.2.6. Permeability of conductive thread ............................................................ 60
2.2.7. FEI Nova NanoSEM ................................................................................ 62
2.2.8. Biocompatibility of rMoS2-thread ............................................................. 62
2.2.8.1. NG108-15 Cell culture .......................................................................... 62
2.2.8.2. AlamarBlue® assay ............................................................................. 62
2.3. Surface functionalization of the coated threads ........................................... 63
2.3.1. Preparation of CDI/Gelatin ................................................................. 63
2.3.2. Determination of amine content by Orange acid II assay ...................... 64
2.3.3. Permeability of sf-rGO thread and sf-rMoS₂ thread ............................ 64
2.3.4. Biocompatibility of surface functionalized threads ............................... 64
2.4. Cell seeding in surface functionalized thread scaffolds ............................. 65
  2.4.1. CMC-TYR synthesis and preparation ............................................. 65
    2.4.1.1. Seeding the cells onto surface functionalized thread scaffolds .......... 65
  2.4.2. Live/Dead cytotoxicity assay .......................................................... 66
2.5. Electrical stimulation of neural cells (NG108-15) .................................. 66
  2.5.1. Electrical stimulation of NG108-15 cells ........................................ 66
  2.5.2. Live/Dead cytotoxicity assay on ES-cells ........................................ 67
  2.5.3. PicoGreen® assay on ES-Cell/Scaffolds .......................................... 67
  2.5.4. Live cell imaging with voltage-sensitive dyes .................................... 67
    2.5.4.1. FluoVolt™ solution preparation .................................................. 67
    2.5.4.2. Preparing 20 mM Glucose +Live Cell Imaging Solution .................. 68
    2.5.4.3. Valinomycin ........................................................................... 68
    2.5.4.4. Voltage-sensitive dyes (VSDs) ...................................................... 68
    2.5.4.5. Optical imaging ......................................................................... 69
CHAPTER THREE ............................................................................................. 70
Biocompatibility of the reduced graphene oxide coated thread for tissue engineering 70
  3.1. Introduction .......................................................................................... 70
  3.2. Materials and methods ........................................................................ 72
    3.2.1. Enhancing adsorption of rGO particles to cotton thread .................. 72
    3.2.2. Graphene Oxide synthesis .............................................................. 73
      3.2.2.1. Preparation of GO solution .......................................................... 74
    3.2.3. Graphene Oxide reduction .............................................................. 74
      3.2.3.1. Electrochemical reduction of graphene oxide ............................... 75
      3.2.3.2. Metal iodide treatment of graphene oxide ................................. 75
    3.2.4. Electrical conductivity of reduced GO threads ................................. 76
    3.2.5. Characterization of reduced GO-thread .......................................... 77
      3.2.5.1. X-ray photoelectron spectroscopy of rGO thread ....................... 77
      3.2.5.2. Scanning electron microscopy .................................................... 77
    3.2.6. Permeability of rGO-thread ......................................................... 78
    3.2.7. Biocompatibility of rGO-threads toward nerve cells ....................... 78
3.2.7.1. Cell culture............................................................................................................ 78
3.2.1. Statistical analysis......................................................................................................... 79
3.3. Results and Discussions.................................................................................................. 80
3.4. Electrical conductivity of rGO-thread........................................................................... 80
3.5. SEM ...................................................................................................................................... 81
3.6. x-ray photoelectron spectroscopy .................................................................................. 84
3.7. Wicking properties of thread samples............................................................................. 86
3.8. Biocompatibility of reduced graphene oxide coated threads ........................................ 87
3.8.1. Metabolic activity assay................................................................................................ 87
3.9. Conclusion ............................................................................................................................ 89

CHAPTER FOUR................................................................................................................................. 91

Biocompatibility of intercalated Molybdenum disulphide coated thread scaffold .................. 91
4.1. Introduction........................................................................................................................... 91
4.2. Methods and materials ........................................................................................................ 96
4.2.1. 2D MoS₂ nanoflakes preparation .................................................................................. 96
4.2.2. Preparation of conductive MoS₂-thread ........................................................................ 96
4.2.2.1. Adsorption of MoS₂ nanoflakes to the cotton thread ............................................ 96
4.2.2.2. MoS₂ coating onto the cotton thread ..................................................................... 97
4.2.2.3. Lithium Ion intercalation of MoS₂-thread ............................................................... 97
4.3. Electrical conductivity of the MoS₂-threads ..................................................................... 98
4.4. Characterization of conductive rMoS₂-thread ................................................................. 100
4.4.1. X-ray photoelectron spectroscopy of rMoS₂-thread.................................................... 100
4.4.2. RAMAN spectroscopy of MoS₂ thread................................................................. 100
4.4.3. FEI Nova NanoSEM ™ .............................................................................................. 101
4.5. Permeability of conductive rMoS₂-thread ..................................................................... 101
4.6. Biocompatibility of conductive rMoS₂-thread ................................................................. 101
4.6.1. Cell culture .................................................................................................................. 101
4.6.2. Metabolic activity assay on rMoS₂-thread towards neural cells ................................. 102
4.6.3. Statistical analysis ........................................................................................................ 102
4.7. Results and discussions .................................................................................................... 103
4.7.1. Lithium ion intercalation of MoS₂ ............................................................................... 103
4.7.2. Raman spectroscopy .................................................................................................. 105
4.7.3. FEI Nova NanoSEM ................................................................................................. 106
4.7.4. Electrical conductivity of rMoS₂-thread ..................................................................... 108
4.7.5. Permeability of rMoS2-thread ..................................................................................... 109
4.7.6. Biocompatibility of rMoS2-threads ............................................................................. 110
4.8. Conclusion .......................................................................................................................... 112
CHAPTER FIVE ........................................................................................................................ 114
Surface functionalization of rGO and rMoS2 thread scaffolds .................................................. 114
5.1. Introduction ......................................................................................................................... 114
5.2. Materials and methods ........................................................................................................ 117
5.2.1. Preparation of gelatin functionalized scaffolds ........................................................... 117
5.2.2. Determination of amine content by Acid Orange II assay .......................................... 117
5.2.3. Characterization of cell morphology and viability ...................................................... 118
5.2.4. Metabolic activity of cell seeded on thread scaffolds .................................................. 118
5.2.5. Permeability assay on surface functionalized threads ................................................. 119
5.2.5.1. Plasma treatment ................................................................................................. 119
5.2.5.2. Wettability assay ................................................................................................. 120
5.2.6. Statistical analysis ....................................................................................................... 122
5.3. Results and discussion ...................................................................................................... 122
5.3.1. Surface functionalization of 3D thread scaffolds ......................................................... 122
5.3.2. Physiochemical properties of surface functionalized rGO-thread and rMoS2-threads 124
5.3.3. Permeability of the thread samples after surface functionalization ............................. 125
5.3.4. Biocompatibility of SF-rGO and SF-rMoS2 thread scaffolds ..................................... 128
5.4. Conclusions ......................................................................................................................... 131
CHAPTER SIX .......................................................................................................................... 132
Electrical stimulation of neural cells within conductive nerve tissue scaffolds ....................... 132
6.1. Introduction ......................................................................................................................... 132
6.2. Materials and methods ........................................................................................................ 136
6.2.1. Electrical stimulation set up ........................................................................................ 136
6.2.2. Electrical stimulation of NG108-15 cells .................................................................... 136
6.2.3. Cell morphology and Live/Dead cytotoxicity assay ................................................... 137
6.2.4. Electrical stimulation of NG108-15 cells seeded onto thread scaffolds ...................... 138
6.2.5. Proliferation culture and assay of electrical stimulated cell-scaffolds ......................... 139
6.2.6. Live cell imaging by voltage-sensitive dye “FluoVolt™ Membrane potential” .............. 141
6.2.6.1. Differentiating cell culture ..................................................................................... 141
6.2.7. FluoVolt™ membrane potential solution preparation ............................................... 141
6.2.8. Preparing live cell imaging solution ............................................................................... 142
**LIST OF FIGURES**

**Figure 1.1** A tissue engineering model that demonstrates cell seeding within porous scaffolds [4]. . . . 26

**Figure 1.2** Schematic of the damaged and regenerated nerve after peripheral nerve injury. [19] ....... 28

**Figure 3.1** Image of three samples of rGO-thread held by transparent polymer film to record the  
wicking rate of liquid dispersed on protruding ends of the threads. ................................................... 78

**Figure 3.2** SEM Micrographs of A) GO 2%-thread B)GO 5%-thread C) ER-GO 2%-thread, D) ER- 
GO5%-thread E) FeI2-GO2%-thread F) FeI2-GO5%-thread G) CPAM-thread H) Blank thread. .......... 83

**Figure 3.3** XPS spectra of the GO on threads before and after reduction; a)GO 2%-thread, b)GO5%- 
thread, c)ER-rGO2% thread, d)ER-GO5% thread, e)FeI2-GO2% thread, f)FeI2-GO5% thread. ........ 86

**Figure 3.4** Metabolic activity of PC12 cells in presence of FeI2-GO thread and ER-GO thread in two 
concentrations compared to cells unexposed to threads (control) reported in percentage of alamarBlue  
reduction. Data are shown as mean values ± standard deviation(n = 3)............................................. 88

**Figure 4.1** XPS spectra of the elements of A-B) Li, C-D) S, and E-F) Mo in MoS2 coated threads  
before and after Li+ ion intercalation. .................................................................................................. 104

**Figure 4.2** Raman spectra of MoS2-thread before and after Lithium ion intercalation. ...................... 106

**Figure 4.3** HRSEM images of A and D) CPAM thread B and E) MoS2-thread C and F) rMoS2-thread  
............................................................................................................................................................ 106

**Figure 4.4** SEM images of A and B) MoS2-thread, C and D) rMoS2-thread E and F) Blank thread 108

**Figure 4.5** Wicking rate of dye solution along the length of rMoS2-thread before and after surface 
functionalization. Standard deviation was calculated from mean value of three different measurements  
............................................................................................................................................................ 110

**Figure 4.6** PC12 cells viability is assessed and compared between MoS2-thread before and after ion  
intercalation and GO thread before and after reduction using alamarBlue assay. PC12 cells’ viability  
was assessed and compared as control. Data are reported as percentage of reduced alamarBlue  
solution and are mean values ± standard deviations n = 3. ............................................................... 111

**Figure 4.7** Comparison of NG108-15 cell viability measured with alamarBlue® assay, following 5  
days of exposure to the rGO-thread and rMoS2-thread, compared to the cells without exposure to  
threads as control. They are reported as percentage of reduced alamarBlue solution and are mean  
values ± standard deviations of three repeat experiments, each consisting of four wells per sample  
each experiment ....................................................................................................................................... 112

**Figure 5.1**. Wettability assay set up for a) rGO thread and b) rMoS2 thread is shown. ................. 120

**Figure 5.2**. Image of wicking of the fluorescent dye up on the sf-rMoS2 thread scaffolds attached to  
transparent polymer film and paper scales .......................................................................................... 121

**Figure 5.3** NG108-15 cells morphology by confocal microscopy. In these figures viability of neuronal  
cells grown on thread scaffolds for 4 days without surface functionalization a) have fewer cells  
attached and b) surface functionalized threads with CDI/Gelatin show better cell attachments. ...... 123

**Figure 5.4**. Amount of absorbed Acid Orange II onto a) sf-rGO thread compared with untreated rGO  
thread scaffold and b) sf-rMoS2 thread scaffold compared with untreated rMoS2 thread scaffold. ... 124

**Figure 5.5**. Graph of wettability rate of thread samples regarding rMoS2-thread: Blank thread, CPAM  
thread, MoS2-thread before and after ion intercalation, rMoS2-thread before and after surface
functionalization and plasma treated rMoS₂ thread. Error bars indicate one standard deviation from the mean $n = 3$.

**Figure 5.6** Graph of wettability rate of rGO-thread scaffolds compared with the blank thread and CPAM coated thread, before and after GO reduction as well as after surface functionalization of rGO-thread and plasma treated rGO thread. Error bars indicate one standard deviation from the mean $n = 3$.

**Figure 5.7** Both samples are hydrophilic; however with plasma treatment hydrophilicity of thread scaffolds increased after surface functionalization.

**Figure 5.8** Metabolic rate of seeded NG108-15 cells after surface functionalization of rMoS₂-thread at day 5, 6 and 7 was determined via alamarBlue® assay. The data are presented as mean $n = 3$ and error bars represent the standard deviation.

**Figure 5.9.** Metabolic rate of NG108-15 cells towards rGO-thread scaffold after surface functionalization using alamarBlue® assay over 7 days after seeding and assessed for 3 consecutive days after culture. Results expressed as mean $n = 3$ ± standard deviation. Significantly different results presented as $p < 0.05$.

**Figure 6.1** Electrical stimulation set up for NG108-15 cells seeded on thread scaffolds.

**Figure 6.2** Image of electrical stimulation set up for sf-rGO threads, sf-rMoS₂-threads and control cells in a 24-well plate.

**Figure 6.3** Confocal laser scanning microscope images of Calcein AM and PI on NG108-15 cells electrically stimulated at 20V with different duration. Analysis of electrical stimulation of NG108-15 cells at 20V potential at different duration of 5, 30 and 60 min are reported, 24 hours post electrical stimulation, bright field images (a), Viable cells are stained with Calcein AM showing in green (b), Dead cells floating are stained with propidium iodide (PI) (C), and finally overlay of all these fields (d). Scale bar represents 100µm.

**Figure 6.4** Immunostaining of NG108-15 neural cells 24 hours after electrical stimulation at 20 V for 5min, 30 min and 60min and non-stimulated cells serve as control. Confocal analysis images (a) Bright field images, (b) Viable cells (green) stained with Calcein AM, (c) Dead cells (red) stained with PI and (d) Overlay image of all these fields. Scale bar represents 100 µm.

**Figure 6.5** NG108-15 cell proliferation after electrical stimulation seeded inside the sf-rGO tissue scaffolds. Quant-iT PicoGreen® ds DNA assay was used to quantify the cell proliferation by the DNA content. The data represent mean ± standard error $n = 4$.

**Figure 6.6** NG108-15 cells proliferation after electrical stimulation seeded inside the sf-rMoS₂ tissue scaffolds.

**Figure 6.7** Confocal laser scanning microscope images of Calcein AM and PI on NG108-15 cells seeded onto the thread scaffolds, electrically stimulated at 60mV for 5 min at 5 Hz frequency. a) non-stimulated Cell/rGO thread scaffold, b) electrically stimulated cell/rGO thread scaffold.

**Figure 6.8** Confocal laser scanning microscope images of Calcein AM and PI on NG108-15 cells seeded onto the thread scaffolds, electrically stimulated at 60mV for 5 min at 5 Hz frequency. c) non-stimulated cell/rMoS₂ thread scaffold and d) electrically stimulated cell/rMoS₂ thread scaffold. Scale bar represents 100µm.

**Figure 6.9** Electrical response of neuron cells on sf-rGO thread scaffolds. Field potentials are calculated by FluoVolt™ membrane potential and laser confocal microscopic live cell imaging. Field
potential traces from cells on sf-rGO thread scaffold are shown and the electrical activity of neuronal network formed on the tissue scaffold is confirmed. X-axis represents Time in msec and Y-axis or transmembrane voltage is presented as fluorescent intensity. ............................................................ 154

**Figure 6.10** Electrical response of neuron cells on sf-rMoS2 thread scaffolds. Field potentials are calculated by FluoVolt™ membrane potential and laser confocal microscopic live cell imaging. Field potential traces from cells on sf-rMoS2 thread scaffold are shown and the electrical activity of neuronal network formed on the tissue scaffold is confirmed. X-axis represents Time in msec and Y-axis or transmembrane voltage is presented as fluorescent intensity. ............................................................ 155
LIST OF TABLES

**Table 3.1.** Electrical resistance, resistivity and conductivity of coated thread samples.........................80

**Table 3.2** Rate of Wicking for Different Types of Thread Samples .................................................................86

**Table 4.1** Electrical resistivity and conductivity of blank thread, MoS$_2$ thread and rMoS$_2$-thread....109
**PUBLICATIONS**

Journal Publications

- **Nooshin S. Taheri**, Peggy Chan, Wei Shen, Andrew Hung, “Biocompatibility of Conductive rGO coated threads towards nerve cells”, Submitted

- **Nooshin S. Taheri**, Peggy Chan, Wei Shen, “Fabrication and characterization of rMoS2 coated thread: its electro-conductivity and biocompatibility on neural cell growth” manuscript to be submitted

- **Nooshin S. Taheri**, Peggy Chan, Wei Shen, Kai Wei Chang, Kourosh Kalantar Zadeh “Molybdenum disulphide scaffolds engineered to promote cell adhesion towards electrical stimulation of NG108-15 cells for peripheral nerve repair”, (Under review)


Conference Publications

ABSTRACT

Millions of patients suffer from peripheral nerve injuries developed as a result of damage to peripheral nervous system. Nerve tissue engineering is a promising approach to repair damaged or malfunctioning peripheral nerves (auto graft) caused by accident or disease. There are different approaches to achieve this; one is to combine a fabricated scaffold made from one or two materials with appropriate properties with specific cells and physical cues to motivate cells to develop into functional tissues. Different kind of tissues has specific challenges and requirements, from the shape of the scaffold (e.g., tubes, sponges, blocks etc) and the physical properties of the biomaterials (e.g., porosity, degradability, stiffness) to the ultimate biological results (e.g., promoting cell proliferation, differentiation, cell invasion).

The field of tissue engineering and biomaterial development demand a range of expertise, since all tissue engineering areas require different materials and fabrication strategies and planning. The form of implants varies between the applications. Some applications such as muscle or fat need the replacement of large blocks of tissues. Others like replacing the skin in healing wound need a membrane. To engineer an implant for nerve or muscle fibers, fibrous structures are considered. After adapting material and scaffold properties to different tissues in the body, the most important property essential for all tissue engineering is biocompatibility. This property means that the material and scaffold does not have toxicity and will not induce any harmful effect towards considered tissues or cells. In this study, two 2D materials (GO, MoS$_2$) with excellent properties are introduced to be combined with common cotton thread as scaffold for the first time in nerve tissue engineering.

The aim of this project is to discover an alternate substrate like thread and other materials that enhance conductivity, permeability and better electrical guidance for nerve tissue scaffolds. Current tissue scaffold system has limited permeability due to the lack of the vascular system. The use of cotton thread can be used to mimic the vascular system to promote permeability,
in terms of moving nutrients, removing waste and other biomolecules. Conductive materials allow electrical stimulation which may be advantageous for nerve tissue regeneration and to control electrical signal direction through wettability of the thread. **Chapter 3** aims to investigate the biocompatibility and conductivity of the reduced graphene oxide coated threads as a tissue engineering scaffold. To achieve this, first graphene oxide was synthesized followed by coating the CPAM treated threads with graphene oxide particles. Next graphene oxide was reduced by two method of reduction; electrochemical reduction and metal iodide treatment. The resulting scaffolds exhibited proper conductivity in both methods and concentrations of graphene oxide, however, electrochemical reduction of GO proved better conductivity compared to metal iodide treatment method. As far as permeability is concerned, electrochemical reduction exhibited improved wicking properties of thread scaffolds, which may play a role in better neurite exchange and guiding electrical activity along the thread scaffolds within the cell culture system. The last step was to investigate biocompatibility of rGO coated thread, with a promising outcome confirming that rGO-thread scaffolds are biocompatible. Overall, this study showed that rGO thread scaffold can be a promising nerve tissue scaffold for nerve tissue engineering applications.

The conception of the second project is initiated in emergent of molybdenum disulphide (MoS$_2$) as its recent progress suggest its new interesting applications in biomedical field and existing graphene with its excellent properties to be investigated and compared for better tissue scaffolds materials. In **Chapter 4**, molybdenum disulphide was synthesized and coated to the treated cotton thread. Molybdenum disulphide was successfully rendered conductive by electrochemical intercalation and characterized by XPS, RAMAN and SEM. The wicking properties of rMoS$_2$-thread were determined. And finally, rMoS$_2$-thread showed biocompatibility to nerve cells.
These developed tissue scaffolds were surface functionalized by CDI treatment conjugated with gelatin to enhance cell adhesion on the tissue scaffolds.

The three-dimensional thread scaffolds were assessed for permeability, cell adhesion and biocompatibility in Chapter 5. First approach was to enhance and promote attachment of nerve cells (NG108-15) seeded onto the coated thread scaffolds to the surface of tissue scaffold. Surfaces and layers of coated (rGO 2%, rMoS₂) thread scaffolds were engineered by treatment of CDI conjugated with gelatin solutions to enhance neural cell adhesion. Before cell seeding onto the surface functionalized thread scaffolds, the accessible amine content on the surface of all samples were investigated using Acid orange II assay. In both tissue scaffolds (rGO2%, rMoS₂) amine content was successfully increased by surface functionalization to promote cell adhesion. By developing a new protocol to optimize CDI/Gelatin tissue scaffold’s surface functionalization cell attachment was improved in both tissue scaffolds. Then cell adhesion was investigated by Immunofluorescence assay under confocal laser microscopy. Second approach was to investigate permeability of coated thread scaffolds after surface functionalization. The permeability of these three dimensional tissue scaffolds was determined by wettability assay. The wettability assay revealed a decrease of permeability in both tissue scaffolds after surface functionalization. Plasma treatment was carried out on all thread samples to facilitated liquid wicking properties of the surface functionalized (rGO2%, rMoS₂) tissue scaffolds in two folds.

Electrical stimulation of NG108-15 cells attached on the thread scaffolds was established and investigated in Chapter 6 for proliferation and cell functionality. The proliferation rates of the electrically stimulated nerve cells were significantly higher than the non-stimulated ones. Excitability of neurons in engineered nerve tissue scaffolds were determined by optical imaging of cell signaling using voltage-sensitive dyes. These findings highlight for the first time the possibility of enhancing nerve regeneration through conductive thread scaffolds with
excellent surface properties and cell proliferation. Our novel integration of engineered cotton thread with reduced graphene oxide (rGO2%) and new biomaterial rMoS₂ as a 3D nerve tissue scaffold provides a favourable nerve conduit for peripheral nerve repair and other neural applications. **Third approach** was to establish electrical stimulation properties for NG108-15 cell line by investigating new electrical stimulation setting, voltage, frequency and duration to properly excite NG108-15 neural cells. A potential of 60 mV/mm for 5 min at 5 Hz was selected with best results to be applied to the cells through customized electrical 6-well plate system using a signal generator. To carry out the electrical stimulation, two platinum plates attached to the customise 6-well plate lid, were in contact with the cells though medium. Direct current was applied to the cells to regulate cell functions. **Fourth approach** is to electrically stimulate neural cells NG108-15 while seeded and attached to the conductive thread tissue scaffolds. After electrical stimulation of the cell-scaffolds, it is required to investigate cell viability, proliferation and functionality of scaffolds. Cell-scaffolds viability was investigated by alamarBlue® assay, and then proliferation rates of the electrically stimulated cell-scaffolds were carried out by PicoGreen® assay, with significantly higher rate than non-stimulated cell-scaffolds. Then for the first time, excitability of our new developed engineered nerve tissue scaffolds was determined and recorded by optical imaging of cell signaling using voltage-sensitive dyes (FluoVolt™ membrane assay kit).

To the best of author’s knowledge, there are no prior reports on employing conductive common cotton thread in nerve tissue engineering and nerve repair. This is the first time that reduced graphene oxide coated thread has been used as a nerve tissue scaffolds. Also, employing molybdenum disulphide to make cotton thread conductive to be used as a nerve tissue scaffold is a novel idea to create a second nerve tissue scaffold for nerve tissue repair.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. OVERVIEW

The aim of tissue engineering is to repair or replace tissues or organs that have been damaged and destroyed by accident or disease. There are different approaches to achieve this; one is to combine a fabricated scaffold made from one or two materials with appropriate properties with specific cells and scaffolds to motivate cells to develop into functional tissues or organs. Different kind of tissues have specific challenges and requirements, from the shape of the scaffold (e.g., tubes, sponges, blocks etc) and the physical properties of the biomaterials (e.g., porosity, degradability, flexibility) to the ultimate biological results (cell proliferation, differentiation, cell adhesion) [1]. The field of tissue engineering and biomaterial development demand a range of expertise, since all tissue engineering areas require different materials and fabrication strategies and planning. The form of implants varies between the applications. Some applications such as muscle or fat need the replacement of large blocks of tissues. Others like replacing the skin in wound healing need a membrane. To engineer an implant for nerve or muscle fibers, fibrous structures are considered. After adapting material and scaffold properties to different tissues in the body, the most important property essential for all tissue engineering is biocompatibility. This property means that the material and scaffold does not have toxicity and will not induce any harmful effect towards considered tissues or cells.
CHAPTER ONE

1.2. SIGNIFICANCE AND INNOVATION

In the present research, our goal is to introduce the common cotton thread as a novel 3D substrate to serve as a nerve tissue scaffold. In order to make the cotton thread a more suitable scaffold for neural regeneration, it is first surface engineered to enhance attachment of the conductive materials. Thread as a scaffold has a surface structure with porosity, permeability, electrical guidance through its wettability, cell alignment and flexibility. Two 2D materials; graphene and molybdenum disulphide; with excellent properties have been considered for the purpose of this research. The use of nanoflakes of molybdenum disulphide in the design of thread tissue scaffolds is considered to enhance neuronal adhesion. Thread substrate with nanoflakes biomaterials facilitates better interactions between neuron tissues and nanomaterial. These biomaterials are made conductive through different approaches to promote the inherent electrical activity of nerve tissues. These electrical conductive materials will encourage increased nerve or axonal regeneration through electrical stimulation. The administration of electrical stimulation encourages the growth of tissue for transplantation. Reduced Graphene oxide and intercalated molybdenum disulphide with high conductivity can be developed as neural prosthesis in nerve tissue engineering applications. This research has combined important elements in design and fabrication of two novel nerve tissue scaffolds to facilitate nerve cells to adhere, proliferate and finally form nerves.
CHAPTER ONE

1.3. OUTLINE OF THE THESIS

In Chapter 1, an extensive literature review has been developed on both graphene and molybdenum disulphide applications and properties. The literature on the cotton thread and its applications in different fields is reported. A background on nerve tissue engineering and different conductive polymers employed for nerve tissue scaffolds developed to date with their limitations and drawbacks. Finally, literature on electrical stimulation of different nerve cells is stated for nerve regeneration applications.

In Chapter 2, general method conducted in the research as well as all the materials used throughout this research project is reported.

Chapter 3 focuses on the synthesis of graphene to obtain graphene oxide (GO) then reducing it through two different methods of reduction in order to obtain conductive graphene oxide (rGO). Then the graphene oxide is coated onto the engineered cotton thread. Next rGO thread is characterized to determine the reduction of graphene oxide. Later its conductivity, permeability and biocompatibility is investigated and reported.

Chapter 4 introduces the new 2D material, Molybdenum disulphide (MoS₂), and the synthesis of MoS₂ nanoflakes followed by ion intercalation of MoS₂ to achieve conductive molybdenum disulfide. After coating the material onto the cotton thread, the scaffold is characterized to determine the transition of MoS₂ from semiconducting phase to metallic phase. The chapter is concluded with the result of conductivity, permeability and biocompatibility of rMoS₂ thread scaffold towards nerve cells.

In Chapter 5, rMoS₂-thread scaffold and rGO-thread scaffold are surface functionalized to promote adhesion of nerve cells onto the scaffolds. First, they are surface engineered to enhance adhesion of nerve cells to the conductive thread scaffolds. Then the characterization
 CHAPTER ONE

of surface functionalized thread scaffolds is undertaken by Acid Orange II assay to determine the accessible amine content on the surface of the thread scaffolds. Then nerve cells seeded onto the conductive thread scaffolds are stained to be observed and assessed for viability and adhesion, by confocal microscopy. To assess the biocompatibility of the surface functionalized thread alamarBlue assay was carried out on surface functionalized rMoS$_2$ and rGO threads. Also permeability of both thread scaffolds was investigated.

In **Chapter 6**, nerve cells seeded onto the conductive thread scaffolds are stained to be observed and assessed for viability and adhesion. Then the neural cells are electrically stimulated at different potentials and frequencies to optimize the action potential of the specific nerve cells NG108-15. Then neural cells are electrically stimulated while being live imaged after staining with voltage-sensitive dyes. Finally, neural cell proliferation rate and their functionality are confirmed while seeded on conductive tissue scaffolds. Combined work of electrical stimulation and 3D structure of engineered thread scaffolds demonstrate the potential employment of these scaffolds for neural tissue regenerations.

In **Chapter 7** general discussion on the achievements of this research is reported and the future work is explained.
1.4. LITERATURE REVIEW

1.4.1. TISSUE ENGINEERING

The purpose of tissue engineering is to repair or replace the damaged or malfunctioning tissue or organ. Tissue engineering has advanced greatly in the recent years as an interdisciplinary field[2]. The idea of scaffold as a prosthesis to grow cells and regenerate tissues was created in 1893[3]. A scaffold should have high porosity, high surface-to-volume ratio, biocompatibility[2]. Tissue scaffolds should be designed to support various cell types and mimic the extracellular matrix [4]. Nerve tissue engineering is a challenging area which involves peripheral nerve regeneration, spinal cord damage and trauma to the brain [1]. One of the most challenging fields of tissue engineering is neural tissue engineering. This involves tissues in the body that are electrically responsive like the brain, skeletal muscle and heart. Hydrogels or electrospun channels have been two common classes of scaffolds in nerve tissue engineering. These electrospun scaffolds are made from natural, synthetic or both of materials [1].
Figure 1.1 A tissue engineering model that demonstrates cell seeding within porous scaffolds [4].

Nerve tissue scaffolds should represent the native architecture and surface properties of injured tissues to be able to promote cellular growth and behavior and aid tissue regeneration. Scaffolds considered for peripheral nerve regeneration should contain these important factors, biocompatibility, biodegradability, mechanical strength and flexibility, permeability and conducting [5]. A variety of synthetic materials like silicone, collagen and chitosan, hyaluronic acid, gelatin and silk fibroin have been fabricated into nerve guides to bond nerve gaps. But some of these materials are non-absorbable and can cause scarring and fibrosis, leading to nerve dysfunction, eventually a second surgery is required to remove them [6, 7]. These natural polymers need complex purification and characterization to become reproducible, with low mechanical strength and fast degradability [7]. Polyesters such as poly (ε-caprolactone (PCL) have been used for fabricating tubular nerve guidance as they are
biocompatible and have appropriate mechanical properties [8]. However, their hydrophobicity and lack of biological activities render them as unfit for cell adhesion, proliferation and migration[9].

Conductive biomaterial scaffolds play an important role in different applications of neural tissue engineering, because neuronal activities have a significant effect in neural regeneration [10]. One approach for fabricating anisotropic engineered tissues are employing aligned fibers or channels ,electrical fields, to generate cellular substrates to facilitate nerve regeneration[11].

1.4.1.1. Peripheral nerve injury

Peripheral nerve injury is a universal health concern and it tremendously affects the patients’ morbidity and mobility[12]. Peripheral nerve injury could result in a nerve gap, if repairing the nerve gap with neurorrhaphy is not possible then some kind of graft should be implanted between the nerve stump and the gap to support axonal regrowth [13]. The basic concept of tissue engineering grafts is that neural substrate guides and protects axonal regeneration in the impaired nerve, and delivers the biochemical cues to the target tissue [14-16]. In injuries with a large nerve gap (>3 cm) autologous nerve graft is used. However, autografting needs two surgical procedures followed by all problems of invasive treatments. one of the problems with autograft treatment is that only 50% of patients gain functional recovery of the damaged nerve [17]. A promising alternative to autograft is employing synthetic nerve conduits for peripheral nerve repair [18]. A nerve conduit is needed to enhance neural growth, axon extension and control of direction, to assure functionality of autografts [12].
Figure 1.2 Schematic of the damaged and regenerated nerve after peripheral nerve injury.[19]

For this research, two of the most common two-dimensional materials; graphene oxide and Molybdenum disulphide are selected to be investigated and employed as conducting material in combination with common cotton thread to perform as nerve tissue scaffolds for neural tissue engineering applications. In this literature review, first common biomaterials used in nerve tissue engineering are reviewed then graphene oxide literature is reported considering its conductivity, biocompatibility, methods of its reduction followed by a brief literature on common cotton thread and its applications.
1.4.2. BIOMATERIALS FOR NERVE TISSUE ENGINEERING

1.4.2.1. Conducting polymers (CPs)

Conducting polymers are the most common materials to be employed as scaffolds for peripheral and central nerve tissue regeneration [20]. There are different types of conducting polymers include natural polymers like chitin, collagen, gelatin and chitosan, and synthetic polymers like silicone, PLGA, PCL, PLLA and conducting polymers (polypyrrole, polyaniline) used in different nerve tissue engineering applications [21].

A vast research is currently carried out on conducting polymers (CPs) specifically for their biomedical applications. The most common CPs studied are Polyaniline, Polypyrrole [10], Polythiophene and Poly (3,4-ethylenedioxythiophene) (PEDOT), for their simple synthesis and modifications [22, 23]. The extracellular matrix is naturally biodegradable and undergoes a massive remodelling during the natural healing process [23]. Conducting polymers are not biodegradable by nature and therefore are not suitable for clinical application as tissue scaffolds. However, it is possible to prepare biodegradable CP-based scaffolds by different methods, like grafting water soluble conducting oligomers to the surface of a biodegradable scaffold [24]. Biological tissues have specific mechanical properties. Incompatibility between the mechanical properties of a tissue scaffold and the tissue in which it is embedded may well cause inflammation of the neighboring tissue. CP-based materials tend to be relatively inelastic due to their limited freedom in 3D, resulting in easy breakage [20]. Another drawbacks on some polyesters like (poly(lactic acid) and poly(glycolic acid) is that they are hydrophobic which makes them unsuitable to employ for tissue scaffolds[25].


1.4.2.2. Carbon Nanotubes CNTs

Carbon nanotubes are one dimensional macromolecules; with outstanding properties such as high tensile strengths, thermal and chemical stability [19]. They also have metallic- and semi-conductive electronic properties which make them promising candidates for biosensors, drug and vaccine delivery and novel biomaterials [26]. However, a great part of research published since 2001 has focused on potential health concerns caused by CNT. They include studies into lung toxicity, skin irritation and cytotoxicity [27]. Lam et al. [28] found that single wall CNT products generated dose-dependent lung lesions, characterized by interstitial granulomas. They also believed that granuloma formation was due to the presence of CNT, while they realized that the SWCNT containing Ni caused more mortality. The research determined that CNT with Ni was more toxic than quartz, and that SWCNT were more toxic that carbon black, which produce lung toxicity. Shvedova et al. [29] focused on CNT cytotoxicity, to determine the effect of SWCNT on immortalized HaCaT (human epidermal keratinocytes) cells. The presence of SWCNT with 18 h incubated HaCaT cells caused accelerated oxidative stress, loss in cell viability and defect in cellular structure. Another study on the effect of CNT-based material on neuronal cells was carried out to show the interactions concerning a series of polyurethane PU/CNF nanocomposites and astrocytes. They concluded that the increased CNF loading in nanocomposite during neural cell seeding showed decreased astrocyte adhesion and retarded neurite growth in (rat pheochromocytoma) PC12 cells [30].

1.4.2.3. Graphene

In last decade, graphene oxide has been investigated widely due to its excellent properties as mechanical, structural and electronic [31]. A single atomic layer of carbon atoms packed into a two dimensional honeycomb lattice, has attracted enormous interest in a varieties of fields,
CHAPTER ONE

such as electronics, energy storage, catalysis, and biomedical field due to the novel physical and chemical properties [32]. Graphene oxide is not conductive and through three predominant methods chemical [33], heat treatment [34] and chemical vapor deposition (CVD) [35] can be reduced to achieve conductive graphene oxide. Graphene has appeared as an environmentally stable electronic material with exceptional thermal, mechanical, and electrical properties because of its two dimensional sp²-bounded structure [36].

1.4.2.3.1. Graphene Oxide synthesis

Several two-dimensional (2D) materials are in bulk form as stacks of bonded layers with weak interlayer attraction, facilitating exfoliation into separate atomically thin layers. One of these 2D materials receiving the most interest today is graphene, the monolayer type of graphite. Graphene has unique electrical and thermal conductive properties. Graphene has emerged as an environmentally stable electronic material with exceptional thermal, mechanical, and electrical properties because of its two dimensional sp²-bounded structure [36, 37]. In 1859 British chemist B.C. Brodie [38] investigated the structure of graphite and determined that it is composed of carbon, hydrogen and oxygen after one of the reactions by adding chlorate of potash (potassium chlorate, KClO₃) to the graphite in KClO₃-fuming nitric acid (HNO₃). In 1899, Staudenmaier L., added the chlorate in multiple steps over the process of reaction instead of a single addition like Brodie, to improve KClO₃-fuming HNO₃. In 1958, Hummers and offeman, [39] established a new oxidation method by reacting graphite with a combination of potassium permanganate (KMnO₄) and concentrated sulphuric acid (H₂SO₄) [40].

While other modified versions have been created, these three prime methods form the original routes for developing GO, and the changes have been insignificant.
CHAPTER ONE

1.4.2.3.2. Graphene Oxide reduction methods

In order to have conductive graphene oxide, it must be reduced; to remove the oxygen groups in the graphene chemical structure, different methods have been introduced to carry out this process in the literature. Over the years so many different methods have been discovered for reduction of graphene oxide. The reduction of graphene oxide monolayers facilitates access to graphene; however complete removing oxygen containing groups restores remaining vacancies in the carbon lattice.

One important chemical method to mass production of rGO involves using strong oxidants to convert graphite into GO, and then employing a reducing agent like hydrazine to reduce the dispersed GO to rGO [41]. To enhance the electrical performance of GO, there are different reports on effective reduction of GO employing both chemical and thermal reductive treatments [33, 42]. In another study the sheet resistance of GO films reduced by hydrazine was compared to those reduced with different concentrations of sodium borohydride (NaBH₄) films, which the results showed much lower sheet resistance of GO films [43]. Dua et al.[44] reported the use of ascorbic acid as a mild and effective alternative to hydrazine to reduce GO into rGO to develop a flexible sensor employing inkjet-printed films of rGO on poly ethylene terphathalate (PET) to detect NO₂ and Cl₂. Another environmentally benign, cost effective and mild method of GO reduction with high conductivity is reported using AlI₃ as a reductive agent to directly achieve flexible reduced GO [45].

Liu et al.[46] showed another simple and low-cost method to directly reduce GO films by Lawson’s reagent, leading to highly conductive graphene films. The reduced graphene oxide also was fabricated on cotton thread as a proof of concept. A green and facile method for reduction of graphene oxide was proposed by employing mussel inspired dopamine (DA) as reducing agent, by one step pH-induced polymerization of DA onto graphene oxide [47].
Recently, Liu et al.[33], reported another green, low cost and simple method for highly efficient reduction of graphene oxide (GO) papers with metal iodide aqueous solutions. Four different metal iodide (MgI₂, AlI₃, ZnI₂, FeI₂) were synthesized directly from metal and iodine powder with water as a catalyzer. The study determined that an extremely high bulk conductivity of 55088 S/m for reduced graphene oxide (rGO) papers was obtained with FeI₂ solution. The catalytic effect of strong Lewis acid for the promotion of the nucleophilic substitution reaction is responsible for the greatly improved bulk conductivity.

In our research, the latter method of reduction has been taken up in order to achieve higher conductivity by some modification to suit cotton thread coatings. Some modification has been carried out to this method to be suitable to successfully reduce the graphene oxide onto the cotton thread with metal iodide solution. As FeI₂ produced highest conductivity at a pH value of 1, it was chosen to reduce GO for our experiments, however we mixed the GO dispersion with the FeI₂ solution, then the pH value of the mixture was adjusted to 1.5 with the help of HCl, then the combined solution is put in water bath for 6 h at 95°C.

The other method of graphene oxide reduction employed in our research suitable for graphene coated thread was electrochemical reduction.

1.4.2.4. Biocompatibility of reduced graphene oxide

Recently, biocompatibility of reduced graphene oxide on different cell types has been studied. In 2013, Asian red ginseng was used for green reduction of graphene oxide. The investigation on the biocompatibility of the ginseng-rGO, showed proliferation of the stem cells after three days, as well as accelerated differentiation of (neural stem cells) hNSCs into neurons after three weeks [48]. The dependency of toxicity of rGO on the type of dispersant and concentration of the particles in the suspensions was reported [49]. The investigation indicated that certain concentration of GO-PEG shows the biocompatibility with mice
fibroblast cells (line L929). The cellular toxicity assay on biopolymer functionalized rGO showed good cyto-compatibility at a high concentration of 100 µg/ml for HUVEC cells [47]. Another toxicity investigation of microbially reduced graphene (M-rGO) showed significant biocompatibility even at high concentration as a potential substrate for PMEF cell growth [50].

Chang et al. [51] have carried out an extensive study on the cytotoxicity of graphene oxide towards A549 cells. Their results confirmed that they don’t go into the cell, and are not toxic to them. However, high concentration of GO showed minor loss of cell viability. Finally, they concluded that GO is quiet safe at cellular level. Liao et al.[52] performed toxicity study on graphene oxide and graphene by measuring the efflux of hemoglobin in suspended red blood cells. Graphene oxide showed better hemolytic activity when compared to aggregated graphene sheets. Another study on biocompatibility of graphene oxide in mice showed no pathological changes in assessed organs. Also, good biocompatibility of GO towards red blood cells was observed. These results imply that GO is a promising candidate for biomedical applications such as targeted drug delivery [53]. Ag/graphene polymer hydrogel used in wound dressing effectively kill bacteria and increased the healing rate of wounds in rats. This graphene composite showed great biocompatibility [54]. A nanotube hydroxyapatite-reduced graphene oxide composite was developed and its biocompatibility was investigated. The results of cell culture and viability showed that the presence of rGO enhanced osteoblast adhesion and proliferation [55]. In another study, the immuno-toxicity of GO with PVP-coating on human immune cells showed good immunological biocompatibility [56]. A study on DNA/RNA aptamers/GO- nanosheets proved them great sensing platform for living cells, since GO-nanosheets showed good biocompatibility to living cells [57]. Shen et al. [58] employed PEGylated-GO as a nanovector for effective delivery of proteins into cells. The results confirmed that PEGylated GO can be a promising candidate for protein
delivery vector due to their high biocompatibility. Yang et al. [59] synthesized a nanocomposite, aptamer-gold nanoparticle-hybridized graphene oxide (Apt-AuNP-GO) to assist in treatment of tumor cells under NIR Illumination. The Apt-AuNP-GO nanocomposite showed excellent biocompatibility with specific targeting ability and tumor cell destruction capability, to be great candidate for application in the photothermal treatment of breast cancer. Song et al. [60] developed a polycaprolactone (PCL)/GO biocomposite nanofiber scaffold for tissue engineering. The biocompatibility of PCL/GO scaffold was investigated using mouse marrow mesenchymal stem cells (mMSCs) and PC12-L cells. The results indicated that cells had good proliferation and viability towards GO.

The critical review of biocompatibility of reduced graphene and graphene materials shows that there is no study on biocompatibility of metal iodide reduced graphene oxide (FeI₂-rGO) or electrochemically reduced graphene oxide (ER-GO). The other advantage of this study is that reduced graphene oxide is coated on cotton threads for the first time to be employed in developing conductive nerve tissue scaffold for peripheral nerve damage and other nerve tissue engineering applications. This research emphasizes on the biocompatibility, availability, low cost, permeability and conductivity of reduced graphene oxide coated thread as a novel idea for tissue engineering field.

1.4.3. THREAD

There are some common and inexpensive materials, such as paper, fabric and thread which are currently discovered to use as alternate substrates for cost sensing applications.[61]

Thread has several characteristics that make it an attractive matrix for the scaffold in tissue engineering; i) it is flexible, lightweight, and porous. ii) it is very cost effective and vastly available, iii) thread is hydrophilic, with high wettability to adhere the cells, iv) it is biocompatible and biodegradable, v) thread can be manipulated easily by sewing, knitting
and weaving to any structure vi) its wicking property and flexibility make it a promising candidate for tissue engineering. One of the essential properties of tissue scaffold’s function is the ability of fiber alignment. Contrasting to rigid substrates, thread can be easily folded and twisted into any complex 3D geometry due to its flexible character. Furthermore, liquid flows through this porous material by capillary action over a short distance, without the need of external (hydrodynamic, electrophoretic or acousto-fluidic) pumping [62]. Voids between the fibers which comprise the thread act as capillaries, enabling liquids to ‘wick’ along threads without the need of external forces or pumps [63].

Threads and yarns have a convenient channel structure to make them suitable for fabricating microfluidic devices. The use of thread as a liquid transporting channel was first demonstrated by the groups of Whitesides [64]. Since then more complex thread-based devices have been reported [62, 65, 66]. Threads have been used as microfluidic devices, in blood grouping [67], and identification of antibody presence [68], biomedical assays and immune-chromatographic assay [69]. Bovine serum albumin and glucose present in blood plasma was successfully detected, as examples of detection of chemical substances relevant in diagnostics. The results show that cotton and silk threads are suitable materials for incorporation into field-deployable disposable chemical reaction and sensing systems [61].

In another study [64], threads made from eight different materials (rayon, hemp, nylon, polyester, wool, 50% cotton, 50% acrylic, acrylic and natural silk) for transporting liquids purpose were examined in thread-based devices. Ultimately, they chose to utilize mercerized cotton- a hydrophilic type of cotton thread- that does not need to undergo plasma oxidation to enable wicking. Their investigation also included the creation of deceives where thread was encapsulated by polymer tapes, enhancing wicking, while reducing liquid evaporation
The idea of using electronic threads for human health monitoring has been reported in many studies [70]. The electronic threads are usually metal-based threads [70], or threads made by carbon nanotubes coated with polyelectrolytes [71].

Gold-nanoparticle coated thread was embedded into fabrics to detect chemical or biological analytes in military and medical applications through SERS (surface-enhanced Raman scattering spectrometry) [72]. Ballerini et al. [72] also showed the use of thread as a substrate for inexpensive, disposable diagnostics for surface-enhanced Raman scattering (SERS) spectrometry.
1.4.3.1. Conductive Thread

The idea of the conductive textiles and threads has been a research interest for a long time. However their properties have been changed over the years to meet important qualifications for their potential applications in portable electronic devices, in healthcare units and wearable displays, warfare, space suits [73-77].

Nowadays conductive textiles made by conjugated polymers, metal powders and carbon nanotubes are still common, however these materials are expensive and treatments of them on textile fabrics involves complicated methods. Also, the shortcoming associated with them includes low open-air stability, flexibility and non-homogeneity of coatings.

Conducting nano-composite fibres of poly (methyl methacrylate) (PMMA) and multi-walled carbon nanotubes (MWCNTs) has been prepared by electrospinning. With electrical conductivity of the MWCNT showing then orders of magnitude improvement to the PMMA [78]. Carbon nanotubes (CNTs) have been used in different studies due to their efficient charge transport owing to the network of nanotubes. CNT-cotton threads has been used for albumin detection as proof of concept [71]. In another study, conductive threads were fabricated for wearable dye-sensitized solar cells by means of common threads (cotton, silk or wool) dip coating it with conductive polymer PEDOT:PSS [79]. Liu et al. [80] have produced a super-capacitor by coating SWCNTs onto cotton thread, then covering it with MnO2 and PPY layers through electrochemical deposition process. Conductive textile is also developed by deposition of conductive polymer (polyaniline (Pan)-polythiophene (PTh/ Poly (ethylene terephthalate) (PET) onto fibers in an organic medium with FeDCL3.

A comprehensive characterization of reduced graphene oxide coated polyester fabrics was carried out by Molina et al. [81]. They also investigated the effect of different layers of graphene coating has on to reduce the resistance of the GO coated polyester fabric. The
increase in the number of rGO layers on the fabrics produced an increase of the conductivity of the fabrics.

A conventional dip and dry method to coat the cotton fabric with graphene oxide was employed to enhance the conductivity by another study[82]. Then different types of reducing agents and their concentrations, reduction time and number of coating process on conductivity of the fabrics was investigated. They concluded that the best conductivity and mechanical performance of the fabrics were obtained using Na$_2$S$_2$O$_4$ as a reducing agent. Electrical conductivity increased by approximately three orders of magnitude with the increase in the number of coating process from 1 to 20 cycles [82]. There is also a study on rGO-nylon textile, coating the GO onto nylon textile with low temperature chemical reduction following GO wrapping by electrostatic self-assembly with bovine serum albumin (BSA), to serve as an adhesive to improve the adsorption of GO sheets onto the textile resulting in higher conductivity [83]. Javed et al.[84] reported reducing the graphene oxide (GO) onto the wool and cotton fibers to create electro-conductive networks by ultraviolet (UV) curing method, to prove anti UV properties of graphene coated cotton fabrics.

These studies from the literature focus on developing conductive textile with conducting thread through reducing graphene oxide onto the fabrics. However, none have used the reduced graphene oxide coated thread for cell culture, cytotoxicity, nerve conduit, suture for nerve surgery or tissue scaffold for nerve tissue engineering as purposed by the present research for the first time.

1.4.3.2. Silk applications in tissue engineering repair

Silk as natural protein polymer has been used as suture for clinical purposes for centuries. Silk has been used in biomedical applications because of its biocompatibility, cell adhesion
and biodegradability. Silk filaments are the most similar to the cotton threads used as neural
tissue scaffold in the present study.

There have been a few studies on silk scaffolds for tissue engineered ligaments. Seo et al.
[85] studied the mechanical properties and biocompatibility of silk and PGA scaffolds as an
artificial ligament in tissue engineering. Park et al.[86] used electrospun silk fibroin conduit
into a 10-mm long sciatic nerve defect in rats with functional recovery.

Conductive silk based materials have been employed for biomedical applications like
electrodes for recoding endogenous signals [87]. Preparation of regenerated silk fibron
solution tends to be a long and complicated process, then is degumming process followed by
centrifuging [87]. The process of fabricating silk fibroin-based scaffolds, commonly involves
toxic spinning solvents, therefore the probability of their cytotoxicity always exists[1].

1.4.3.3. Electrospun nanofibers

Several studies have pursuit to synthesize conductive nanofibers using electrospun methods,
for tissue engineering applications. Lelkes et al. [88] electrospun polyaniline-gelatin blends
to produce conductive nanofibers. Gomez et al.[89] fabricated conductive topographic
substrate for neural interfacing PPy microchannels by electrochemical synthesis. PPy
microchannels promote axon formation of rat embryonic hippocampal neurons.

Electrospun nanofibers are made by polymeric solutions or polymer melt. However, they use
high-priced and toxic solvents and if the polymer concentration is low, the production of fiber
will be at slow rate. Electrospinning allows us to have nanofibrous scaffolds from different
polymer solutions [1].

Thread scaffolds can direct the growth of neurites in the direction of the long axis of fibers,
analogues to the role of natural topographical cues found in the ECM of the peripheral
 CHAPTER ONE  

nervous systems. This conductive thread scaffold allow nerve regeneration of nervous system that guide development of neurons its 3D structure and facilitate electric stimulation of the cells on the substrates [20].

After reviewing these thread scaffolds (silk and polymer electrospun threads), it is concluded that common cotton thread has the advantages of these thread scaffolds without the disadvantages. Cotton thread can be made conductive with any conductive biomaterial to become conductive. The flexibility, permeability and durability of rGO thread or rMoS₂ thread are significant properties when designing neural tissue implants.

1.4.4. MOLYBDENUM DISULPHIDE

Because of significant properties of graphene oxide, there has been a great attention to explore of ultrathin two dimensional analogues of graphene, Molybdenum disulphide. Transition-metal dichalcogenides (TMDCs), with a general formula of MX₂, in which M represents any transition-metal element from group IV, V, or VI (e.g., Ti, Zr, V, Nb, Mo, and W) in the periodic table and X represents a chalcogen (S, Se, or Te)[90], are a family of around 60 materials, some of which exist as naturally occurring minerals. Two-dimensional semiconductors such as MoS₂ and others offer an important advantage when compared with classical 3D electronic materials. Since 1960s studies on TMDs have been done and revealed interesting properties, such as insulating, semiconducting, metallic and even superconducting[90]. These properties depend on the character of the transition metal and chalcogen present in the material. Due to their significant properties, TMDCs applications vary between batteries [91], electrocatalysts [92], lubricants [93] and solar cells [94].

Molybdenum disulphide is a quasi 2D transition metal dichalcogenides (TMD) with layered structure. The bulk MoS₂ is a semiconductor with indirect band gap with an energy gap of 1.29 eV. Because the van der Waal bonds between the S-Mo-S layers are weak, exfoliation of
the crystal into individual layers with different properties to the bulk MoS$_2$ is a possibility[95].

In the past decade, molybdenum disulphide (a monolayer two-dimensional TMDC) has emerged in the center of extensive research due to its superior electronic and structural properties. These properties include the sizable and direct band gap of monolayer MoS$_2$ making it suitable to be employed in field effect transistors [90, 96]. The monolayer MoS$_2$ shows photoluminescence property dependent on its thickness as well, which makes them fit for photosensors [97]. Another significant characteristics of the MoS$_2$ is their high elasticity, as found by research on MoS$_2$ nanosheets [98]. Flexibility and transparency are also desirable features for next-generation electronics. Although MoS$_2$ has been extensively investigated for decades, its near-atomically thin material [90] characteristic is new, especially its role in biomedical applications.

This monolayer 2D TMDCs (MoS$_2$, WS$_2$) can be synthesized by top-down exfoliation or bottom-up synthesis methods[99], which in parts are similar to those used for graphene preparations. For this research, layered MoS$_2$ is prepared by means of top-down exfoliation of the bulk powder.

1.4.4.1. Applications of MoS$_2$

Synthesis of layered MoS$_2$/Graphene composites by an L-cysteine assisted solution phase showed excellent electrochemical performances as anode materials for Li-ion batteries [100]. In another study, a composite film of MoS$_2$/graphene flake was characterized for Pt-free solar cells [101]. Inorganic fullerene-like nano-materials of TMDCs, (especially IF-MoS$_2$, IF-WS$_2$) have been incorporated into coatings of medical devices and drug delivery due to their lubricant behavior. One of these applications is coating orthodontic wires which are inserted to brackets, endodontic files and dental cream [102].
1.4.4.2. Molybdenum disulphide in tissue engineering applications

Molybdenum disulphide nanoplatelets were employed to reinforce biodegradable polymeric nano-composites of polymer polypropylene fumarate (PPF) with the intention of developing implants with improved mechanical properties for bone tissue engineering applications [103]. Wan et al. [104] fabricated artificial nacre with high electrical conductivity and excellent toughness, by employing synergic toughening of graphene oxide and molybdenum disulfide nanosheets through a vacuum-assisted filtration self-assembly process. They have suggested that this composite can be used in tissue engineering. However, research involving MoS$_2$ in tissue engineering is very limited, specifically none in nerve tissue engineering to our knowledge today.

1.4.4.3. Molybdenum disulphide lithium ion intercalation

The intercalation reaction involves charge transfer between the intercalated and host material, which follows change of the electronic properties of the host materials. Li$^+$ ion intercalation causes modification of the MoS$_2$ structure as a result of the electron transfer between the lithium compound and MoS$_2$ nanoparticles. This results in the development of metallic property of MoS$_2$ followed by creation of two different phases (2H and 1T) within the single layer nanoparticles [105].

Tachibana et al.[106] have fabricated highly conductive and stable hybrid ultrathin films containing MoS$_2$ by employing the intercalation-exfoliation method in addition to the Langmuir-Blodgett (LB) technique. The lateral conductivity was measured in the range of $10^1$ to $10^2$ S cm$^{-1}$ in 10-layer LB films.

In another study [101], MoS$_2$/graphene composite counter electrode (CE) was used for Pt-free-synthesized solar cells (DSSCs). The presence of graphene with MoS$_2$ improves the
electro-catalytic activity of the resultant composite, attributable to high conductivity from graphene flakes. MoS$_2$-polyaniline (PANI) composites were synthesized by a facile hydrothermal method and a simple in situ polymerization procedure.

1.4.4.4. Biological applications of Molybdenum disulphide

Molybdenum disulphide (MoS$_2$) has recently gained wide attentions in different fields; however, studies on its cytotoxicity towards different mammalian cells are in its infancy which needs vast research to make sure that the use of it is safe for the humans. A few studies have recently been reported on cytotoxicity of MoS$_2$ towards cells.

Liu et al.[107] have modified chemically exfoliated MoS$_2$ nanosheets with lipoic acid-terminated polyethylene glycol (LA-PEG), obtaining PEGylated MoS$_2$ (MoS$_2$-PEG) with high stability in physiological solutions and no obvious toxicity. They utilized MoS$_2$ nanosheets to deliver anticancer drug molecules for chemo and photothermal combination therapy, achieving remarkable synergistic effects both in vitro and in vivo. Ce6 is loaded on MoS$_2$-PEG which exhibits enhanced cellular uptake, that could promote under mild NIR photothermal heating to achieve even more effective cancer cell killing.

Inorganic fullerene like molybdenum disulphide nanoparticles was synthesized by pulsed laser ablation in water. The prepared MoS$_2$ nanoparticles showed good biocompatibility and solubility towards human embryonic epidermal fibroblast cells CCC-ESF-1 cell [108]. MoS$_2$ also showed good biocompatibility, reducing cytotoxicity with HepG2 cell line as a 2D nanocarrier for gene delivery without serum interference and cancer therapy [109]. Ou et al. [110] investigated two biological models affected by the ion exchange behaviour in molybdenum disulphide. First one assesses the MoS$_2$-glucose system for its sensitivity toward different glucose concentrations. Second system reveals the viability of yeast cells by the aid of the PL modulation of MoS$_2$. Teo et al. [111] assessed cytotoxicity of three different
transition metal dichalcogenides including MoS$_2$. They employed MTT viability assay to assess the biocompatibility of MoS$_2$ towards human lung carcinoma epithelial cells (A549). They concluded that MoS$_2$ nanosheets cause very low cytotoxicity to A549 cells even at high concentrations. However, they reported that MTT assay was not suitable due to overstated results. Yin et al. [32] reported application of MoS$_2$ nanosheets with controlled size decorated by chitosan as a chemotherapeutic drug nano-carrier for NIR photothermal triggered drug delivery.

The biocompatibility research on MoS$_2$ is in its early stages, thus it will be vital to investigate their toxicity on different cell lines by different viability assays towards decoding their effects on our health to ensure the potential risks before employing them.

1.4.5. CELL ADHESION

In developing a new tissue scaffold, the most significant concern is cell adhesion, followed by cell behavior when it is in contact with the scaffold surface which is a significant consideration in tissue engineering [112]. Most tissue engineering scaffolds are made from polymer matrices which should demonstrate some basic properties, like biocompatibility, strength, biodegradability and surface properties to aid cell adhesion and proliferation [113]. Although, these mentioned surface properties are the main factors that influence the absorption of proteins onto material surface, the scaffolds porosity is an important factor in cell adhesion. Rigogliuso et al.[114] used scaffolds that were treated with type-I collagen or plasma treatment to modify surface chemistry of the scaffold material. The other method to increase cell affinity onto the surface of polymeric scaffold materials was plasma surface modifications. HUVECs cells showed better adhesion to the electrospun polymer fibres with smaller diameter (0.3µm and 1.16µm) than larger diameter fibers (7µm). They were well attached and proliferated on the highly contained nano and micron scaled polymeric fibers.
CHAPTER ONE

[115]. In another study, Surface functionalization of scaffolds with the mussel-inspired poly(dopamine) proved to be efficient on a variety of biomaterials and useful since its synthesis is simple, solvent free and non-toxic [116]. Poly(dopamine) coating showed increase in cell adhesion and viability on different material surfaces, and the cells didn’t adhere on non-coated surfaces.

Chen et al.[117] investigated NG108-15 cells for their adhesion to the scaffolds. Cell adhesion affects cell proliferation, differentiation and finally the characteristics and role of the engineered tissue. Badami et al.[25] used human fibronectin in PBS to promote cell adhesion in their electrospun substrates. Wan et al. [118] showed that plasma treatment increased cell adhesion to poly (lactide-co-glycolide) (PLGA) films. The results were accredited to the surface morphology of PLGA and surface chemistry during oxygen plasma treatment. They also showed that OCT-1 osteoblast-like cells attach and spread better on patented surface compared to the cells on a smooth surface.

1.4.6. ELECTRICAL STIMULATION OF NEURAL CELLS SEEDED ON NERVE SCAFFOLDS

Electrical stimulation of cells have been proven to promote nerve regeneration [119]. It is believed that electrical stimulation improves neuritis and axon extension in vitro and nerve regeneration in vivo [120]. Electrical stimulation has demonstrated valuable results in improving nerve regeneration. Conductive polymers in tissue scaffolds also have been involved in delivery of electrical stimulation [121].

Huang et al. [122] showed that electrical stimulation can increase neurite outgrowth in Schwann cells by means of conductive polymers and progress peripheral nerve regeneration in animal models of nerve injury. Earlier studies showed that electrical charges play a significant role in stimulating either the proliferation or differentiation of various cell types.
[24]. Schmidt et al. showed that electrical stimulation caused significant increase in neurite length in PC12 cells cultured on Polypyrrole (PP) films. They electrically stimulated PC12 cells at a steady potential of 100 mV for 2h. For comparison, the constant potential scenario of 100 mV corresponds to a current of about 100 µAmp given that the resistance of the PP film is about 1 KΩ [24]. The use of this magnitude of current is justified based on previous electrical stimulation studies in rats using currents of 0.6 µAmp, 10-30 µAmp and 400 µAmp [123-125].

Scherman et al.[126] employed the longitudinal suture model for bridging nerve defects and compared with autologous nerve graft. They concluded that longitudinal sutures are suitable scaffolds to repair short nerve gaps and appropriate alternative to nerve grafting.

Kim et al.[127] electrically stimulated rat calvarial osteoblasts with biphasic electric current of 1.5 µA/cm2 at 3000 Hz, and observed 31% proliferation increased with continuous stimulation of 2 days. They concluded that electrical stimulation enhanced cell proliferation and generated vascular endothelial growth factor. Lee et al.[128] developed conductive meshes using PPy on electrospun poly(lactic-co-glycolic acid)(PLGA) nanofibers. Then the PC12 cells grown on the PPy-PLGA electrospun meshes were electrically stimulated with applying a potential of 10mV/cm. They observed 40-50% longer neurites and more neurite-bearing cells, when compared to non-stimulated cells on the same scaffolds.

PC12 cells were electrically stimulated with a potential of 10 V/cm, while nested on conductive PPy-PLAG random and aligned electrospun scaffolds. Their result showed 40-50% longer neuritis and 40-90% more neurite formation in electrically stimulated cells when compared to unstimulated cells on the same scaffolds [128].

To fabricate conductive topographic substrates for neural interfacing, PPy microchannels were synthesized. These microchannels promoted axon formation of rat embryonic
hippocampal neurons. Electrical stimulation of a potential of 100 mV was applied for a total time of 2 h per sample [89].

Clearly, the ability of NG108-15 hybrid cells to form synapses affords many opportunities to correlate biochemical aspects of synaptogenesis.

Ghasemi-mobarakeh et al. [129] fabricated an electrospun conductive nanofibrous polyaniline (PANI) with poly (e-caprolactone)/gelatin (PG) scaffold. PANI/PG scaffolds seeded with nerve stem cells were electrically stimulated with a steady potential of 1.5 V for 15, 30 and 60 min. They have shown increased cell proliferation and neurite outgrowth when compared to the non-stimulated PANI/PG scaffolds. The negatively charged phosphate groups within the membrane of NG108-15 cells tend to create a strong interaction to the positively charged polymer coated nanotubes [130].

In another study it was found that Laminin-single wall nanotubes (SWNT) thin films are conductive to neural stem cells and are able to successfully excite them. Generation of action potential of neural stem cells were observed upon applying current through the SWNT substrate by calcium imaging [131].

NG108-15 neural cell line is commonly used in vitro co culture models of myotubes to observe synapse formation. They display various characteristics of mammalian nerve cells [132]. This model cell was selected to investigate the effect of electrical stimulation on neuromuscular junctions (NMJs) formation. The cells were electrically stimulated after 2-3 days of co culture, at 50V and 0.5 Hz for 5, 30 and 60 min and were analysed after 4 days. Immunofluorescence experiments showed increase of aggregation of acetylcholine receptors and neural outgrowth in 30 and 60 min groups.[133].
Next study, Schwan cells were considered to be seeded on PPy chitosan substrate and allowed adhesion for 20 h. Later a constant potential 100 mV/mm was applied to the cells for 4 h through the membrane followed by another 12, 24, and 36 h culture. Their results confirmed that electrical stimulation enhances neurite outgrowth using conductive polymers and accelerated peripheral nerve regeneration in vivo models of nerve injuries [122]. Wilson et al. showed that electrical stimulation of nerve cells can significantly enhance the regeneration of peripheral nerve injuries after several animal experiments [134]. Hardy et al. [87] prepared silk films with interpenetrating polypyrrole and polystyrene sulphonate as conductive scaffolds. The size of growth cones of the growing axon is around the size of 10-20 µm, therefore micro scale patterns are more desirable for scaffolding.

However, these electrical stimulation studies involve mostly planar substrates which are optimal model surfaces for characterizing cell responses to electric fields, but essential three dimensional architectures are absent for design of a functional nerve tissue system.

1.4.6.1. Electrical activity recording

At the present, key technologies to record the electrical activity of neural cells involve intracellular recordings with sharp or patch electrodes, extracellular recordings by means of multi-electrode arrays (MEAs), and optical imaging through voltage-sensitive dyes (VSDs) [135]. Patch electrodes are mostly used to record intracellular activity of single cell behavior (action potential). Multi-electrode arrays (MEAs) have been employed to record the electrical activity of nerve tissues to observe the effect of different drugs and their dosage on field potential of nerve tissues in drug screening.

There are different methods of recording extracellular and intracellular activities of excitable cells. These methods include multi electrode arrays, patch clamp, voltage sensitive dyes. By employing microelectrodes to measure these activities, only a few positions in a sample will
be recorded. In cases of recordings from neurons in layers of thick samples, however, there are limitations to record beyond 50µm below the surface by light scattering. Therefore, one of the advantages of voltage sensitive dyes is the use of them in recording action potentials and voltages deep in sample. Recording electrical activities of excitable cells is crucial to understanding their network and fundamental properties [136].

For this study, optical imaging was chosen to obtain extracellular recordings of field potential (FP) generated by action potential, by using voltage-sensitive dyes (VSDs). Electrical activity of neural cells seeded within the conductive tissue scaffolds is recorded to confirm functionality of our new developed tissue scaffolds. Spira et al. [137] recorded the intracellular potential of excitable cells by a MEA device. The electrical stimulation of nerve cells could be employed in neural transplant for treatment of nerve injuries[131].
CHAPTER TWO

MATERIALS AND METHODS

2.1 INTRODUCTION

Two of the 2D materials, graphene oxide and Molybdenum disulphide are employed as conductive materials to be coated onto the cotton thread for neural tissue engineering purposes. To achieve this, they are converted to the reduced graphene oxide and 2H phase MoS$_2$ to increase their electrical conductivity. Next, common cotton thread is chosen as nerve tissue engineering scaffold to be electrically conductive through rGO and rMoS$_2$ coating and be functionalized and seeded with neuronal cells for further study and finally to be electrically stimulated. This chapter reviews the methodology involve to achieve two new neural tissue scaffolds for nerve tissue engineering. This methodology contains, graphene oxide synthesis, graphene oxide reduction methods, thread preparation and adsorption, MoS$_2$ synthesis, ion intercalation of MoS$_2$ (rMoS$_2$), coating of rMoS$_2$ onto thread, biocompatibility of GO, rGO, MoS$_2$, conductivity of GO thread and MoS$_2$ thread, wettability of the thread samples, characterization of both conductive materials, surface functionalization of the samples, cell adhesion.

2.1.1 SYNTHESIS OF GRAPHENE OXIDE

Graphene oxide was synthesized from graphite powder employing a modified Hummer and offeman methods (Hummers and Offeman 1958). The experimental details are as follows: Graphite oxide is synthesized from graphite powder (SP-1 grade 325 mesh, Bay Carbon Inc.) by a modified Hummers method. The graphite is first pre-treated in order to fully oxidize it to graphite oxide (GO). To accomplish this concentrated H$_2$SO$_4$ (50 mL) is heated to 90°C in a 300 mL beaker with K$_2$S$_2$O$_8$ (10 g) and with P$_2$O$_5$ (10 g) added with stirring until all of the reactants are completely dissolved. The mixture is then cooled to 80°C. Graphite powder (12
CHAPTER TWO

is then added to the H$_2$SO$_4$ solution resulting in bubbling which subsides within 30 min. The mixture is kept at 80°C for 4.5 h using a hotplate after which the heating is stopped and the mixture diluted with 2 L of DI water and left overnight. The following day the mixture is filtered and washed using a 0.2 µ Nylon Millipore filter to remove all traces of acid. The solid is transferred to a drying dish and allowed to dry in air overnight. For the oxidation step of the synthesis, H$_2$SO$_4$ (460 mL) is placed into a 2 L Erlenmeyer flask and chilled to 0°C using an ice bath. The pre-treated graphite is then added to the acid and stirred. KMnO$_4$ (60 g) is added slowly and allowed to dissolve with the aid of stirring, while the temperature is closely monitored so as not to allow the mixture to go above 10°C. This mixture is then allowed to react at 35°C for 2 h after which distilled water (920 mL) is added, initially in 20-30 mL aliquots. Since the addition of the water causes the temperature of the mixture to rise rapidly, water addition is carried out in an ice bath so that the temperature does not climb above 50°C. As more water is added, the mixture becomes less reactive until the final ~700 mL can be poured in with no observable resulting rise in temperature. After adding all of the 920 mL of DI water, the mixture is stirred for 2 h at which time 2.8 L of DI water is added. Shortly after the dilution with 2.8 L of water, 50 mL of 30% H$_2$O$_2$ is added to the mixture resulting in a brilliant yellow color along with bubbling. The mixture is allowed to settle for at least a day after which the clear supernatant is decanted. The remaining mixture is centrifuged and washed with a total of 5 L of 10% HCl solution followed by 5 L of DI water to remove the acid.

2.1.2. PREPARATION OF GO SOLUTION

To prepare two concentration of GO solution of 2% wt and 5% wt, 0.04 g /0.1g graphene oxide respectively, was added to 2 mL milli-Q water each. Then the solution was soft ultra-
sonicated using Ultrasonic cleaner (Ultrasonic, Australia) for 2 h to achieve a homogeneous dispersion of GO.

2.1.3. COTTON THREAD PREPARATION

Cotton thread was kindly supplied by the school of Fashion and Textiles, RMIT University, Melbourne, Australia. The threads were soaked in 70% ethanol for 1 h then were washed 3-5 times with PBS and dried at room temperature.

2.1.4. CATIONIC POLYACRYLAMIDE PREPARATION

Threads were coated with cationic polyacrylamide (CPAM) to engineer their surface charge and increase absorbent of graphene onto the threads. On the day of experiment, the cationic polyacrylamide (CPAM) solution was prepared by diluting CPAM dry powder to 0.01% with milli-Q water (0.1mg/mL or 2mg/20mL). Then the mixture was stirred using a magnetic stirrer (IKA® C-MAG HS 4-South East Asia) for 1 h to aid the dissolution process. The threads which were washed with 70% Ethanol (Ethanol 96% (74% v/v) was diluted with sterile dH2O) of 20 mm length were immersed into the CPAM solution for another 1 h, to make the threads cationic. The prepared cotton threads were rinsed with DI water and air dried. Then the threads were soaked individually in GO and FeI₂-GO solutions overnight. Later they were put in -80°C freezer, to prepare the threads for freeze drying in freeze dryer (PowerDry, PL6000, by Thermo Scientific) for 12 h.

2.1.5. COATING GO ONTO THE CPAM TREATED THREADS

Then the treated thread samples with CPAM were soaked in GO 2% and GO 5% solutions for 2 h. Then the GO-threads were rinsed thoroughly with distilled water to remove loosely bound GO particles and then were ultra-sonicated for 30 min in ultrasonic cleaner for better adsorption of GO particles into the thread’s filaments.
CHAPTER TWO

2.1.6. GRAPHENE OXIDE REDUCTION

Graphene oxide is electrically insulating by nature, in order to make it conductive it has to be reduced. It means that oxygen functional groups are to be removed from its chemical structure. In this study, metal iodide (FeI₂) treatments of the graphene oxide as well as electrochemical reduction of graphene oxide has been chosen to reduce GO.

2.1.6.1. Metal iodide treatment with FeI₂

Metal iodide, FeI₂ was synthesized straight from the reaction of iodine/metal powder with a few drops of water as a catalyzer. The detail of experiment is as followed: 0.25 g iodine particles (99.99% trace metals basis, Sigma-Aldrich, Australia) were pestled into powder in an agate mortar and mixed with 0.25 g iron powder (99%, reduced powder, Aldrich, Australia) in a beaker. Then a few drops of DI water were added into the beaker. Shortly, the reaction between iodine and metal powder strongly began and finished in 10 s. The metal iodide was obtained. Then, 5 mL DI water was added into the beaker, which was stirred until all of the resulting metal iodide was dissolved. Then the pH value of the supernatant was tuned to the critical pH value which was at 1.64, by adding HCl (1mol/L) under the measurement of a pH meter (Sper Scientific Benchtop Ph/Mv Meter, Ic860031, Australia).

Then the supernatant was transferred to another beaker. To finish, the GO solution was poured over the supernatant in water bath for 6 h at 95°C to obtain rGO solution. Two GO samples with different concentrations were prepared to check the reproducibility and standard deviation of conductivity was obtained.

2.1.6.2. Electrochemical reduction of graphene oxide

After GO synthesis the treated cotton thread with CPAM is soaked in GO solution overnight and then is sonicated for 30 minutes. Then the GO coated thread is rinsed with water to
remove excess graphene oxide form the thread surface. The GO-thread is then kept in a -80°C overnight and later freeze dried using freeze dryer for another 12 h.

The electrochemical reduction was performed by carrying out cyclic voltammetry in phosphate-buffered saline (PBS) solution (one sachet of PBS was dissolved in 100 mL of dH2O and then sterilized by autoclaving under standard conditions) to reduce GO and call it electrochemically reduced graphene oxide (ER-GO).

The GO thread is attached to copper wire with conductive adhesive and let to be air dried. The other electrode is copper wire connected to the positive electrode on a 200 W, DC power supplier (Agilent HP E3649A). Both electrodes are inserted in the electrolyte that is PBS, with a pH7. Then 10-20 V of potential is applied to the electrodes for 5-10 min and during this time the current will drop and then will be steady which means the GO is reduced. Precipitation of black particles is seen around the thread sample electrode in the PBS. This could be attributed to the GO reduction and the π network restoration within the carbon structure of the achieved ER-GO.

2.1.7. SCANNING ELECTRON MICROSCOPY –SEM

The morphology of the GO-thread and rGO-thread were observed by scanning electron microscopy using a Philips XL 30 SEM (SEMTech Solutions) system operating at 30kV. The blank cotton thread, CPAM-thread, GO-thread and rGO-thread at both concentrations of GO were prepared with gold sputtering using SPI-Module™ Sputter Coater and Vacuum Base (SPI Supplies®, PA, U.S.A.) to make their surfaces conductive.

2.1.8. ELECTRICAL CONDUCTIVITY OF THE THREAD SAMPLES

Electrical conductivity of the cotton thread after coating with conductive material presents a challenge. Threads have one very long dimension and the other two very small. Therefore,
determining their physical properties are difficult. The electrical resistivity of a cotton thread can be measured by two-probe method according to the literature [138], using a source-meter (Keithley 2400, up to 200V, 1A, 20W Power output). A uniform current density is applied across the sample thread clamped between two electrodes placed on parallel faces. Then the potential drop between the electrodes is measured on a source-meter.

Conductive materials obey Ohm’s law, where the current through a conductive material between two points is directly proportional to the potential difference, and hence a linear (I-V) curve is the actual representation of a conductive material. Current-voltage (I-V) plots for GO-thread and rGO-thread were determined to assess the conductivity of threads.

To calculate the electrical resistivity of the thread samples first the electrical resistance is measured. The electrical resistance measured by two-point probe, between two electrodes because the geometry of the thread is known. The resistance is obtained using Ohm’s law since the applied current is uniform, then the electrical resistivity is calculated.

Electrical resistance is measured at specific intervals of thread length of 2 mm, at 4 different places on the thread to obtain the best estimate. The slope of a plot of electrical resistance against length gives the resistivity. The electrical resistivity denoted by \( \rho \) (unit: \( \Omega \text{m} \)) is given by:

\[
\rho = \frac{RA}{L} = \frac{\pi d^2 R}{4L}
\]

The distance \( L \) represents the distance between the two probes.

Then using the electrical resistivity of thread formula,

\[
\rho = \frac{RA}{L} = \frac{\pi d^2 R}{4L}
\]

Where
CHAPTER TWO

\[ \rho \] is the electrical resistivity (Ω m)

\( A \) is the cross-sectional area of the thread

\( d \) the thread diameter (m)

\( L \) is the thread length (m),

\( R \) is the electrical Resistance (Ω)

The electrical resistivity of all thread samples with different methods of reduction, concentration was compared and reported. Also the electrical conductivity \( \sigma \) of all thread samples is to be obtained by

\[ \sigma = \frac{1}{\rho} \]

To determine the electrical resistivity of the reduced graphene oxide coated thread, followed by electrical conductivity measurements, the two probe method was conducted. First the resistance of each sample (GO thread, FeI2-GO thread, ER-GO thread and blank thread) was calculated (\( R = V/I \)) using the V/I (voltage/current) curve after each measurement.

2.1.9. X-RAY PHOTOELECTRON SPECTROSCOPY-XPS

The X-ray photoelectron spectroscopy (XPS) spectra were carried out on X-ray photoelectron spectrometer (K alpha-, Thermo Scientific, USA) with AL K\(_\alpha\) radiation as source.

2.1.10. BIOCOMPATIBILITY OF RGO-THREAD

2.1.10.1. PC12 cell culture

The PC12 cells were obtained from cryostored stocks in the School of Chemical Engineering, Monash University.

PC12 cells were grown and maintained in Dulbecco’s Modified Eagle Medium (DMEM), (Gibco\(^\circledR\), Life Technologies Pty Ltd., Australia) supplemented with 50 units per mL Horse
serum, 1% Penicillin-Streptomycin (Gibco®, Life Technologies Pty Ltd., Australia) and 10% Fetal Bovine Serum (FBS) (Gibco®, Life Technologies Pty Ltd., Australia) at 37°C in a 5% humidified carbon dioxide incubator.

2.1.10.2. AlamarBlue® Assay

The rGO-thread samples were sterilized in the 70% ethanol for 1 h. Cell seeding was carried out with a density of $10^4$ cells per well in a 24-well plate. The length of the rGO-thread sample put in each well was 5 mm. The well-plates were incubated at 37°C in humidified 5% CO₂ incubator for 1, 3, 5 and 7 days. At each time, the well plates were observed under an inverted microscope (Nikon Eclipse TS100) to observe the cells viability and growth. The viability of PC12 cells in the presence of reduced graphene coated threads was studied by means of the alamarBlue® assay (Invitrogen, Mulgrave, VIC, Australia) on 4 days for duration of 7 days (day 1, 3, 5 and 7). At each time point, 200 µL of 10% (v/v) alamarBlue dye (diluted in DMEM supplemented with FBS (9%)) was dispersed to each well after removing the thread samples from the cells and medium containing cells exposed to threads and each well with control cells (cells cultured without threads). The cells which did not exposed to the thread samples served as control. The 96-well plate was then incubated at 37°C for 4 h in a humidified of 5% CO₂ incubator. Fluorescence analysis using an excitation wavelength of 570 nm absorbance and 600 nm emissions was performed using micro plate reader (BioTek Synergy Mx, Winooski, VT). The metabolic activity of cells was stated in terms of the percentage of alamarBlue® reduction. The samples investigated were blank thread, GO 2%-thread, GO 5%-thread, FeI₂-GO2%-thread, FeI₂-GO5%-thread, ER-GO2%-thread and ER-GO5%-thread.

2.2. MOLYBDENUM DISULFIDE

2.2.1. MoS₂ NANOFLAKES PREPARATION
CHAPTER TWO

One gram of MoS$_2$ powders (99% purity, Sigma Aldrich) was added to 0.5 mL of N-Methyl-2-pyrrolidone NMP (99% anhydrous, Sigma Aldrich) solvent in a mortar and ground for 30 min. the mixture was put in a vacuum oven to dry overnight at room temperature then was collected and redispersed into a 10 mL NMP solvent. The obtained solution was sonicated with ultrasonic cleaner (Ultrasonic Processor GEX500) for 90 min at the power of 125 W, and the supernatant containing 2D MoS$_2$ nanoflakes was collected after being centrifuged for 45 min at the speed of 4000 rpm.

2.2.2. MoS$_2$ COATING ONTO THE COTTON THREAD

The treated cotton thread with CPAM as was explained in the last section is cut to 5cm pieces then is placed in 5 mL of MoS$_2$-NMP solution. The thread soaked in the MoS$_2$-NMP solution is ultra-sonicated for 30 min. The thread in the solution is soaked overnight to adsorb MoS$_2$ nanoflakes. Later the threads removed from the MoS$_2$-NMP solution are washed with PBS 3 times to remove any unattached particles. Then the MoS$_2$-thread is kept in -80ºC freezer overnight. To stabilize the attachment of MoS$_2$ nanoflakes onto thread the MoS$_2$-threads are then freeze dried in freeze dryer for another 12h.

2.2.3. LITHIUM ION INTERCALATION OF MoS$_2$-THREAD

The lithium (Li$^+$) ion intercalation was carried out using a three-electrode cell on CHI 760D electrochemical workstation (CH Instruments, Austin USA), with lithium perchlorate, LiCLO$_4$ (0.1 M, ACS reagent, >95.0%, Sigma-Aldrich) and Poly(Pyprolene-carbonate) PC, (97% anhydrous, Sigma Aldrich), as electrolyte solution. MoS$_2$-thread was chosen as working electrode, platinum wire as counter electrodes and Ag/AgCl as reference electrode. Electrodes were cycled at least 40 cycles prior to any measurements. Various potentials ranged from ±2 to ±12 V were applied across the electrodes.
CHAPTER TWO

Another method of Li$^+$ ion intercalation was used with employing a DC power supply (Keithley, 2400, USA). Various potentials ranged from 2 V to 12 V were applied across a two-electrode configuration via a DC power supply where the cathode was connected to the MoS$_2$-coated thread cut to 3 mm and the anode was a platinum (Pt) wire. The electrolyte was 0.1M lithium perchlorate in polypropylene carbonate.

2.2.4. ELECTRICAL CONDUCTIVITY OF RMOS$_2$-THREAD

To determine the electro-conductivity of MoS$_2$-thread before and after Li$^+$ ion intercalation two-point probe method was employed, the same procedure was carried out as mentioned in section 2.8. These experiments were repeated three times on different parts of the MoS$_2$-thread sample to have more precise results. These MoS$_2$-thread scaffolds will eventually be under wet conditions (cellular environment). Therefore, the conductivity experiments were performed in two stages dry and hydrated states. To measure the conductivity in wet state the MoS$_2$-threads were first washed with sterile phosphate buffered saline (PBS, pH7) solution.

2.2.5. X-RAY PHOTOELECTRON SPECTROSCOPY OF MO$_2$S THREAD

It is believed that high concentration of the intercalated Li$^+$ ions ultimately forces the transition of the MoS$_2$ crystal from stable hexagonal semiconducting phase (2H phase) to a metastable metallic phase (1T phase). In X-ray photoelectron spectroscopy measurements this phase transition is verified.

XPS measurements were carried out on a VG-310F instrument using Al non-monochromated X-rays (20 kV, 15 mA) with the hemispherical energy analyzer set at a pass energy of 20eV for the peak scans. The peaks related to the Mo and S were selected and analysed.

2.2.6. PERMEABILITY OF CONDUCTIVE THREAD
CHAPTER TWO

The wicking rates of eight different samples of cotton thread were measured, to determine and compare the wicking property of the rGO-thread in different methods of reduction and GO concentrations. The color of graphene oxide threads (black) made it hard to identify the level of liquid wicking along the samples. In these experiments, 8 µL of aqueous solution of fluorescent dye 1 mg/mL concentration diluted with 5% methanol was prepared. The water-isopropyl alcohol (IPA) solution was prepared to measure the wicking of the water-IPA solutions in untreated cotton thread. The surface tension of water-IPA is known therefore is a convenient way to measure the wicking property of the thread. Since the cotton thread is porous, the wicking onset, in the thread, can be determined by measuring the wicking speed of the liquid within different GO-thread samples. We examined the wicking property of eight samples of cotton threads; blank, CPAM coated thread, GO 2% thread, GO 5% thread, FeI2-rGO 2% thread, FeI2-rGO 5% thread, ER-rGO 2% thread, ER-rGO 5% thread.

The prepared solution was applied to one end of the thread \((length \, l = 2cm)\) with a 10 µL pipette. The threads were kept vertical attached to a transparent polymer film beside a paper ruler. Using a digital camera (Dino-Lite Digital Microscope AM4815ZT) the wicking motion of the liquid moving along the threads were recorded and measured. Measurements for each class of samples were repeated 3 times, and were recorded for visual appraisal.
2.2.7. FEI NOVA NANOSEM

To reveal the micrograph of the MoS$_2$ nanoparticles coated threads before and after Lithium ion intercalation, high-resolution field emission scanning electron microscopy (FEI Nova Nano-SEM) using low vacuum operating at 30kV acceleration voltage was employed. Samples were gold sputtered SPI-Module™ Sputter Coater and Vacuum Base (SPI Supplies®, PA, U.S.A.) with before SEM using Philips XL30 SEM (with Oxford X-MaxN 20 EDXS Detector and HKL EBSD system) imaging.

2.2.8. BIOCOMPATIBILITY OF RMoS$_2$-THREAD

2.2.8.1. NG108-15 Cell culture

NG108-15 cell line was selected for electrical stimulation experiment, because they symbolize a commonly accepted neuronal model system for in vitro studies after differentiation, as they exhibit many characteristics of mammalian nerve cells [132]. NG108-15, a neural cell line is employed to observe cellular functionality. NG108-15 cell line (Mouse neuroblastoma × Rat glioma hybrid) were grown and maintained in Dulbecco’s Modified Eagle Medium (DMEM), low Glucose, GlutaMAX (Gibco®, Life Technologies, Pty Ltd., Australia) supplemented with 10% fetal bovine serum (FBS), and 50 units/mL Penicillin-Streptomycin (P/S) at 37°C in a 5% humidified carbon dioxide incubator.

2.2.8.2. AlamarBlue® assay

AlamarBlue® assay was selected to evaluate the metabolic activity of NG108-15 cells. Cell seeding was performed at a density of ($5 \times 10^4$) per well. The prepared MoS$_2$-thread before and after intercalation besides ER-GO 2% are sterile by placing them in the 70% ethanol for 1 h. The sterile thread samples were cut to 2 mm in length and put in a 48-well plate with 3 repeats each samples along with NG108-15 cells and medium. The wells with cells only
serve as control. The 48-well plate with four samples and control is incubated for 3 days. The scaffolds were incubated at days 1, 2 and 3 for 4 h with 10%(v/v) alamarBlue® (Life Technologies Pty Ltd., Australia) in DMEM medium. A volume of 200 µL of the media from each sample was then pipetted into a 96-well plate and a microplated reader (Synergy TM Mx, Biotek) was used to measure excitation at 570 nm and emission 600 nm. The metabolic activity of cells was stated as the percentage of alamarBlue® reduction. These experiments were carried out in three replicates.

2.3. SURFACE FUNCTIONALIZATION OF THE COATED THREADS

To investigate the ability of the thread scaffolds structure to aid cell adhesion, the coated threads with lithium ion intercalated MoS2 and electrochemically reduced GO with 2% concentration were engineered to improve cell adhesion to their surfaces.

2.3.1. PREPARATION OF CDI/GELATIN

The thread scaffolds were sterilized in 70% ethanol for 1 h. Sterilized CPAM treated thread, rMoS2-thread and ER-GO2%-thread scaffolds were activated in infiltrated 40 nM 1,1’-carbonyldiimidazole (CDI) (Sigma-Aldrich, NSW, Australia, with the molecular formula (C3H3N2)2CO. It is a white crystalline solid, it is often used for the coupling of amino acids for peptide synthesis and as a reagent in organic synthesis) in acetone (Sigma-Aldrich, NSW, Australia) at room temperature for 3 h. The activated thread scaffolds were subsequently washed in acetone for five times in order to remove any unreacted residues of CDI then were soaked in acetone for 5 min to become completely dried. Then gelatin powder (Sigma-Aldrich, NSW, Australia) (10 mg/mL) was mixed in a sodium bicarbonate (NaHCO3) buffer (50 mM, pH9) at room temperature and then the pH of the solution was adjusted to 9 by adding sodium hydroxide (NaOH), then the gelatin solution was stirred and heated to 80°C over a magnetic stirrer (IKA® C-MAG HS 4-South East Asia) to dissolve gelatin. The gelatin
solution was filtered followed by soaking the coated threads with CDI in gelatin solution for 24-48 h at 4ºC (fridge).

2.3.2. Determination of Amine Content by Orange Acid II Assay

To determine the amine content of cotton thread surface after surface functionalization, Acid Orange II assay was carried out. In brief, surface functionalized threads (rMoS$_2$, rGO 2%) were cut into 2 mm samples. The untreated thread samples served as control. Samples were incubated in 1 mL of Acid Orange II (Dry content 60%, Sigma-Aldrich) solution in DI water (500 µM, pH3) overnight at room temperature. Then the samples are washed with copious water at pH3 (adjusted with HCl) to remove unbound Acid Orange II from the threads. Next the thread samples are incubated in 1 mL of DI water at pH12 (adjusted with NaOH) overnight to remove the bound dye from the samples. A series of Acid Orange II standard solutions (100 µM-500 µM) were prepared in DI water at pH12 and the data used to create the standard curve. The amount of the bound dye was quantified by measuring the optical density using a plate reader at 492 nm.

2.3.3. Permeability of SF-RGO Thread and SF-rMoS$_2$ Thread

Blue food color was used to identify the level of liquid wicking, along the samples. In these experiments, 8 µL of aqueous solution of food dye 1mg/mL concentration diluted with 5% methanol was prepared. The water-isopropyl alcohol (IPA) solution was prepared to measure the wicking of the water-IPA solutions in untreated cotton thread. The prepared solution was applied to one end of the thread ($length \ l = 2cm$) using 10 µL pipette. The rest of experiments are as stated in section 3.6.

2.3.4. Biocompatibility of Surface Functionalized Threads
CHAPTER TWO

AlamarBlue® assay was used to determine the biocompatibility of the thread scaffolds after surface functionalization towards NG108-15 cells. After sterilizing the surface functionalized thread in 70% ethanol for 1 h. The cultured cells were seeded on 5 mm length of these samples: sf-rMoS2-thread, sf-rGO2%-threads, rMoS2-threads and rGO2%-threads. Cells that are not exposed to the threads served as control.

2.4. CELL SEEDING IN SURFACE FUNCTIONALIZED THREAD SCAFFOLDS

2.4.1. CMC-TYR SYNTHESIS AND PREPARATION

CMC-TYR hydrogel is used to coat the tissue culture well plate to encourage cells to attach to the thread scaffolds instead of bottom of the dish. The lyophilized CMC-TYR was dissolved in PBS at concentration of 3% (v/v) followed by filter-sterilized using a 0.22 µm sterile syringe filter. Crosslinkers H2O2 and HRP are filter-sterilized then added to the solution at 1% to the CMC-TYR gel. In each well 300 µL gel is pipetted to the bottom of culture dish to set.

2.4.1.1. Seeding the cells onto surface functionalized thread scaffolds

Then the activated thread scaffolds are taken out of the fridge and rinsed with sterile PBS three times. Threads are washed with acetone five times to make sure they are dry. The surface functionalized thread scaffolds are then laid on top of the gel in the culture dish before adding the NG108-15 (1.5×10^5) cells with medium to the scaffolds. NG108-15 cells were seeded onto the scaffolds and incubated at 37°C in a 5% CO2 humidified incubator for 4-5 days.
2.4.2. **Live/Dead Cytotoxicity Assay**

The viability of the re-cultured NG108-15 cells grown on thread samples was assessed by staining the cells with Live/Dead cytotoxicity kit (Life Technologies, VIC, Australia) containing Calcein AM and Propidium Iodide (PI) according to the provided protocols. The surface functionalized thread scaffolds are washed with PBS three times, after 5 days of culture in incubator. The thread scaffolds are stained first by Calcein AM 1:1000, and incubated for further 15-20 min. Then the Calcein AM is removed by washing three times with PBS and replaced with PI and incubated for further 5 min at room temperature. All the samples are washed three times with PBS. Then they are visualized under the confocal laser scanning microscope (Nikon Instruments Inc., Japan) to observe the cell adhesion to the scaffolds and compare the adherent of them to rMoS$_2$ or ER-GO2% threads.

**2.5. Electrical Stimulation of Neural Cells (NG108-15)**

**2.5.1. Electrical Stimulation of NG108-15 Cells**

NG108-15 cells were cultured in a 6-well plate for 24 h at a density of ($5\times10^4$) cells. Next day, one of the wells were kept as control and the other one was electrically stimulated with a signal generator (Rhode & Schwarz, 2625, Germany) set at 5 Hz frequency with a pair of platinum electrodes (eDAQ, NSW, Australia) connecting to the threads touching cell medium.

Two different voltages: 60 mV and 20 V for three different durations, 5 min, 30 min and 60 min were investigated on the NG108-15 cells.
2.5.2. Live/Dead Cytotoxicity Assay on ES-cells

The next day both control and ES-cells were stained for viability assessments. Then Live/Dead cytotoxicity assay is carried out. First, Calcein AM solution 1:1000 was added to the cells (3 µL Calcein AM to 3 mL medium) and were left for 15-20 min in incubator. Then they were stained with PI 1:1500 (1.5 µL to 3 mL of medium) were kept at room temperature for 5 min. Then confocal microscopy was carried out to assess the viability of the ES-cells against control.

2.5.3. PicoGreen® Assay on ES-Cell/Scaffolds

To carry out proliferation assay on NG108-15 cells seeded onto sf-rMoS2 thread and sf-rGO thread, PicoGreen® assay was chosen. NG108-15 cells were seeded at the density of 5×10^4 cells/mm in a 24-well plate. After 5 days of culture to promote cell adhesion, half of the samples were electrically stimulated at amplitude of 60 mV; 5 Hz for 5 min in each well and the other half were kept as control without ES.

2.5.4. Live Cell Imaging with Voltage-Sensitive Dyes

2.5.4.1. FluoVolt™ Solution Preparation

FluoVolt™ Loading Solution is a voltage-sensitive dye and is prepared as follows, in brief, to a 15 mL tube, 100µL of 100X PowerLoad™ Concentrate (Component B) and 10 µL of FluoVolt™ dye, 1000X (Component A) were mixed using vortex in order to prepare fresh solution. Then add 10 mL of PBS to tube to mix. The medium from adherent cells were removed and cells were washed twice in sterile PBS. Then 2 mL of FluoVolt™ loading solution was added to cells, and incubate cells at room temperature for 15-30 minutes. Then FluoVolt™ loading solution is removed and cells are washed twice in PBS. For optimal
results, a filtered-sterile, 2M Glucose Stock Solution were also prepared to support cell health in longer hours’ experiments, to study primary or differentiated neural cell types.

2.5.4.2. Preparing 20 mM Glucose + Live Cell Imaging Solution

First 2M Glucose Stock Solution is diluted 1:100 into LCIS for a final glucose concentration of 20mM.

2.5.4.3. Valinomycin

To visualize the membrane staining of FluoVolt™ dye, standard FITC settings is used on confocal microscope. To confirm positive responses from the dye, NG108 cells were treated with 10 µM Valinomycin (a potassium ionophore) for 30 min, and an equal volume of isotonic potassium chloride (KCl) solution was added to depolarize the cells.

Isotonic KCl is composed of 140mM KCl, 5 mM NaCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 20 mM HEPES, 20 mM Glucose, and pH is adjusted to 7.4 using NaOH.

2.5.4.4. Voltage-sensitive dyes (VSDs)

NG108-15 cells plated in a 35-mm dish at a density of (3×10⁵) cells with 2 mL of culture medium were stained with FluoVolt™ Membrane Potential Kit (Life Technologies, Carlsbad CA, USA). As the FluoVolt™ solution preparation was stated before. The dye was added to the culture medium and the cells were incubated at 37°C under an atmosphere of 5% CO₂ for 15-20 min. Confocal live images were obtained through a 100X silicone oil immersion objective which needed glass chambers Slide (Nunc® Lab-Tek® Chamber Slide™ System, Sigma-Aldrich) with an optically clear polystyrene plate bottom. The clear base permits better microscopic viewing. Membrane staining was visualized with Sapphire 488-100, 488 nm, 100 mW solid state laser system (Coherent, Inc, CA, U.S.A.) by using standard fluorescein isothiocyanate filter setting (excitation, none; emission, 520/35). Images were
recorded with an exposure time of 10 ms at 93 -100 frames per second with a confocal laser scanning microscope and an EMCCD camera, Andor iXon DU897 (Andor Technology Ltd, Belfast, UK), and then analysed by using the Andor iQ (ver.2.9.1), Andor and Cspro software.

2.5.4.5. Optical imaging

Standard FITC setting is used to visualize the membrane staining of FluoVolt™ dye. Short exposure (10 ms or less) are possible with pixel 2×2 binning or greater. However, it depended on hardware configurations to measure rapid or successive depolarization. To confirm positive responses from voltage-sensitive dye, cells were treated with 10 µM Valinomycin (Potassium Ionophore, Sigma-Aldrich) for 30 min, and then an equal volume of isotonic potassium chloride (KCl) solution was added to depolarize the cells before live cell imaging with confocal laser microscope.
CHAPTER THREE

BIOCOMPATIBILITY OF THE REDUCED GRAPHENE OXIDE COATED THREAD FOR TISSUE ENGINEERING

3.1 INTRODUCTION

Current lack of success in the present methods of nerve injury treatments has led to numerous studies on nerve tissue engineering [21, 126, 139]. Most tissue engineering techniques present in the literature use a tissue scaffold seeded with cells, biomolecules to encourage the regeneration of tissues or their repair [140-142]. These scaffolds have specific task to carry out and are made from a range of materials such as conductive polymers[2, 6, 20, 143], biomaterials[10], carbon nanotubes[7, 19, 30, 144], polyesters[8] or metals[145]. The disadvantages surrounding these materials to name a few, are low stability, permeability [146], flexibility and porosity [141].

In the field of nerve tissue engineering, scaffolds could be fabricated from different materials however they should have specific characteristics such as biocompatibility, porosity[141], permeability[141, 146] and electrical properties [142]. The most important is the permeability of the scaffold to easily transport nutrient, remove waste within the scaffolds [141]. In the normal situation, in vivo vascular provides most of the nutrients essential for cells to function. However, in most tissue engineering constructs such blood supply is not available either in vitro or in vivo after implantation. Therefore, the ability of scaffold to deliver sufficient nutrients to cells is vital for a successful tissue engineering scaffold.

The present work has developed a reduced graphene oxide conductive thread in an attempt to establish the possibility of using coated cotton thread with reduced graphene oxide as a conductive neural scaffold for nerve tissue engineering.
CHAPTER THREE

Graphene has achieved high interest in research for its excellent electronic, mechanical [147], optical [148, 149] and thermal properties [44]. Graphene is superior to carbon nanotube, conjugated polymers and conductive materials like metal powders for these properties. Graphene is a two dimensional one-atom-thick sp²-bonded carbon networks [84], which looks like a honeycomb lattice. Its outstanding characteristics have led to its rapid development in different fields such as the electrochemical bio-sensing of glucose [150] in photothermal therapy of cancer cells [48, 151], tumor marker [150].

However, Graphene oxide (GO) has electrical insulating properties. In order to achieve its electrical conductivity, it is necessary to remove almost all of the oxygen-containing groups from its chemical structure [152, 153]. Different methods of reducing graphene oxide have been reported in the literature [33, 42, 43, 46, 154], these methods include chemical, electrochemical, high temperature annealing [34, 43, 151, 153, 155]. Ultraviolet light has been used recently to reduce graphene oxide on wool and cotton, to increase the electrical conductivity of the graphene oxide coated thread [84].

Thread is a common and inexpensive material, which is currently discovered as alternate substrates for low cost and field based sensing applications [64, 156]. Unlike rigid substrates, threads can be easily twisted and folded into complex three-dimensional geometries because of their flexible nature. Furthermore, thread substrates do not need external pumping for liquid flow over short distances[72]. The use of thread as a liquid transporting channel was first demonstrated by the group of Whitesides [64]. Liquid flows through the porous structure of thread by capillary action. Thread is physically strong, flexible with good permeability and availability at low cost. Porosity [157]of cotton thread is a another significant features that promotes cell adhesion [158], pore interconnectivity [157] and nutrient mixing.
CHAPTER THREE

The current literature on thread-based devices has predominantly aimed at characterizing dye/ink flows or diagnostic applications such as blood typing [67], detection of clinically relevant substances in artificial urine[159], immunoassays[64].

Herein, graphene oxide was reduced onto the common cotton thread to realize its conductivity, through metal-iodide (FeI₂) treatment and electrochemical reduction. Cotton thread was treated with Cationic polyacrylamide (CPAM) to enhance adsorption of graphene oxide particles to the filaments of thread.

Our approach was to associate conductive reduced graphene oxide with flexibility and biocompatibility and one directional control of the cotton thread to promote cell adhesion, its permeability similar vascular systems and biocompatibility [16]. This research aimed to investigate permeability, conductivity and biocompatibility of reduced graphene oxide coated thread towards PC12 cell lines.

3.2 MATERIALS AND METHODS

3.2.1. ENHANCING ADSORPTION OF RGO PARTICLES TO COTTON THREAD

Cationic polyacrylamide (CPAM) is one of the most often used flocculants for liquid/solid separation, preservation and drainage in papermaking [160]. To encourage more adsorption and accumulation of GO particles to achieve higher conductivity, thread substrates were treated with CPAM to manipulate their surface charge [72]. On the day of experiment, the CPAM solution was prepared by diluting CPAM dry powder to 0.01% with milli-Q water (0.1 mg/mL or 2 mg/20 mL). Then the mixture was stirred over a magnetic stirrer (IKA® C-MAG HS 4-South East Asia) for 1h to aid the dissolution process. The threads of 20 mm length were washed with ethanol then immersed into the CPAM solution for another 1h, to
CHAPTER THREE

make the threads cationic. The prepared cotton threads were rinsed with DI water and air dried.

Then the threads were soaked individually in ER-GO and FeI₂-GO solutions separately overnight. Later they were rinsed and kept in a -80°C freezer, followed by immediate freeze drying using a freeze dryer (PowerDry, PL6000, by Thermo Scientific) for 12 h.

3.2.2. GRAPHENE OXIDE SYNTHESIS

Graphene oxide was synthesized from graphite powder employing a modified Hummer and Offeman methods [39]. The experimental details are as follows: Graphite oxide is synthesized from graphite powder (SP-1 grade 325 mesh, Bay Carbon Inc.) by a modified Hummers method [39]. The graphite is pre-treated in order to fully oxidize it to graphite oxide (GO). To accomplish this concentrated H₂SO₄ (50 mL) is heated to 90°C in a 300 mL beaker with K₂S₂O₈ (10 g) and with P₂O₅ (10 g) added with stirring until all of the reactants are completely dissolved. The mixture is then cooled to 80°C. Graphite powder (12 g) is then added to the H₂SO₄ solution resulting in bubbling which subsides within 30 min. The mixture is kept at 80°C for 4.5 hours using a hotplate after which the heating is stopped and the mixture diluted with 2 L of DI water and left overnight. The following day the mixture is filtered and washed using a 0.2-micron Nylon Millipore filter to remove all traces of acid. The solid is transferred to a drying dish and allowed to dry in air overnight. For the oxidation step of the synthesis, H₂SO₄ (460 mL) is placed into a 2 L Erlenmeyer flask and chilled to 0°C using an ice bath. The pre-treated graphite is then added to the acid and stirred. KMnO₄ (60 g) is added slowly and allowed to dissolve with the aid of stirring, while the temperature is closely monitored so as not to allow the mixture to go above 10°C. This mixture is then allowed to react at 35°C for 2h after which distilled water (920 mL) is added, initially in 20-30 mL aliquots. Since the addition of the water causes the temperature of the mixture to rise
rapidly, water addition is carried out in an ice bath so that the temperature does not climb above 50°C. As more water is added, the mixture becomes less reactive until the final ~700 mL can be poured in with no observable resulting rise in temperature. After adding all of the 920 mL of DI water, the mixture is stirred for 2 h at which time 2.8 L of DI water is added. Shortly after the dilution with 2.8 L of water, 50 mL of 30% H$_2$O$_2$ is added to the mixture resulting in a brilliant yellow color along with bubbling. The mixture is allowed to settle for at least a day after which the clear supernatant is decanted. The remaining mixture is centrifuged and washed with a total of 5 L of 10% HCl solution followed by 5 L of DI water to remove the acid.

3.2.2.1. Preparation of GO solution

To prepare two concentrations of GO solution of 2% wt and 5% wt, 0.04 g and 0.1 g GO respectively, was added to 2 mL milli-Q water each. Then the solution was ultra-sonicated using Ultrasonic Cleaner (Ultrasonics, Australia) for 2 h to achieve a homogeneous dispersion of GO.

3.2.3. Graphene Oxide Reduction

In the present research chemical and electrochemical reduction methods to reduce GO is employed in order to achieve its conductivity. The chemical approach is attractive because it allows to deposit graphene as solution on any surface [161]. First method of GO reduction is simple, fast and non-hazardous by electrochemical method. The second method is metal iodide treatment of graphene oxide, expected to produce highly conductive rGo. These two methods are going to be compared for better conductivity of rGO, so that the rest of research will continue with the best method of reduction.
CHAPTER THREE

3.2.3.1. Electrochemical reduction of graphene oxide

The treated cotton thread with CPAM is soaked in GO solution overnight and then is sonicated for 30 min. Then GO-thread is rinsed with DI water to remove excess graphene oxide from the thread surface. The GO-thread is then kept at -80ºC freezer overnight and later freeze dried for another 12 h.

The electrochemical reduction was performed by carrying out cyclic voltammetry in Phosphate-buffered saline (PBS) solution. To do this, GO-thread is attached to copper wire with conductive adhesive and left to be air dried. The other electrode is only copper wire connected to the positive electrode on a 200 W, DC power supplier (Agilent HP E3649A, U.S.A.). Both electrodes are inserted in the PBS solution, with a pH value of 7. Then 10-20 V of potential is applied to the electrodes for 5-10 min and during this time the current will drop and then will be steady which means the GO is reduced on the thread. Precipitation of black particles is seen around the thread sample electrode in the PBS electrolyte. This could be attributed to the GO reduction and the π network restoration within the carbon structure of the achieved electrochemically reduced GO (ER-GO).

3.2.3.2. Metal iodide treatment of graphene oxide

Metal iodide FeI₂ was synthesized straight from the reaction of iodine/metal powder with a few drops of water as a catalyzer. The detail of experiment is as followed: 0.25 g iodine particles were pestle into powder in an agate mortar and mixed with 0.25 g iron powder in a beaker. Then a few drops of DI water were added into the beaker. Shortly, the reaction between iodine and metal powder strongly began and finished in 10 s. The metal iodide was obtained. Then, 5 mL of DI water was added into the beaker, which was stirred until all of the resulting metal iodide was dissolved. Then the pH value of the supernatant was tuned to the critical pH value which was at 1.64, by adding HCl (1 mol/L) under the measurement of a pH
CHAPTER THREE

meter (Sper Scientific Benchtop Ph-mV meter, AZ, U.S.A.). Then the supernatant was transferred to another beaker and FeI\textsubscript{2} solution is obtained. To finish, GO solution was poured over the FeI\textsubscript{2} supernatant with 1/1 ratio and mixed. Then the mixed solution was placed in water bath (Lab Companion BW-20H, FL, U.S.A.) for 6 h at 95ºC to obtain metal iodide reduced graphene oxide (FeI\textsubscript{2}-GO) solution. Two rGO samples with different concentrations were prepared to check the reproducibility and standard deviation of conductivity.

For the first time, GO has been reduced onto the cotton thread with metal iodide reduction treatment. As prepared thread is put into 10 mL of FeI\textsubscript{2}-GO solution then is placed in water bath for 6 h at 95ºC. Next, rGO-thread is removed from the FeI\textsubscript{2} solution, after a few second wash with DI water to remove excess residue, afterward coated threads are kept in -80ºC freezers overnight. Then the samples are freeze dried in freeze dryer overnight to have a stable FeI\textsubscript{2}-GO coated thread.

3.2.4. ELECTRICAL CONDUCTIVITY OF REDUCED GO THREADS

After reducing Graphene oxide through chemical reduction methods like metal iodide (FeI\textsubscript{2}) treatment or electrochemical reduction, the surface resistivity of all cotton thread samples coated with GO and rGO were measured by two point probe method [138]. This method applies a constant current density across the sample held between two electrodes placed on parallel to each other and then the potential drop across the latter electrodes is calculated. Electrical resistance of each rGO-thread is measured at specific intervals of 2 mm length and each sample three times in different places. This quantity is obtained from the current/voltage, I-V, curve measured by a Source Meter (Keithley 2400, Ohio, U.S.A.). The apparent electrical resistivity of each rGO-threads denoted by \( \rho \) (unit: \( \Omega \text{m} \)) is given by:

\[
\rho = \frac{RA}{L} = \frac{\pi d^2 R}{4L}
\]
CHAPTER THREE

Where R is electrical resistance of the rGO-thread samples, A is the cross-sectional area of the cotton thread fiber, d the fiber diameter, which is assumed to be uniform along the length, and L is the length of the thread held between the two probes. First the resistance of each sample (GO-thread, FeI\textsubscript{2}-GO thread, ER-GO thread and blank thread) was calculated $R = \frac{V}{I}$ using the voltage/current, V/I, curve produced by two point probe after each measurement.

Next the electrical conductivity of the rGO-thread is calculated from following equation.[82]

$$\sigma = \frac{1}{\rho}$$

The electrical conductivity (\(\sigma\)) was calculated from electrical resistivity (\(\rho\)) data obtained of rGO-thread.

3.2.5. CHARACTERIZATION OF REDUCED GO-THREAD

3.2.5.1. X-ray photoelectron spectroscopy of rGO thread

X-ray photoelectron spectroscopy was carried out on all thread samples. The XPS spectra were obtained using a monochromated Aluminium K–Alpha (hv=1486.6 eV) as source.

3.2.5.2. Scanning electron microscopy

The morphology of the GO-thread, and rGO-thread were observed by scanning electron microscopy using a Philips XL 30 SEM (SEMTech Solutions) system operating at 30 kV. Blank thread, CPAM-thread, GO-thread and rGO-thread at both concentrations of GO were prepared with gold sputtered using SPI-Module\textsuperscript{TM} Sputter Coater and Vacuum Base (SPi Supplies\textsuperscript{®}, PA, USA) to make their surfaces conductive.
3.2.6. Permeability of rGO-thread

The color of graphene oxide threads (black) made it hard to identify the level of liquid wicking (usually food dyes or ink are used) along the samples. In these experiments, 8 µL of aqueous solution of fluorescent dye 1 mg/mL concentration diluted with 5% methanol was prepared. The water-isopropyl alcohol (IPA) solution was prepared to measure the wicking of the water-IPA solutions in untreated cotton thread. The prepared solution was applied to one end of the threads (length = 2 cm) using 10 µL pipette. The threads were kept vertical and attached by the ends to a ruler as shown in Figure 3.1. The wicking motion of the liquid moving along the threads were recorded and measured using a digital camera (Dino-Lite Digital Microscope AM4815ZT) connected to the computer recording the movement of liquid along the samples. Measurements for each class of samples were repeated 3 times, and were recorded for visual appraisal.

![Figure 3.1 Image of three samples of rGO-thread held by transparent polymer film to record the wicking rate of liquid dispersed on protruding ends of the threads.](image)

3.2.7. Biocompatibility of rGO-threads toward nerve cells

3.2.7.1. Cell culture

To assess the biocompatibility of rGO-thread to support cell growth and spreading PC12 cells were cultured with rGO-threads for 7 days. The PC12 cells were obtained from Sigma
Aldrich. PC12 cells were grown and maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 50 units per mL Horse serum, 1% Penicillin-Streptomycin (P/S) and 10% Fetal Bovine Serum (FBS) at 37°C in a 5% humidified carbon dioxide incubator.

The rGO-thread samples were sterilized in the 70% ethanol for 1 h. Cell seeding was carried out with a density of $10^4$ cells per well in a 24-well plate. The length of the rGO-thread sample in each well was 5 mm. The well-plates were incubated at 37°C in humidified 5% CO₂ incubator for 1, 3, 5 and 7 days. At each time, the well plates were observed under an inverted microscope (Nikon Eclipse TS100) to observe the cells viability and growth. The viability of PC12 cells in the presence of reduced graphene coated threads was studied by means of the alamarBlue® assay (Invitrogen, Mulgrave, VIC, Australia) on 4 days for duration of 7 days (day 1, 3, 5 and 7). At each time point, 200 µL of 10% (v/v) alamarBlue dye (diluted in DMEM supplemented with FBS (9%)) was dispersed to each well after removing the thread samples from the cells and medium containing cells exposed to threads and each well with control cells (cells cultured without threads). The cells which did not exposed to the thread samples served as control. The 96-well plate was then incubated at 37°C for 4 h in a humidified of 5% CO₂ incubator. Fluorescence analysis with an excitation wavelength of 570 nm and an emission wavelength of 600 nm was performed using a micro plate reader (BioTek Synergy Mx, Winooski, VT). The metabolic activity of cells was stated in terms of the percentage of alamarBlue® reduction. The samples investigated were blank thread, GO 2%-thread, GO 5%-thread, FeI₂-GO 2%-thread, FeI₂-GO 5%-thread, ER-GO 2%-thread and ER-GO 5%-thread.

3.2.1. Statistical Analysis

All the experiments were performed in triplicates to obtain statistical evidence for cells proliferation. Results are given as average value ± standard deviation. Substantial differences
among groups were analyzed by one-way analysis of variance (ANOVA). The mean values of each sample were then compared using ANOVA. The differences were considered as statistically significant for the $p$ values equal or smaller than 0.05.

3.3 RESULTS AND DISCUSSIONS

3.4 ELECTRICAL CONDUCTIVITY OF RGO-THREAD

The conductivities of threads are reported for both cases of reduction treatment in two different concentrations of graphene oxide coated on the cotton thread. The conductivities of ER-GO2%-thread and ER-GO5%-thread have increased by one order of magnitude when compared with that of GO 2% and GO 5%, respectively. Also conductivities of FeI$_2$-GO2%-thread and FeI$_2$-GO5%-thread have improved by one order of magnitude compared to the GO 2% and GO 5% coated threads, respectively. After evaluating the outcome, it can be seen that electrochemical reduction of GO proved better conductivity by 70% in 2% concentration and 23% in 5% concentration compared to metal iodide reduction method. These conductivity data are in an acceptable range which is required for physiological stimulation.

Table 3.1. Electrical resistance, resistivity and conductivity of coated-thread samples

<table>
<thead>
<tr>
<th>Different threads</th>
<th>Electrical Resistance(R) ohm</th>
<th>Electrical Resistivity (ρ)ohm.m</th>
<th>Electrical Conductivity (σ) S/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank thread</td>
<td>1.78×10$^{10}$</td>
<td>1.16×10$^6$</td>
<td>8.62×10$^{-7}$</td>
</tr>
<tr>
<td>GO 2%</td>
<td>1.95×10$^{9}$</td>
<td>1.29×10$^5$</td>
<td>7.75×10$^{-6}$</td>
</tr>
<tr>
<td>GO 5%</td>
<td>2.27×10$^{9}$</td>
<td>1.48×10$^5$</td>
<td>6.75×10$^{-6}$</td>
</tr>
<tr>
<td>ER-GO 2%</td>
<td>3.53×10$^{10}$</td>
<td>2.30×10$^4$</td>
<td>4.34×10$^{-5}$</td>
</tr>
<tr>
<td>ER-GO 5%</td>
<td>3.07×10$^{9}$</td>
<td>2.00×10$^4$</td>
<td>5×10$^{-5}$</td>
</tr>
<tr>
<td>FeI$_2$-GO 2%</td>
<td>1.79×10$^{9}$</td>
<td>8.77×10$^4$</td>
<td>1.14×10$^{-5}$</td>
</tr>
<tr>
<td>FeI$_2$-GO 5%</td>
<td>5.24×10$^{8}$</td>
<td>2.56×10$^4$</td>
<td>3.90×10$^{-5}$</td>
</tr>
</tbody>
</table>
CHAPTER THREE

The results show that the range of electrical conductivity of these conductive threads is consistent with the results from previous studies in the literature. Baniasadi et al. developed a Chitosan/gelatin scaffold with a conductivity less than $10^{-7}$ S/cm when was hydrated in DI water [120]. Javed et al. [84] rendered textiles conductive through Ultra Violet reduction of graphene oxide onto thread and the resistivity of their sample thread measured to $(3.3 \times 10^5)$ $\Omega$m which is higher than the resistivity of FeI$_2$-GO 5% thread.

Therefore, FeI$_2$-GO and ER-GO threads show better conductivity compared to conductive textile made from UV reduced graphene oxide. Even though, an electrical current ranging between 0.6-400 µA for the physiological stimulation has been shown to be effective in both in vitro and in vivo experiments [162]. There is a comprehensive literature on electrical conductive textiles. Martin et al. [27] have measured the conductivity of Chitosan to be $(7.4 \times 10^{-9})$ S/m in dry state and $(3 \times 10^{-2})$ S/m when hydrated. Molina et al. [81] reported different surface resistivity measured at different coating of rGO on fabrics range from $(1.6 \times 10^9)$ $\Omega$/m , to the highest value at $(20.85 \times 10^3)$ $\Omega$/m resulting from more coating of rGO. These fabrics with the measured surface resistivity can serve as an antistatic material. Sheet resistance of rGO obtained by hydrazine reduction, measured by four point probe has been reported [42] to be at $(1 \times 10^7)$ and $(5 \times 10^5)$ $\Omega$/sq. Another study reported [154] electrical conductivity of reduced graphene to be $(3.82 \times 10^{-3}) - (9.38 \times 10^{-1})$ S/m.

Nonetheless, all these studies report electrical resistivity of the conductive threads for conductive textile purposes not intended for peripheral nerve repair in tissue engineering.

3.5. SEM

The morphology of GO-thread, before and after reduction was observed using scanning electron microscopy (SEM). Figure 3.2 shows the micro morphology of all samples; blank thread, CPAM treated thread, ER-GO threads and FeI$_2$-GO threads in both concentrations.
CHAPTER THREE

Figure 3.2 (A-B) revealed that graphene oxide was evenly coated on the threads’ filaments with some graphene particles over the threads. From Figure 3.2 (C-F) it is observed that both ER-GO and FeI₂-GO evenly cover the interspace and the exterior of each strand of fibers as a result this even distribution, good connection of GO particles are achieved. Increasing the concentration of GO resulted in better GO particle coverage. The result showed that rGO at 5% concentration produced by both reduction methods significantly improves the distribution of rGO particles on the cotton thread. Figure 3.2 (C and E) revealed that concentration of 2% coating had morphology of agglomeration with larger particles when compared to Figure 3.2 (D and F) for concentration of 5% of reduced graphene oxide. Comparing the threads in two cases of reduction, Figure 3.2 shows that metal iodide treatment (FeI₂) produce more GO particles on the fiber with more uniformly coverage compared to electrochemical method. These results explain why FeI₂-GO threads have better electrical conductivity by one order of magnitude compared to ER-GO threads.
Figure 3.2 SEM Micrographs of A) GO 2%-thread B) GO 5%-thread C) ER-GO 2%-thread, D) ER-GO5%-thread E) FeI₂-GO2%-thread F) FeI₂-GO5%-thread G) CPAM-thread H) Blank thread.
CHAPTER THREE

3.6 X-RAY PHOTOELECTRON SPECTROSCOPY

To verify the reduction of GO by electrochemical reduction (ER) and metal iodide (FeI₂) treatment onto the cotton threads, X-ray photoelectron spectrometry (XPS) was employed to compare the chemical composition of the GO threads after reduction. The samples investigated are; GO-thread, FeI₂-rGO thread and ER-rGO thread with two concentrations of GO2% and 5%.

Also, surface analysis carried out by fitting the XPS curve to a Gaussian-Lorentzian function after correction for Shirley background. Elements considered for high-resolution scan analysis were C and O spectra.

The spectrum of GO exhibits four types of carbon bonds as shown in Figure 3.4 (a-b): C-C (284.6 eV), C-OH (285.6 eV), C-O-C (286.9 eV) and C=O (288.4 eV). By reduction of GO, within the chemical structure of rGO, the C-C band peak becomes dominant, however peaks associated to the oxidized carbon species are significantly weakened.

The composition of GO coated thread samples were calculated founded on the areas of the XPS peaks. The atomic ratio of carbon to oxygen (C1s/O1s ratio), found from broad-scan XPS spectra, shows that the C1s/O1s ratio was 1.97 for FeI₂-GO and 1.51 for ER-GO, consistent with previous studies. These studies indicated that reduction of graphene oxide with metal iodide results in better electrical conductivity which is consistent with the electrical conductivity value of FeI₂-GO.

Depending on the method of GO reduction different carbon bonds appear differently. On the other hand, the significant intensity decreases in the carbonyl and carboxylate peaks for rGO as compared to GO which verifies that GO is well reduced. The convolutions of C1s peaks at 284.8 eV (C-C), 285.6 eV(C-OH), 286.0 eV (C-O-C) and 287.5 eV (C=O), corresponding to
carbon atoms bonded with different oxygen groups become smaller after ER and FeI₂ reductions as shown in Figure 3.3 (c-f).

Another characteristic of the composition analysis is the strong decrease in the oxygen content by 36% in ER-GO reduction in Figure 3.3 (c-d) and 48% in FeI₂-GO treatment revealed in Figure 3.3 (e-f), revealing considerable de-oxygenation from GO particles on the threads.
CHAPTER THREE

Figure 3.3 XPS spectra of the GO on threads before and after reduction; a) GO2%-thread, b) GO5%-thread, c) ER-rGO2% thread, d) ER-rGO5% thread, e) FeI2-GO2% thread, f) FeI2-GO5% thread.

3.7. WICKING PROPERTIES OF THREAD SAMPLES

Table 3.2 showed that the wicking rate of liquid in FeI2-GO 2% and FeI2-GO 5% as well as GO 5% threads are significantly slower (by 50%) than ER-GO 2% and 5%, owing to the high concentration of graphene oxide (5%) and FeI2 particles. CPAM thread wicks the fluid faster than blank thread and GO 2% coated thread. Electrochemical reduced graphene oxide in both concentrations exhibited better wicking behavior compared to metal iodide reduced graphene oxide.

Table 3.2 Rate of Wicking for Different Types of Thread Samples

<table>
<thead>
<tr>
<th>Thread</th>
<th>Rw (mm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank thread</td>
<td>0.43± 0.03</td>
</tr>
<tr>
<td>CPAM thread</td>
<td>0.55± 0.03</td>
</tr>
<tr>
<td>GO 2%-thread</td>
<td>0.35± 0.07</td>
</tr>
<tr>
<td>GO 5%-thread</td>
<td>0.19± 0.03</td>
</tr>
<tr>
<td>ER-GO 2%-thread</td>
<td>0.50± 0.05</td>
</tr>
<tr>
<td>ER-GO 5%-thread</td>
<td>0.51± 0.07</td>
</tr>
<tr>
<td>FeI2-GO 2%-thread</td>
<td>0.17± 0.05</td>
</tr>
<tr>
<td>FeI2-GO 5%-thread</td>
<td>0.13± 0.01</td>
</tr>
</tbody>
</table>
CHAPTER THREE

In considering rGO-thread as a tissue scaffold, the threads with most rapid rate of wicking are preferred. Therefore, even though FeI₂-GO thread showed better conductivity, ER-GO method of reduction is selected for the remainder of the research.

3.8. BIOMATIBILITY OF REDUCED GRAPHENE OXIDE COATED THREADS

3.8.1. METABOLIC ACTIVITY ASSAY

Figure 3.4 shows the metabolic activities of PC12 cells cultured with the reduced graphene oxide coated threads from both methods of reduction in two concentrations to be compared with the cells that cultured on tissue culture plate (control). Chemical reduction of alamarBlue® reagent is used as an indicator of the cell metabolic activity and cellular health and as a result this redox indicator changes color[163].

The results show that cells grown on FeI₂-GO 2% display higher metabolic activity \( (p = 0.0395) \) when compared to the FeI₂-GO 5%. In the case of electrochemical reduction, ER-GO 2% shows higher \( (p < 0.1022, n = 3) \)metabolic activity compared to ER-GO 5% until the seventh day when the metabolic activity of the ER-GO 2% falls below 5% concentration\( (p < 0.9571, n = 3) \). Overall cells grown on ER-GO 2% exhibits highest metabolic activities compare to those from other thread samples.

The rise in the percentage of alamarBlue reduction in all samples over 7 days is a good indication of biocompatibility of thread scaffolds FeI₂-GO 2% \( (p < 0.00006, n = 3) \), FeI₂-GO 5%\( (p < 0.00001, n = 3) \), ER-GO 2%\( (p < 0.00083, n = 3) \), ER-GO 5%\( (p < 0.0063, n = 3) \) towards PC12 cells. Therefore, these results confirm that cells were not affected by exposing to the reduced graphene oxide coated threads.
Figure 3.4 Metabolic activity of PC12 cells in presence of FeI2-GO thread and ER-GO thread in two concentrations compared to cells unexposed to threads (control) reported in percentage of alamarBlue reduction. Data are shown as mean values ± standard deviation ($n = 3$).
3.9 Conclusion

In brief, common cotton thread has been introduced for the first time for preparation of rGO-thread tissue scaffolds by coating the reduced graphene oxide onto the treated thread. Graphene oxide was successfully synthesized and reduced using two different methods of reduction onto the common cotton thread with two different concentrations to compare. First method was electrochemical reduction and metal iodide (FeI$_2$) treatment as a novel method to be combined with thread. Next the electro-conductivity of the threads was evaluated by XPS and two-point probe assessment and the results show that rGO-threads are conductive. Also permeability of the thread samples was determined, with electrochemical reduction exhibiting better wicking rates. In particular, the cells showed high metabolic activities when cultured with the rGO-threads which confirm the biocompatibility of rGO-thread towards nerve cells. It is concluded that conductive and biocompatible rGO-thread as a 3D structure has a great potential for nerve tissue scaffold in nerve regeneration applications.
CHAPTER FOUR

BIOCOMPATIBILITY OF INTERCALATED MOLYBDENUM DISULPHIDE COATED THREAD SCAFFOLD

4.1 INTRODUCTION

Thousands of peripheral nerve repair procedures are performed each year around the world. The cause of peripheral nerve injuries varies between trauma, cancer or congenital defects. Nerve defects with gaps less than 10 mm can be reconnected surgically with micro suture, however longer defects are more challenging to treat. Regardless of the need, the existing choices to regenerate short nerve gaps are limited. The current standard is the autologous nerve graft, but there are some shortcomings such as limited donor sources, donor site morbidity, and multiple surgical sites. Therefore there is a substantial clinical necessity to address short nerve gap defects [164].

Nerve tissue engineering is an auspicious path to aspire this need with synthetic nerve conduit. A significant amount of work has been dedicated to develop and improve synthetic nerve conduits which have resulted in promising regeneration and functional restoration of nerve defects (11-15). Without regard to all the effort, there is a need to improve their performance compared to autologous nerve grafts.

One of the most important properties of synthetic nerve conduit is its ability to conduct electricity. Hence, a considerable effort has been focused on employing conductive materials in nerve tissue engineering. The aim is to develop a novel material that fulfills the conductivity requirements of neural tissue and the concerns of tissue engineering. The ideal properties for this nerve conduit are biocompatibility, biodegradability and electro conductivity. Researchers for years have studied electro conductive polymers, such as poly(ε-caprolactone)[8], polypyrrole (PPy)[128], polyanaline[165], and polyphosphazene. These
polymers have demonstrated excellent electrical conductivities as well as positive biocompatibility especially PPy. However, there are some shortcomings with polymers, like their poor solubility and degradation profile, or fragility.

In this research, the prospects of integrating molybdenum disulphide (MoS$_2$) into cotton thread are introduced to develop a novel conductive scaffold as a whole for neural tissue regeneration. The recent presence of the thread microfluidics in biomedical field [61, 63, 65, 67, 69] has inspired our research for potential applications of cotton thread in nerve tissue engineering.

Molybdenum disulphide is a semiconducting transition metal dichalcogenides, with a chemical structure of S-Mo-S layers that are maintained together along the third lateral dimension by reasonably weak van der Waals forces [166, 167]. Due to its great electronic and magnetic properties, MoS$_2$ has been employed in a range of different applications varied between batteries [91], electrocataysts [92], lubricants [93] and solar cells [94].

In our research, after coating the MoS$_2$ nanoflakes onto the cotton thread as presented in details in methodology, Li$^+$ ion intercalation is carried out in order to obtain conductive MoS$_2$-coated threads, which from now on we call it rMoS$_2$-coated thread. In the intercalation process, an external voltage is applied across the working cathode (cotton thread coated with the 2D MoS$_2$ nanoflakes) and platinum wire as the counter electrode. The electrolyte solution is the mixture of PC with LiClO$_4$.

Molybdenum disulphide is a layered transition metal dichalcogenides (TMDCs) material which are structured in groups of hexagonal, bonded with Van der Waal forces. Molybdenum disulphide has the molecular formula MoS$_2$ and space group P63/mmc. Monolayer molybdenum disulphide is a new emergent material with a promising future in nanoelectronics and optoelectronics [90, 168]. Molybdenum disulphide with its excellent
properties, including its direct inherent band gap, high mobility, and quantum confinement, has been used in different fields of research, including nanoelectronics, catalysis, optoelectronics, and lithium batteries, solar cells, biosensors, [169-172]. This thin Nano flake material depending on its chemical composition and structural configuration can be metallic, semi-metallic or semiconducting. In this study, molybdenum disulphide is coated onto the cotton thread by soaking, sonication and then freeze drying, and is made electrically conductive by lithium ion intercalation method [109].

Before employing any material for tissue engineering applications or any biomedical purposes, it must be evaluated for its biocompatibility and cytotoxicity towards the considered cell line. Comprehensive toxicity analysis is required on new materials to make sure that they are safe before using them for medical application. There is only a handful of research done on studying the cytotoxicity of molybdenum disulphide in the literature [173]. Wu et al. [108] reported good biocompatibility of MoS₂ towards A549, and K652, and CCC-ESF-1 cell lines using MTT assay. In the recent study, Liu et al. [107] used MoS₂ nanosheets to deliver anticancer drug molecules for chemo and photothermal combined therapy, they reported great harmonious results in vitro and in vivo. Zhang et al. fabricated MoS₂-based DNA sensors [174]. Also, MoS₂ nanosheets are employed as photothermal agents for cancer treatment [175]. Next lithium ion intercalated MoS₂ nanosheets were functionalized by lipoic acid-terminated polyethylene glycol (LA-PEG). These PEGylated MoS₂ nanosheets were then loaded with a PS agent, chlorine e6 for photodynamic therapy. In another study, PEGylated MoS₂ were used for gene delivery and therapy of cancer using HepG2 cell line [109].

Here, we report a simple, and low cost method for creating a nerve tissue scaffold, using cotton thread for its biocompatibility, permeability and flexibility, as well as its porous structure [64]. It has a 3D structure which allows cell growth and cell adhesion.
CHAPTER FOUR

In order to employ thread as nerve conduit, suture or 3D scaffolds for nerve tissue engineering, it is advantageous to render it metallic to transmit electrical conductivity. In the present research, the thread scaffold is further modified by coating it with MoS\(_2\) nanoparticles. Lithium ion intercalation has been carried out on MoS\(_2\) nanoparticles attached to the cotton thread to make them metallic to achieve electrical conductivity in MoS\(_2\)-thread.

Many studies relating to optical properties of MoS\(_2\) use mechanical exfoliation methods to produce high quality MoS\(_2\) crystals [97, 176]. The major disadvantage of this method is the low yield, which limits their use in large scale fabrication of MoS\(_2\) photoluminescence [90]. To overcome this problem, vapor synthesis techniques have been suggested, although it requires high temperature during processing (700-1000\(^\circ\)C), unsuitable with common integrated circuit fabrication practice [177].

The 2D MoS\(_2\) nanoflakes used here are synthesized using a grinding aid liquid-phase exfoliation technique related to that reported by Yao et al. [178]. Liquid phase exfoliation techniques, is the better approach to feasible mass synthesis of 2D MoS\(_2\) moderately lower temperatures [179]. Previous liquid-phase exfoliation methods were generally hazardous, long processes and not environment friendly [99]. A new method of liquid-phase exfoliation proposed by Coleman et al. [180] offers less time consuming exfoliation process, compared to the earlier techniques [90, 177]. Coleman et al. suggested liquid-phase exfoliation of 2D MoS\(_2\) flakes in a proper organic solvent assisted with high power sonication [180]. The 2D MoS\(_2\) nanoflakes used here are synthesized using a grinding aid liquid-phase exfoliation technique related to that reported by Yao et al. [178].

The electronic and magnetic properties of this layered compound changes considerably by incorporating of lithium (Li) ions into the van der Waals gap of MoS\(_2\) chemical structure. The molecular layers of MoS\(_2\) are bounded with weak van der Waals forces that aid the
intercalation of foreign molecules in between the layers. The intercalation process is associated with charge transfer from the lithium ions to the lowest unoccupied conduction band of the MoS$_2$ structure. This reaction consequently increases ‘d’ band filling followed by electronic property change of MoS$_2$ [166]. The intercalation makes it possible to have semiconductor state transition to metallic state.

Intercalation of Li$^+$ ion in MoS$_2$ shows significant properties in different applications [101, 106]. According to the literature, there are different methods of the insertion reaction of Li ion into the van der Waals gap, such as alkali solutions [181], direct synthesis and electrochemical methods [166].

The electrolytic property of PC in combination with LiClO$_4$ as solution has been studied from different aspects over the years. One study examined the conductivity of the electrolyte PC mixed with amide-solvents along with LiClO$_4$ as solution. The report supports the increase of electrolyte conductivity affected by the reaction of the solvents with respect to Li$^+$. The results show that the PC alone shows more Li cycling efficiencies in a Li-on-Pt half-cell test when compared with PC/DMA, PC/DMAA and PC/DMF [182].

It is been proven that, this process in bulk MoS$_2$ follows the intercalation of the x quantity ($(0 \leq x \leq 1)$ of Li$^+$ ions and injection of an equal quantity of electrons (e$^-$) into MoS$_2$ [167]. The outcome of this intercalation and injection of charge is the formation of Li$_x$MoS$_2$, resulting in modification of their original crystal phase (from trigonal prismatic 2H to octahedral 1T phase). It is proposed that high concentration of intercalating Li$^+$ ions ultimately causes the transition of MoS$_2$ crystal from stable hexagonal semiconducting phase (2H phase) to a metastable metallic phase (1T phase).

The MoS$_2$ coated thread was tested in vitro for its conductivity, permeability and biocompatibility to support neuronal proliferation and viability of NG108-15 cell line. The
ultimate goal of this study is to create a high performance tissue scaffolds for nerve tissue engineering.

4.2. METHODS AND MATERIALS

4.2.1. 2D MoS$_2$ NANOFLAKES PREPARATION

The MoS$_2$ nanoflakes were prepared from MoS$_2$ bulk powder using a grinding-assist liquid phase exfoliation technique [99]. In brief, the bulk MoS$_2$ powder (99% purity, Sigma Aldrich) is mechanically grounded with the N-nethyl-2-pyrrolidone (NMP, 99% anhydrous, Sigma Aldrich) solvent and further treated with high power sonication. 2D MoS$_2$ nanoflakes are collected from the supernatant after centrifugation. Lithium ion intercalation of MoS$_2$ nanoparticles

One gram of MoS$_2$ powder (99% purity, Sigma Aldrich) was added to 0.5 mL of N-Methyl-2-pyrrolidone NMP (99% anhydrous, Sigma Aldrich) solvent in a mortar and ground for 30 min. Then the mixture was put in a vacuum oven to dry overnight at room temperature then was collected and redispersed into a 10mL NMP solvent. The obtained solution was sonicated (Ultrasonic Processor GEX500) for 90min at the power of 125W, and the supernatant containing 2D MoS$_2$ nanoflakes was collected after being centrifuged for 45 min at the speed of 4000 rpm.

4.2.2. PREPARATION OF CONDUCTIVE MoS$_2$-THREAD

4.2.2.1. Adsorption of MoS$_2$ nanoflakes to the cotton thread

Cationic polyacrylamide (CPAM) is one of the most used floculants for liquid/solid separation, preservation and drainage in papermaking [160, 183]. To encourage more adsorption and accumulation of MoS$_2$ nanoflakes to achieve higher conductivity, thread
substrates were treated with CPAM to manipulate their surface charge. On the day of experiment, the CPAM solution was prepared by diluting CPAM dry powder to 0.01% with milli-Q water (0.1 mg/mL or 2 mg/20 mL). Then the mixture was stirred for 1h to aid the dissolution process. The threads which were washed with ethanol of 5cm length were immersed into the CPAM solution for another 1h, to make the threads cationic. The prepared cotton threads were rinsed with DI water to remove any unattached particles and air dried.

4.2.2.2. MoS\textsubscript{2} coating onto the cotton thread

The treated cotton thread with CPAM is placed in 5 mL of MoS\textsubscript{2}-NMP solution. The soaked thread in the MoS\textsubscript{2}-NMP solution is ultra-sonicated for 30 min using ultrasonic cleaner (Ultrasonic Processor GEX500 Australia). The thread in the solution is soaked overnight to adsorb MoS\textsubscript{2} particles. Later the threads removed from the MoS\textsubscript{2}-NMP solution are washed with PBS 3 times to remove any unattached particles. Then the MoS\textsubscript{2}-thread is kept in -80°C freezer overnight. To stabilize the attachment of MoS\textsubscript{2} nanoflakes onto thread MoS\textsubscript{2}-threads are immediately freeze dried using freeze dryer (PowerDry, PL6000, by Thermo Scientific) for another 12 h.

4.2.2.3. Lithium Ion intercalation of MoS\textsubscript{2}-thread

High electrical resistivity limits the charge transfer kinetics of the semiconducting 2H MoS\textsubscript{2} phase nanosheets. Liquid-phase-exfoliated 2D MoS\textsubscript{2} nanoflakes are transformed from 2H into 1T crystal phase by employing electrochemical manipulation of intercalating lithium (Li\textsuperscript{+}) ions into MoS\textsubscript{2} crystal structure. The Li\textsuperscript{+} ion intercalation was carried out using a three-electrode cell on CHI 760D electrochemical workstation (CHI Instruments, Austin USA), with 0.1M LiClO\textsubscript{4} and Poly (Pyprolene-carbonate) (PC) electrolyte solution. MoS\textsubscript{2}-thread was chosen as working electrode, platinum wire as counter electrodes and Ag/AgCl as reference. Various potentials ranged from ±2 to ±12 V were applied across the electrodes.
Another method of Li$^+$ ion intercalation was used with employing a DC power supply (Keithley, 2400, USA). Various potentials ranged from 2V to 12V were applied across a two-electrode configuration via a DC power supply where the cathode was connected to the MoS$_2$-coated thread cut to 3mm and the anode was a platinum (Pt) wire. The electrolytes were 0.1M LiClO$_4$ (98% purity, Sigma Aldrich), in polypropylene carbonate (97% anhydrous, Sigma Aldrich).

4.3 ELECTRICAL CONDUCTIVITY OF THE MoS$_2$-THREADS

To experimentally determine electrical conductivity of cotton thread presents a challenge. Threads have one very long dimension and the other two very small. Therefore, determining their physical properties are difficult. The electrical resistivity of a cotton thread was measured by two probe method [138] using a Source-Meter (Keithley 2400, up to 200V, 1A, 20W Power output). In this method, a uniform current density is applied across the sample thread clamped between two electrodes placed on parallel faces. Then the potential drop between the electrodes was measured. Electrical resistivity of rMoS$_2$-thread was measured in both hydrated and dry state. Since the threads are going to serve as tissue scaffold in wet conditions. The values of surface resistivity (Ω/m) were calculated using the electrical resistance of the samples followed by obtaining electrical conductivity of the MoS$_2$-threads.

Current to Voltage, I-V plots for MoS$_2$-thread were determined to assess the conductivity of scaffolds before and after lithium ion intercalation. Conductive materials obey Ohm’s law, where the current through a conductive material between two points is directly proportional to their potential difference, and hence a linear (I-V) curve is the actual representation of a conductive material. The resistance measured between two electrodes follows the calculation of the electrical resistivity of the thread sample, since its geometry is of known dimensions.
The resistance is obtained using Ohm’s law then the electrical resistivity is calculated given that the applied current is uniform.

Electrical resistance is measured at specific intervals of length of 2mm, at 4 different places on the thread to obtain the best estimate. The slope of a plot of electrical resistance against length gives the resistivity. The electrical resistivity denoted by $\rho$ (unit: $\Omega m$) is given by:

$$\rho = \frac{RA}{L} = \frac{\pi d^2 R}{4L}$$

The distance $L$ represents the distance between the two probes.

Then using the electrical resistivity of thread formula,

$$\rho = \frac{RA}{L} = \frac{\pi d^2 R}{4L}$$

Where

$\rho$ is the electrical resistivity ($\Omega m$)

A is the cross-sectional area of the thread

d the thread diameter (m)

L is the thread length (m),

R is the electrical Resistance ($\Omega$)

The electrical resistivity of all thread samples were compared and reported. Electrical conductivity $\sigma$ of all thread samples is to be obtained by

$$\sigma = \frac{1}{\rho}$$
The electrical conductivity is perhaps the best indicator of the extent to which semiconducting MoS$_2$ coated thread has transmitted to its metallic state rMoS$_2$.

Two-point probe method was used to measure electrical resistance ($\Omega$m) of all samples. Thread samples were measured by connecting the probes with a distance of 2mm. Electrical resistivity of rMoS$_2$-thread was measured by two probe methods in both hydrated and dry state. Since the threads are going to serve as tissue scaffold in wet conditions. The values of surface resistivity ($\Omega$/m) were calculated using the electrical resistance of the samples followed by obtaining electrical conductivity of the MoS$_2$-threads.

4.4. CHARACTERIZATION OF CONDUCTIVE rMoS$_2$-THREAD

4.4.1. X-RAY PHOTOELECTRON SPECTROSCOPY OF rMoS$_2$-THREAD

It is believed that high concentration of the intercalated Li$^+$ ions ultimately forces the transition of the MoS$_2$ crystal from stable hexagonal semiconducting phase (2H phase) to a metastable metallic phase (1T phase) [105]. The phase transition can be verified by X-ray photoelectron spectroscopy measurements. XPS measurements were carried out on a VG-310F instrument using (Thermo K-alpha X-ray Photoelectron Spectrometer) Al non-monochromated X-rays (20 kV, 15 mA) with the hemispherical energy analyzer set at a pass energy of 20eV for the peak scans. The peaks related to the Mo and S were selected and analysed.

4.4.2. RAMAN SPECTROSCOPY OF MoS$_2$ THREAD

Raman microscopy was carried out on a Horiba (TRIAX 320 spectrometer fiber coupled to an Olympus BX41 confocal microscope with 532 nm 90$\mu$W excitation).
4.4.3. FEI NOVA NANOSEM™

To reveal the micrograph of the MoS₂ nanoparticles coated threads before and after lithium ion intercalation, high-resolution field emission scanning electron microscopy (FEI Nova Nano-SEM, with Oxford X-MaxN 20 EDXS Detector) using low vacuum operating at 30kV acceleration voltage was employed. Samples were gold sputtered using SPI-Module™ Sputter Coater and Vacuum Base (SPI Supplies®, PA, U.S.A.) before SEM imaging. Also scanning electron microscopy was done using Philips XL30 SEM (with Oxford X-MaxN 20 EDXS Detector and HKL EBSD system).

4.5. PERMEABILITY OF CONDUCTIVE RMoS₂-THREAD

Blue color food dye was used to identify the level of liquid wicking, along the samples. In these experiments, 8µL of was applied to one end of the thread (length($l = 2cm$)) using a pipette. The threads were kept vertical and attached by the ends to a ruler. A digital camera (Dino-Lite Digital Microscope AM4815ZT) is used to record the wicking motion of the liquid moving along the threads were recorded and measured. Measurements for each class of samples were repeated 3 times, and were recorded for visual appraisal.

4.6. BIOCOMPATIBILITY OF CONDUCTIVE RMoS₂-THREAD

4.6.1. CELL CULTURE

The NG108-15 neuroblastoma×glioma hybrid culture cells were obtained from the school of biomedical engineering, Swinburne University, and routinely cultured in the base medium with Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum (FBS) (Gibco®, Life Technologies Pty Ltd., Australia), 2 mM L-glutamine, and 50 unit/mL penicillin-streptomycin (P/S) at 37°C in a 5% humidified carbon dioxide incubator. The
culture medium was replaced every 2 days and sub culturing of cells was done every 4-5 days.

4.6.2. METABOLIC ACTIVITY ASSAY ON rMoS$_2$-THREAD TOWARDS NEURAL CELLS

AlamarBlue® assay was employed to evaluate the metabolic activity of NG108-15 cells grown on rMoS$_2$-thread. Cell seeding was performed at a density of $5 \times 10^4$ per well. The MoS$_2$-thread and rMoS$_2$-thread are made sterile by soaking them in the 70% ethanol for 1h. The sterile thread samples were cut to 2 mm in length and put in a 48-well plate with 4 repeats each samples along with NG108-15 cells and medium. The wells with cells only was served as control. The 48-well plate with samples and control is incubated for 3 days. At day 1, day 2 and day 3, the scaffolds were incubated for 4h with 10%(v/v) alamarBlue® (Life Technologies) in DMEM medium. A volume of 200 μL of the media from each sample was then pipetted into a 96-well plate and a microplated reader (Synergy™ Mx, Biotek) using an excitation wavelength of 570 nm and emission wavelength of 600 nm performed fluorescence analysis. The metabolic activity of cells was represented as the percentage of alamarBlue® reduction. These experiments were carried out in three replicates.

4.6.3. STATISTICAL ANALYSIS

Statistical analysis was carried out to determine the differences between different samples. The significance was obtained using one-way analysis of variance with a student’s t-test, to analyse differences between groups, using GraphPad Prism version 6.01 (GraphPad Prism software Inc., La Jolla, CA, U.S.A.). Experiments were performed independently at least three times. All values are expressed as mean ± standard deviation ($n = 3$). Differences between treatments were considered significant at $p < 0.05$. 
CHAPTER FOUR

4.7. RESULTS AND DISCUSSIONS

4.7.1. LITHIUM ION INTERCALATION OF MoS₂

After deposition of Li⁺ onto MoS₂-thread by electrochemical process, XPS measurements of clean and intercalated (MoS₂-thread and rMoS₂-thread) were carried out. Here, the results represent the change in Li level before and after Li⁺ ion intercalation as well as a series of XPS of Mo3d levels of MoS₂ when increased by Li deposition. The Mo3d core levels is made of two different photoemission peaks (3/2 and 5/2). According to Figure 4.1, Mo3d₃/₂, Mo3d₅/₂, S2p₁/₂ and S2p₃/₂ peaks are noted at 232.08, 228.88, 163.18, and 161.98 eV, respectively, stating 2H MoS₂ phase prevailing in the crystal structure of the 2D MoS₂ nanoflakes. After Li⁺ ion intercalation at 10 V, new peaks are showing at lower binding energies when compared to 2H phase peaks. These new peaks can be recognized as 1T phase peaks. Nonetheless, the peaks indicating the 2H phase are still seen after the intercalation process, revealing the coexistence of 1T and 2H phase in the structure of intercalated MoS₂.

Overall, these results confirmed that the intercalation process has transformed the MoS₂ crystal structure from 2H phase to metastable metallic 1T phase on the thread. Therefore, semiconducting MoS₂ has successfully transformed to metallic/conducting MoS₂ (rMoS₂).
Figure 4.1 XPS spectra of the elements of A-B) Li, C-D) S, and E-F) Mo in MoS$_2$ coated threads before and after Li$^+$ ion intercalation.
CHAPTER FOUR

4.7.2. RAMAN SPECTROSCOPY

Raman spectroscopy is carried out to further examine the crystal structure of the 2D MoS$_2$ nanoflakes attached to the cotton thread before and after intercalation. Raman spectral of 2D MoS$_2$ thread (black line) and 2D intercalated MoS$_2$ nanoflakes onto thread, indicating both $E_{2g}^1$ and $A_g^1$ Raman modes.

As Figure 4.2 shows two Raman peaks can be seen at ~384 and ~412 cm$^{-1}$ for MoS$_2$ nanoflakes coated onto cotton thread corresponding to in-plane ($E_{2g}^1$) and vertical plane ($A_{1g}$) vibrations of Mo-S bonds in 2H MoS$_2$, respectively. After normalizing both the Raman spectra taken from the 2H MoS$_2$ thread, and after ion intercalation 1T MoS$_2$ thread, in order to investigate the effects of Li$^+$ ion intercalation in MoS$_2$ nanoflakes coated on thread. Figure 4.2 shows that both $E_{2g}^1$ and $A_{1g}$ Raman modes indicate shifting to the right, revealing an increase in the vibration frequency, when compared to the initial Raman spectrum. The shift in $A_{1g}$ mode, corresponding to vertical plane vibrations of Mo-S bonds, is observed to be much larger than that of $E_{2g}^1$ mode, which represents the in-plane vibrations. The peaks observed in the Raman spectrum can be related to the strain introduced in the lattice and the effect of increase van der waals forces from the intercalating Li$^+$ ions suppressing the vibrations of Mo-S bonds.
Figure 4.2 Raman spectra of MoS₂-thread before and after Lithium ion intercalation.

4.7.3. FEI NOVA NANOSEM

To reveal the micrograph of MoS₂ nanoflakes coated onto threads before and after Li ion intercalation,

Figure 4.3 HRSEM images of A and D) CPAM thread B and E) MoS₂-thread C and F) rMoS₂-thread
CHAPTER FOUR

The morphologies of CPAM thread, MoS$_2$-thread before ion intercalation and after were visualized using SEM. The SEM images reveal that MoS$_2$ nanoflakes uniformly cover the surfaces of all filaments of the cotton thread. There is significant change after Li ion intercalation on the surface coating of MoS$_2$-thread. The rMoS$_2$ nanoflakes are adhered to the surface of all single filaments of the cotton thread as a very thin sheet. Figure 4.3 demonstrates that rMoS$_2$ loads MoS$_2$ sheets in the interspaces of thread fibers and surfaces of each filament but also can immerge into the interior of the thread due to evenly distribution of MoS$_2$ onto the thread fibers.
Molybdenum disulphide is a semiconductor 2D material, however after lithium intercalation it changes to a metallic state. Here the electrical conductivity of the MoS$_2$-thread as well as rMoS$_2$-thread is measured and compared with the CPAM-threads and blank thread as control. Both dry state and wet state conductivities measurement were obtained since these thread
substrates are going to be employed as nerve tissue scaffolds mostly in hydrated situations within cellular environment.

Table 4.1 Electrical resistivity and conductivity of blank thread, MoS\textsubscript{2} thread and rMoS\textsubscript{2}-thread.

<table>
<thead>
<tr>
<th>Different threads</th>
<th>Electrical Resistance (R)</th>
<th>Electrical Resistivity (ρ)</th>
<th>Electrical Conductivity</th>
<th>Electrical Conductivity (σ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ohm</td>
<td>ohm.m</td>
<td>S/m</td>
<td>S/m</td>
</tr>
<tr>
<td>Blank thread</td>
<td>$1.78 \times 10^{10}$</td>
<td>$1.16 \times 10^{6}$</td>
<td>$2.45 \times 10^{-5}$</td>
<td>$8.62 \times 10^{-7}$</td>
</tr>
<tr>
<td>MoS\textsubscript{2}-Thread</td>
<td>$2.59 \times 10^{9}$</td>
<td>$5.08 \times 10^{4}$</td>
<td>$2.29 \times 10^{-2}$</td>
<td>$1.96 \times 10^{-5}$</td>
</tr>
<tr>
<td>rMoS\textsubscript{2}-Thread</td>
<td>$5.60 \times 10^{6}$</td>
<td>$1.10 \times 10^{-2}$</td>
<td>$2.79 \times 10^{3}$</td>
<td>$9.54 \times 10$</td>
</tr>
</tbody>
</table>

As shown in Table 4.1 it is seen that, ion intercalation improves electrical conductivity of MoS\textsubscript{2}-thread by 5 order of magnitudes. Conductive silk film prepared with coating of polypyrrole and polystyrene sulfonate showed $(1.24 \times 10^{5})$ ohm/sq. sheet resistance [87], similar to the resistance of rMoS\textsubscript{2}-thread in this study.

4.7.5. Permeability of rMoS\textsubscript{2}-thread

The results in Figure 4.5 show that blank thread wicks the fluid (food coloring) faster than CPAM thread. Electro-chemical reduced molybdenum disulfide show better wicking properties compared to intercalated molybdenum disulfide. In considering rMoS\textsubscript{2}-thread as a tissue scaffold, the threads with most rapid rate of wicking are preferred.
After lithium ion intercalation the wicking rate of MoS₂-thread has decreased \((p < 0.0020, n = 3)\) compared to MoS₂-thread. The permeability of rMoS₂-thread \((p < 0.0001, n = 3)\) also shows decrease against blank thread. These results show that wicking rate of rMoS₂-thread is better than that of rGO-thread. Even though, electrochemical intercalation of MoS₂ has decreased the wicking properties of MoS₂, but wicking rate of 1.25 mm/s is a reliable rate for the extracellular matrix environment that these threads are going to be used for tissue scaffolding.

4.7.6. **BIOCOMPATIBILITY OF RMOS₂-THREADS**

Biocompatibility is vital concern when considering the development of tissue scaffold for biomedical purposes. Here, metabolic activity of PC12 cells through percentage of reduction of alamarBlue reagent was investigated after exposure to rGO-thread and rMoS₂-thread over 3 days. **Figure 4.6** reveals that rMoS₂-thread \((p = 0.0016, n = 3)\) and rGO-thread \((p = 0.0757, n = 3)\) show good metabolic rate when compared to the control (cells only). The results also indicate that the number of cells cultured with rMoS₂-thread \((p = 0.0001, n = 3)\)
3) and rGO-thread \((p = 0.0001, n = 3)\) increased gradually over time. Both thread scaffolds show good biocompatibility which makes it a promising biomaterial for conductive tissue scaffold.

Figure 4.6 PC12 cells viability is assessed and compared between MoS2-thread before and after ion intercalation and GO thread before and after reduction using alamarBlue assay. PC12 cells’ viability was assessed and compared as control. Data are reported as percentage of reduced alamarBlue solution and are mean values ± standard deviations \((n = 3)\).

Since cell adhesion to the fibers of tissue scaffold is of significance importance, another cell line is chosen to be investigated for nerve tissue scaffold development. The hybrid cell NG108-15 cell line is a well-studied neuronal model cell for nerve regeneration scaffolds.

Figure 4.7 shows that after 5 days of culture the viability of NG108-15 cells sited on rMoS2-thread \((p = 0.0907, n = 3)\) and on rGO-thread \((p = 0.8415, n = 3)\) are close to that of control; indicating that both rMoS2-thread and rGO-thread have similar effect on cells in terms of viability. The results show that NG108-15 cells cultured with conductive rMoS2-
CHAPTER FOUR

thread \((p = 0.0005, n = 3)\) and rGO-thread \((p = 0.0001, n = 3)\) had gradually grow over time which show high biocompatibility of threads.

![Figure 4.7](image)

**Figure 4.7** Comparison of NG108-15 cell viability measured with alamarBlue® assay, following 5 days of exposure to the rGO-thread and rMoS\(_2\)-thread, compared to the cells without exposure to threads as control. They are reported as percentage of reduced alamarBlue solution and are mean values ± standard deviations of three repeat experiments, each consisting of four wells per sample each experiment.

4.8 CONCLUSION

Molybdenum disulphide for the first time was employed to serve as a biomaterial incorporating into the common cotton thread as nerve tissue scaffold. First semiconducting molybdenum disulphide was coated onto the treated cotton thread following lithium ion intercalation of molybdenum disulphide to make it conductive. XPS study confirmed that the intercalation process used has converted the MoS\(_2\) from semiconducting to conducting material on thread. The molybdenum disulphide was found to covering the thread evenly after lithium ion intercalation. The wettability assay confirmed the good permeability of the rMoS\(_2\)-thread which is an important property of tissue scaffolds. Then the biocompatibility of the rMoS\(_2\)-thread was assessed towards two different neural cell lines, PC12 and NG108-15 cell. The rMoS\(_2\)-thread is biocompatible to both cell lines, and can therefore be considered as a promising candidate for nerve tissue scaffold.
CHAPTER FIVE

SURFACE FUNCTIONALIZATION OF RGO AND RMoS$_2$ THREAD SCAFFOLDS

5.1 INTRODUCTION

In this present chapter, surface of rGO-thread and rMoS$_2$-thread layers have been engineered, in order to enhance the attachment of nerve cells onto their surface to serve as nerve scaffolds. NG108-15 cell line has been chosen as our nerve cell model. To develop a functional vital engineered tissue scaffold, one should consider an appropriate design and fabrication of a scaffold. A temporary scaffold works as a provisional cell adhesion and proliferation foundation until a nerve tissue is generated by seeded cells to create a tissue morphology similar to that of a natural tissue[115]. Surface modification can influence the degradation rate and enhance cell adhesion properties [184]. It is proven that the process of cell adhesion, proliferation and differentiation on a polymeric material depends on surface properties such as the chemical and physical properties, charge, roughness and hydrophilicity/hydrophobicity. It is known that cell adhesion behavior is affected by different surface morphologies of tissue scaffolds [130]. The presence of positively charged surfaces allow for better adhesion of cells to the substrate [131]. Also, surface characteristics such as wettability rate and charge density have a significant role in protein adsorption and the interactions between the cell and substrate [24]. Cell attachment and proliferation is significantly defined by the chemical and physical properties of the tissue scaffolds’ surface [118]. The physical properties of scaffolds allow them to be modified for a particular application by binding biologically important molecules into the polymer by means of one of various methods for their functionalization [121].

Cells do not merely attach together to develop tissues, but are arranged into varied and characteristic patterns. A diverse range of cell attachment mechanisms are accountable for
accumulating cells together as well as linking their internal cytoskeleton to establish the architecture of the tissue [185].

There is a comprehensive literature regarding surface functionalization of tissue scaffolds to make them suitable for cell adhesion, proliferation and differentiation. Sofia et al. used silk nanofibers as substrate for cell attachments and tissue scaffolds. Adhesion of human osteoblast-like cells was investigated in the presence of serum and no serum on decorated silk films with integrin recognition sequences (RGD), parathyroid hormone (PTH) and modified PTH 1-34 (mPTH). They concluded that in the absence of serum, more cells attached to RGD and PTH than to plastic, and the silk and mPTH substrates had the fewest cells attached. The serum enhanced cell adhesion on almost all substrates investigated [186]. Albrecht et al. investigated the surface functionalization of poly(etherimide) (PEI) membranes, employing wet-chemical approach by a reaction between imide groups and amines [187]. Cheng et al. evaluated PC12 cells’ affinity to the developed chitosan-gelatin composite films. Their results showed that the elastic combination of chitosan and gelatin proved better affinity to nerve cells compared to chitosan [188]. Lee et al. found that electrospinning is a potential path to create nanofiber structures that are capable of supporting adhesion and guiding extension of neurons for nerve regeneration [128]. Low et al. used amino salinization to cover the surface of porous silicon scaffolds with collagen to promote cell adhesion and spreading [184]. Ku et al. investigated coating poly(dopamine) to enhance cell attachment on different materials surfaces even the anti-adhesive substrate; poly(tetrafluoroethylene). Their results indicated that poly(dopamine) coated substrates increased the affinity of cells to the modified surfaces when compared to the unmodified non-wetting surfaces [116].

In the literature different methods has been investigated to promote cell adhesion on the surface of a variety of scaffolds. Badami et al. incubated electrospun substrates with human fibronectin in PBS to promote cell adhesion [25].
Comprehensive studies have been done to render materials biomimetic [188]. The surface functionalization of biomaterials with bioactive molecules is a simple way to make biomimetic materials. The early work has used long chains of ECM proteins such as fibronectin and laminin for surface functionalization. Biomaterials can be coated with these proteins, which usually have promoted cell adhesion and proliferation. The short peptide sequences are relatively more stable during the modification process than long chain proteins such that nearly all modified peptides are available for cell binding. The biomimetic materials modified with these bioactive molecules can be used as a tissue engineering scaffold that potentially serves as an artificial ECM providing suitable biological cue to guide new tissue formation [189].

The most commonly used peptide for surface modification is Arg-Gly-Asp (RGD), the signaling domain derived from fibronectin and laminin. [190]. It is known that gelatin contains Arginine-Glycine-Aspartate (RGD) motifs, RGD peptides provide a high affinity site for cell binding, and are often incorporated in biomaterials to promote cell adhesion [189, 191]. Gelatin derived from collagen has been used for pharmaceutical and medical applications due to its abundant availability, cost-effectiveness, excellent biodegradability and biocompatibility, and non-immunogenic properties.

Gelatin is commonly used natural polymer which is derived from collagen. The isoelectric point of gelatin can be modified during the fabrication process to yield either negatively charged acidic gelatin, or positively charged basic gelatin at physiological pH. This theoretically allows electrostatic interactions to take place between a charged biomolecule and gelatin of the opposite charge, forming polyion complexes [190].

In the present research gelatin has been employed for surface functionalization of both thread scaffolds. This technique guaranties simplicity, low cost operation and a good adhesion[187].
5.2. MATERIALS AND METHODS

5.2.1. PREPARATION OF GELATIN FUNCTIONALIZED SCAFFOLDS

The thread scaffolds were sterilized in ethanol (70%, v/v) for one hour. Sterilized CPAM treated thread, rMoS\textsubscript{2} coated thread and ER-GO2% coated thread scaffolds were activated in infiltrated 40 nM 1,1’-carbonyldiimidazole (CDI) (Sigma-Aldrich, NSW, Australia) in acetone at room temperature for 3h. The activated thread scaffolds were subsequently washed in acetone for 5 times in order to remove any unreacted residues of CDI then were soaked in acetone for 5 min to become completely dried. Then gelatin powder (Sigma-Aldrich, NSW, Australia) (10 mg/mL) was mixed in a sodium bicarbonate (NaHCO\textsubscript{3}) buffer (50 mM, pH 9) at room temperature and then the pH of the solution was adjusted to 9 by adding sodium hydroxide (NaOH), then the gelatin solution was stirred and heated to 80ºC over a magnetic stirrer (IKA® C-MAG HS 4-South East Asia) to dissolve gelatin. The gelatin solution was filtered followed by soaking the coated threads with CDI in gelatin solution for 24-48 h at 4ºC (fridge).

5.2.2. DETERMINATION OF AMINE CONTENT BY ACID ORANGE II ASSAY

Acid Orange II assay is employed to calculate and determine the amount of surface amine on the surface functionalized threads. In brief, surface functionalized (SF) threads (rMoS\textsubscript{2}, rGO 2%), SF-rMoS\textsubscript{2} and SF-rGO threads, were cut into 2 mm samples. The untreated thread samples serve as controls. Samples were incubated in 1 mL of Acid Orange II solution in DI water (500 µM, pH3) overnight at room temperature. Then the samples are washed with copious water at pH3 (adjusted with HCl) to remove unbound Acid Orange II from the threads. Next the thread samples are incubated in 1 mL of DI water at pH12 (adjusted with NaOH) overnight to remove the bound dye from the samples. A series of Acid Orange II
standard solutions (100 µM-500 µM) were prepared in DI water at pH12 and the data used to create the standard curve. The amount of the bound dye was quantified by measuring the optical density with a plate reader at 492 nm.

5.2.3. CHARACTERIZATION OF CELL MORPHOLOGY AND VIABILITY

NG108-15 cells (4×10⁴ cell/mm) were seeded onto sf-rGO and sf-rMoS₂ thread and non-surface functionalized rGO and rMoS₂ threads for 4 days, changing the medium every 2 days to promote cell adhesion on the thread scaffolds. Cell viability was investigated using the Live/Dead® viability/cytotoxicity Kit (Molecular Probes®, Invitrogen™, Life Technologies, CA, U.S.A.). To determine cell morphology and viability on the scaffolds, the cells were stained with Calcein AM (4mM diluted in anhydrous dimethyl sulfoxide; DMSO) and incubated for 15-20 min at 37°C in 5% humidified CO₂ incubator. Then samples were washed with PBS to remove non-adherent cells. Intracellular esterase’s activity converts non-fluorescent, hydrophobic Calcein AM to a hydrophilic, strong fluorescent compound, and Calcein AM is well-retained in the intact live cell cytoplasm. Then the samples are stained with Propidium Iodide (PI) for 2-5 min at room temperature. When PI binds to DNA (nucleic acid) of cells with damaged membrane its fluorescence is enhanced 20-30 fold, exhibiting a bright red fluorescence in dead cells. After staining, the cells were observed by laser scanning confocal microscope (Nikon, TE2000U inverted fluorescence microscope, DS-Fi1 USB CCD camera, NIS elements imaging software).

5.2.4. METABOLIC ACTIVITY OF CELL SEEDED ON THREAD SCAFFOLDS

Metabolic activity of NG108-15 nerve cells grown on sf-rGO and sf-rMoS₂ thread were investigated by alamarBlue® assay. NG108-15 cells were seeded on scaffolds at a density of 5×10⁴ cells/mm and were incubated at 37°C in humidified CO₂ incubator for 4 days to promote cell adhesion. On Day 5, 6 and 7 after adding 10% (v/v) alamarBlue® solution
CHAPTER FIVE

(Invitrogen, CA, U.S.A.) diluted with culture medium, to each well containing the cell/scaffolds, the plate is incubated at 37°C in humidified CO₂ incubator for 4 h. After removing the thread samples, the absorbance was measured at both 570 nm and 600 nm by microplate reader (Perkin Elmer, MA, U.S.A.).

5.2.5. PERMEABILITY ASSAY ON SURFACE FUNCTIONALIZED THREADS

One important criteria required for the thread to be employed as a tissue scaffold is to be hydrophilic to transfer neurite and waste to and from the scaffold that is acting as extracellular matrix. Next the liquid wicking property should be homogenous across full length of the thread to provide the same wet condition for all the cells attached to the thread. The cotton thread has pores to aid liquid penetration. Wettability indicates how easy an aqueous spread along the thread fibers. To examine the wicking properties of rGO and rMoS₂ thread, the rate of transport of reagents or in our case media before and after surface functionalization was determined through wettability assay.

5.2.5.1. Plasma treatment

Preliminary results of thread scaffolds’ permeability proved surface functionalization of thread scaffolds decreased the wicking speed of liquid through the thread filaments in both thread scaffolds. To overcome this problem, plasma treatment was chosen to be done on all thread samples to improve wicking properties of thread scaffolds.

Vacuum plasma is used to enhance the fiber surface oxygen concentration and polarity in treated cotton thread [63]. This process significantly improves the wettability of the cotton fibers after surface functionalization, making thread based microfluidic devices more reliable. Reches et al. reported that plasma treatment improved aqueous liquid wicking rates on natural fibers [64].
CHAPTER FIVE

To improve the permeability of surface functionalized rGO and rMoS$_2$ thread scaffolds, the threads were treated with plasma surface modification to promote wettability[114]. The plasma process was carried out in a plasma cleaner (Expanded Plasma cleaner-PDC-002-Harrick Plasma, NY, U.S.A.) to break down organic contaminants.

All thread samples; rGO-thread, sf-rGO thread, MoS$_2$-thread, sf-rMoS$_2$ thread, were put into plasma vacuum for 10 min at 400 mmHg to enhance their wettability and their consistent wicking rate over their full length after surface functionalization with CDI/Gelatin.

5.2.5.2. Wettability assay

All of these thread samples are inherently hydrophilic, meaning that capillary action facilitates fluids to wick along the narrow channels between the voids between fibers.

The threads were cut to 4 cm in length. A scale printed on paper and is fixed on a polymer film with inlets to allow the threads to be held in parallel to the paper scale to provide a convenient measure of lengths of the color change on the threads. In Figure 5.1 the permeability test set up is shown, three samples of each thread scaffolds a) rGO thread and b) rMoS$_2$ thread are attached to scale to measure the wicking of liquid through the thread scaffolds.

Figure 5.1. Wettability assay set up for a) rGO thread and b) rMoS$_2$ thread is shown.
In these experiments, 8 µL of aqueous solution of fluorescent dye 1 mg/mL concentration diluted with 5% methanol was prepared. The water-isopropyl alcohol (IPA) solution was prepared to measure the wicking of the water-IPA solutions in untreated cotton thread. The surface tension of water-IPA is known therefore is a convenient way to measure the wicking property of the thread. Since the cotton thread is porous, the wicking onset, in the thread, can be determined by measuring the wicking speed of the liquid within different GO-thread samples. 10 µL of aqueous solution of fluorescent dye was applied to the end of each thread sample cut to 4 cm in length.

Using a digital camera (Dino-Lite Digital Microscope, AM4815ZT), the wicking motion of the liquid moving along the threads were recorded and measured. In the Figure 5.2 the wicking of fluorescent dye is shown along the rMoS\textsubscript{2} thread.

![Figure 5.2](image-url)

**Figure 5.2.** Image of wicking of the fluorescent dye up on the sf-rMoS\textsubscript{2} thread scaffolds attached to transparent polymer film and paper scales.

Measurements for each class of samples were repeated 3 times and were recorded for visual appraisal. Data were reported by mean($n = 3$) ± standard deviation.
CHAPTER FIVE

5.2.6. Statistical Analysis

All experiments (alamarBlue®, Acid Orange II and permeability test) were carried out at least three times, the results are stated as average values ± standard deviation. Multiple groups of data were statistically analyzed using ANOVA (analysis of variance). In each experiment two groups of data were statistically analyzed using the unpaired Student t-test, probability (p) values lower than 0.05 (95%) were considered statistically significant.

5.3. Results and Discussion

5.3.1. Surface Functionalization of 3D Thread Scaffolds

Cell adhesion is essential for growth and proliferation of cells seeded on tissue scaffolds. One of the important factors affecting cell adhesion is the scaffolds porosity degree as well as, its surface properties. Thread as a 3D scaffold provides an assortment of small and large pores, to optimize cell growth and function [114].

The amine functionalized threads form a conformal monolayer on negatively charged rGO-thread and rMoS₂-thread filament surface. Surface functionalized rGO and rMoS₂ threads were investigated as scaffolds for neuronal cell culture. Gelatin, a model biomolecule, was grafted onto CDI treated rGO and rMoS₂ thread to promote cell-substrate interactions.

In this work, adhesion and morphology of NG108-15 cells were valued on scaffolds, whose surfaces were modified by gelatin surface functionalization. We analyzed the influence of surface modifications on the morphology of adherent cells, in particular, thread scaffolds treated with CDI/Gelatin[114]. These surface modified scaffolds were compared with untreated ones after seeding neural cells. NG108-15 cells were seeded on the scaffolds that were modified with surface modification process, described before, the cell cultures were carried out for 4 days and the morphology and the cell interaction/adhesion on the scaffolds
were analyzed by confocal laser scanning microscope. Live/dead staining was carried out by using Calcein AM to determine the viability of neuronal cells inside the microfibers of sf-rGO and sf-rMoS₂ thread after 4 days of culture. **Figure 5.3** shows that most of the cells are alive (green fluorescence showing live cells) inside the sf-rGO and sf-rMoS₂ thread (red fluorescence showing the thread) and reveals that the thread scaffolds support cell cultivation. The results indicate that cells have a preference for attaching to surface functionalized thread when compared to the untreated thread scaffolds. The proliferation of cells indicates that the functionalized thread surfaces are biocompatible with cells.

**Figure 5.3** NG108-15 cells morphology by confocal microscopy. In these figures viability of neuronal cells grown on thread scaffolds for 4 days without surface functionalization a) have
fewer cells attached and b) surface functionalized threads with CDI/Gelatin show better cell attachments.

5.3.2. PHYSICOCHEMICAL PROPERTIES OF SURFACE FUNCTIONALIZED rGO-THREAD AND rMoS₂-THREADS

Acid Orange II assay was carried out to establish and assess the amount of amine groups activated on surface of sf-rGO and sf-rMoS₂ thread scaffolds. Figure 5.4 a) shows that the surface functionalized threads have more amine present than that on the untreated rGO and rMoS₂ thread, revealing that the surface functionalization process was successful.

The acid orange concentration for each of data point has been calculated for both thread scaffolds before and after surface functionalization, instead of calculating the average absorbance and convert it to concentration.

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 5.4.** Amount of adsorbed Acid Orange II onto a) sf-rGO thread compared with untreated rGO thread scaffold and b) sf-rMoS₂ thread scaffold compared with untreated rMoS₂ thread scaffold.

The results indicate that both thread scaffolds have adequate amount of amine group on their surface. Figure 5.4 a) shows that after surface functionalization, rGO-thread has 15% more amine group on its surface when compared to untreated rGO-thread ($p < 0.11, n = 9$) revealing. Acid orange II assay on rMoS₂-thread confirms that surface functionalization of the rMoS₂-thread has been done successfully. Figure 5.4 b) shows that after surface functionalization the amine content on the thread scaffold have increased ($p < 0.0.126, n = $)}
3) compared to untreated rMoS$_2$-thread. The outcome of this assay indicates that rMoS$_2$-thread contains 13% more amine group during surface functionalization into its surfaces compared to rGO-thread. The results of student t-test show that the surface functionalized rMoS$_2$ thread and non-functionalized rMoS$_2$ thread are significantly different ($p < 0.0007, n = 9$).

The results of an unpaired t-test performed on surface functionalized rGO thread and non-functionalized rGO thread shows the probability of this result, assuming the null hypothesis is 0.0062.

5.3.3. Permeability of the Thread Samples After Surface Functionalization

Wicking properties of all thread samples blank thread, CPAM treated thread, MoS$_2$-thread before and after ion intercalation and finally rMoS$_2$-thread before and after plasma treatment were tested. Figure 5.5 and 5.6 demonstrates the comparison between all thread samples’ wicking behavior.
Figure 5.5 Graph of wettability rate of thread samples regarding rMoS$_2$-thread: Blank thread, CPAM thread, MoS$_2$-thread before and after ion intercalation, rMoS$_2$-thread before and after surface functionalization and plasma treated rMoS$_2$ thread. Error bars indicate one standard deviation from the mean ($n = 3$).

Plasma treatment creates wicking properties by oxidizing the thread surface and removing any contaminants. Figure 5.5 shows that the wicking speeds of plasma treated sf-rMoS$_2$ thread (PT-sf-rMoS$_2$ thread) has improved in comparison with that of untreated sf-rMoS$_2$ thread as well as other samples, indicating that the plasma treatment enhances permeability of the thread surface making the thread more suitable as a tissue scaffold candidate. Figure 5.5 shows that CPAM thread, MoS$_2$-thread and PT-sf-rMoS$_2$ thread had close permeability rate to the blank thread ($p < 0.0707, n = 3$), ($p < 0.0.432 , n = 3$), ($p < 0.201, n = 3$) respectively. However, the permeability rate of rMoS$_2$-thread ($p < 0.0002, n = 3$), and sf-rMoS$_2$ thread ($p < 0.0002, n = 3$), is considered quite lesser than that of blank thread.
The ability of a scaffold to facilitate the delivery of nutrients to cells is vital for the scaffolds' proper function. Transport within a scaffold is mainly done with diffusion. Scaffold permeability rules issues such as oxygen and nutrient delivery, protein transfer, and waste removal [141]. Permeability is a crucial characteristic of nerve tissue scaffolds, to promote cell-to-cell communication, allow neurite exchange, and potential guide vascularization [17].

These results are in consistence with the studies from literature, Li et al. [192] showed that plasma oxidation of cotton thread can enhance wicking and allow liquid transport in thread microfluidic devices. Reches et al. proved that plasma treatment increased the rate of wicking [64].

Figure 5.6 shows that plasma treatment significantly enhances permeability of the thread surfaces; in rGO-thread with 81% and 63% in rMoS2-thread. Therefore, plasma treatment is a reliable method to improve permeability of thread surfaces intended to be employed as tissue scaffolds.
Both samples are hydrophilic; however, with plasma treatment hydrophilicity of thread scaffolds increased after surface functionalization.

Plasma treatment has significantly improved permeability rate of sf-rMoS₂ thread ($p < 0.0147, n = 3$) and sf-rGO thread ($p < 0.0172, n = 3$).

5.3.4. **BIOCOMPATIBILITY OF SF-RGO AND SF-RMO₂ THREAD SCAFFOLDS**

To evaluate viability and growth of NG108-15 cells grown on sf-rGO thread and sf-rMoS₂ thread, alamarBlue® assay was chosen, a colorimetric assay that works by means of enzymatic reduction. NG108-15 cells were seeded onto surface functionalized threads and untreated threads into a well-plate and cultured over 7 days, and were evaluated starting from day 4.

**Figure 5.8** shows that metabolic activity of the NG108-15 cells seeded on scaffold after surface functionalization of rMoS₂-thread scaffold. Over 7 days of culture, there were increased in metabolic activity, as indicated by percentage of alamarBlue reduction, for all samples including surface functionalized threads($p < 0.00001, n = 3$), untreated threads.
(\( p < 0.0018, n = 3 \)) and control cells(\( p < 0.00001, n = 3 \)). The result also indicates that surface functionalized rMoS2-threads are biocompatible. This is most likely due to the introduction of gelatin which promotes cells attachment onto the thread scaffolds.

**Figure 5.8** Metabolic rate of seeded NG108-15 cells after surface functionalization of rMoS2-thread at day 5, 6 and 7 was determined via alamarBlue® assay. The data are presented as mean (\( n = 3 \)) and error bars represent the standard deviation.

A handful of studies recently have shown low cytotoxicity of MoS2 towards cells such as, A549 cells [111], human Bronchial epithelial cells [193], as well as good biocompatibility towards CCC-ESF-1 cells[108]. Our results are similar to these findings on biocompatibility of MoS2 on different cell types.

In **Figure 5.9** the same metabolic activity study is carried out on NG108-15 cells seeded into both surface functionalized rGO-thread and untreated rGO-thread. The results show that over
time, cells seeded into sf-rGO thread had an increase in metabolic activity as indicated by alamarBlue reduction for surface functionalized rGO-threads ($p < 0.00001, n = 3$) and untreated rGO-threads ($p < 0.00001, n = 3$) and finally the control cells($p < 0.00001, n = 3$). These results confirm the feasibility and credibility of surface functionalization of thread scaffolds by CDI/Gelatin to promote cell adhesion, growth and proliferation.

**Figure 5.9.** Metabolic rate of NG108-15 cells towards rGO-thread scaffold after surface functionalization using alamarBlue® assay over 7 days after seeding and assessed for 3 consecutive days after culture. Results expressed as mean ($n = 3$)± standard deviation. Significantly different results presented as ($p < 0.05$).

Yuan *et al.* carried out MTT assay on relative cell proliferation of HepG2 cells treated with reduced GO. Their results suggested that GO is biocompatible and could be a good candidate for bio-linked applications [194]. Our results are consistent with other studies on biocompatibility of rGO towards mammalian cells.

To compare two scaffolds’ biocompatibility after surface functionalization, the results show that both sf-rMoS2 thread scaffolds and sf-rGO thread scaffold are biocompatible for NG108-5 cells.
The electrical conductivity of rGO-thread and rMoS$_2$-thread after surface gelatin functionalization stayed almost the same. Electrical conductivity of SF-rGO was measured to be $1.1 \times 10^{-5}$ S/m, and SF-rMoS$_2$ was calculated to be $1.90 \times 10^{-10}$ S/m.

### 5.4 Conclusions

This study determined the feasibility of surface functionalization of thread scaffolds by using CDI/Gelatin solution to promote cell adhesion. Surface modification of rMoS$_2$-thread scaffold increased neural cells affinity to the surface of the scaffolds. Optimum cell number attached to both thread scaffolds was obtained after 4 days of culturing NG108-15 cells on the proximity of them. Our results confirm that plasma treatment significantly increased wettability of the thread scaffolds after surface functionalization.

Given the concept of conductive thread as tissue scaffolds are novel, comparisons with other similar surface modifications are not possible as a result of lack of similar studies. However, excellent results in the present work proved that the CDI/Gelatin surface functionalization enhances cell adhesion, growth and division. Biocompatibility evaluation confirmed great biocompatibility of the scaffolds after surface functionalization of both rGO-thread and rMoS$_2$-thread.
CHAPTER SIX

ELECTRICAL STIMULATION OF NEURAL CELLS WITHIN CONDUCTIVE NERVE TISSUE SCAFFOLDS

6.1 INTRODUCTION

The field of nerve tissue engineering has been inspired by integrating conductive biomaterials into biomimicking three-dimensional constructs to replace the natural extracellular matrix at different levels of tissue behavior to promote cell and tissue growth [195]. Nerve conduits are a promising alternative to autograft for repair of nerve injuries. Biocompatibility, biodegradability, flexibility and permeability are important factors for creating a nerve tissue scaffold. Most commonly used biomaterial for the nerve tissue scaffolds are conductive polymers such as polypyrrole (PPy) and polyaniline [143]. Conductive polymers have been used in different biomedical applications including biosensors, actuators, and nerve tissue replacement [8, 10, 18]. However, the drawback of these materials is that they are not biodegradable on their natural state. Non-biodegradability of these conductive polymers limits their use as neural scaffold materials [13]. Conductive polymer based scaffolds can be prepared biodegradable through different methods like grafting water soluble conducting oligomers to the surface of a biodegradable scaffold [24].

Several studies have pursuit to synthesize conductive nanofibers (using electrospun methods) for tissue engineering applications. Lelkes et al. [88] and colleagues electrospun polyaniline-gelatin blends to produce conductive nanofibers, with showing good conductivity. Nowadays conductive textiles made by conjugated polymers, metal powders and carbon nanotubes are still common, however these materials are expensive and treatments of them on textile fabrics involves complicated methods. Also, the shortcoming associated with them includes low open-air stability, flexibility and non-homogeneity of coatings.
Gomez et al.[89] fabricated conductive topographic substrate for neural interfacing PPy microchannels by electrochemical synthesis. They have also been used to regulate cell functions by applying electrical stimulation (ES) through conductive polymers, especially for electrically excitable cells such as neuronal or muscle cells [13]. A lot of studies have shown that ES through a conductive polymer significantly enhanced neurite outgrowth and cell spreading. Park et al. [196] investigated the effects of electrical stimulation upon cytotoxicity and differentiation of hMSCs. The hMSCs co cultured with mature neuronal cells on AuNP-coated tissue culture, showed significant neurite outgrowth after subjecting to electrical stimulation of 250 mV for 1000 s once every 3 days. Feng et al. [197] electrically stimulated the cells and observed accelerated growth and development of primary motor neurons. Also they reported two-fold increase in speed of neurite growth on the G-NFs. 100 mV pulse electrical signal stimulation was applied by GNFs to the neurons in vitro. Weng et al. [198] reported that electrical stimulation of PC12 cells grown on inkjet-printed PPy/collagen scaffold enhanced neurite outgrowth and orientation. The cells were subjected to a charge-balanced biphasic pulsed current (250 Hz) for 2 h after changing to differentiation medium.

Electrical signals after nerve injury improves the amount and precision of motor and sensory activity [199-202]. Genchi et al. [203] showed that differentiated PC12 cells exhibited highly aligned and longer neurites on parallel poly (3-hydroxybutyrate) (PHB) fibers with respect to random fibres. The significance of topographic properties of extracellular environments is essential to promote cell division, especially in case of tissue repair or nerve injury.

For this study, NG108-15 a neural cell line is chosen as it is usually employed in co culture models of muscle cells to detect and monitor synapse formation [133].

As indicated in previous chapters, GO and MoS$_2$ has made conductive through electrochemical reduction and lithium ion intercalation respectively and have been coated
onto cotton thread. Reduced graphene oxide and Molybdenum disulphide have been used in various applications; this is the first time rGO and rMoS2 have been used in nerve tissue engineering as a conductive biomaterial. These thread scaffolds (rGO-thread and rMoS2-thread) have been successfully made conductive towards nerve cells.

Then, surface functionalization of both sample threads is carried out to promote cell adhesion onto the scaffolds. It is known that gelatin contains arginine-glycine-aspartate (RGD) motifs; RGD peptides provide a high-affinity site for cell binding and are often incorporated in biomaterials to promote cell adhesion [189, 191].

Electrical stimulation on NG108-15 neural cell line is carried out to observe electrical activity of neural cells within the scaffolds. Experimental conditions, as cell seeding density, voltage, duration of stimulation and frequency are important for achieving consistent results.

Frequency should be selected according to the type of cell, so that it doesn’t interfere with the sensing neural spikes when they are electrically stimulated or the fast polarization phase of the action potential, because the activation kinetics duration is limited to several milliseconds.

The nerve cells were electrically stimulated at different voltages to investigate cells viability, health and spreading, followed by establishing the proper voltage and duration of stimulation for NG108-15 cells, before seeding them into the thread scaffolds.

NG108-15 cells were cultured in 12-well plates at cell density of $4 \times 10^4$ cells/well. Then they were incubated for 24h. On the day of experiments, half of the cells were kept as control (not electrically stimulated) to be compared to the electrically stimulated cells. Two different voltages were considered to be applied to the cells, 60mV and 20V at 5 Hz. Each set to be electrically stimulated for the durations of 5, 30 and 60 min.
CHAPTER SIX

First the frequency of 0.5 Hz at 5, 30 and 60 min duration at three different voltages mentioned was applied, however after staining the cells and observing them under confocal microscope, the results showed cells have died.

Next frequency of 5 Hz at 5, 30 and 60 min duration at three different voltages were applied, and the results showed cells viability and spreading similar to control.

Here for the first time surface functionalized conductive reduced graphene oxide and molybdenum disulphide coated thread scaffolds have been investigated for cell viability, extent of proliferation and differentiation of NG108-15 within these novel conductive tissue scaffold before and after electrical stimulation. Finally show that sf-rGO thread and sf-rMoS$_2$ thread scaffold seeded with neuronal cells promotes cell functionality and are suitable for nerve regeneration system and cell transplantation in nerve injuries.

Electrical activity of cells was investigated using laser confocal microscopy live cell imaging. Also alamarBlue® assay was carried out to determine cells viability after electrical stimulation.

The ability of electrically stimulated NG108-15 cells to proliferate on the scaffolds was evaluated by quantifying the deoxyribonucleic acid (DNA) content using PicoGreen® assay.

This project was designed to investigate the interaction of nerve cells and surface engineered rGO-thread and rMoS$_2$-thread scaffolds in promotion of cell adhesion, viability, proliferation and functionality. Furthermore, examination of the possibility of growth and differentiation of nerve cells in the scaffold during electrical stimulation, followed by electrical excitation.
6.2. MATERIALS AND METHODS

6.2.1. ELECTRICAL STIMULATION SET UP

The electrical stimulation method used here is biphasic (AC) [204, 205] with square wave [206, 207] and these signals are generated by signal generators [206, 208, 209]. Direct coupling is the method used for delivering the stimulus; the platinum electrodes are in direct contact with the cell culture medium. For live cell imaging using laser confocal microscope, the platinum plates are attached to the custom made electrical cell culture plate connected to the signal generator (Rhode & Schwarz, 2625, Germany) through wires. Direct current was applied to the cells on the scaffolds, according to the literature [31]; direct current has proven to regulate cell functions.

6.2.2. ELECTRICAL STIMULATION OF NG108-15 CELLS

The NG108-15 neuroblastoma×glioma hybrid culture cells were obtained from cryostored stocks in the school of biomedical engineering, Swinburne University, and routinely cultured in the base medium with Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum (Gibco®, Life Technologies Pty Ltd., Australia) and 1% penicillin and streptomycin, 1% Glutomax at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was generally replaced every 2 days with fresh medium and subculturing of cells was done every 4 days. When neural NG108-15 cells are 95% confluence, they were washed and resuspended in general DMEM.

Then a potential of 60 mV/mm for 5 min at 5 Hz using signal generator was applied to the cells through the customized electrical 6-well plate system mentioned earlier.
After electrical stimulation, the cells were incubated for another 24 h at 37°C in 5% CO₂ incubator, and then Live/Dead cytotoxicity assay with Calcein AM and PI was carried out to investigate the effect of electrical stimulation on NG108-15 cells viability. NG108-15 cells grown on non-stimulated thread scaffolds served as control.

6.2.3. CELL MORPHOLOGY AND LIVE/DEAD CYTOTOXICITY ASSAY

NG108-15 cells (4×10⁴ cell/mm) were seeded onto sf-rGO and sf-rMoS₂ thread scaffolds and non-surface functionalized rGO and rMoS₂ threads for 4 days, changing the medium every 2 days to promote cell adhesion on the thread scaffold. Cell viability was investigated using the Live/Dead® viability/cytotoxicity Kit (Molecular Probes®; Invitrogen™, Life Technologies, CA, U.S.A.). To examine cell morphology and viability on the scaffolds, the cells were stained with Calcein AM (4mM diluted in anhydrous dimethyl sulfoxide; DMSO) and incubated for 15-20 min at 37°C in 5% humidified CO₂ incubator. Intracellular esterase’s activity converts non-fluorescent, hydrophobic Calcein AM to a hydrophilic, strong fluorescent compound, Calcein that is well-retained in the intact live cell cytoplasm. The samples were stained with Propidium Iodide (PI) for 2-5 min at room temperature. On the other hand, the fluorescence of PI is enhanced 20-30 fold when PI binds to DNA (nucleic
acid) of cells with damaged membrane, exhibiting a bright red fluorescence in dead cells. After staining, the cells were observed by laser scanning confocal microscope (Nikon, TE2000U inverted fluorescence microscope, DS-Fi1 USB CCD camera, NIS elements imaging software).

6.2.4. ELECTRICAL STIMULATION OF NG108-15 CELLS SEEDED ONTO THREAD SCAFFOLDS

To seed NG108-15 cells into the thread scaffolds, first fast-gelling carboxymethyl cellulose (CMC) based hydrogel, specifically carboxymethyl cellulose tyramine (CMC) TYR gel is prepared to avoid adherent of the cells to the bottom of the tissue culture dish.

CMC is a commonly used degradable pharmaceutical material approved by the Food and Drug Administration (FDA) and is cost effective and commercially available. CMC Tyr gel (3%) is prepared and mixed well. Cross linkers H$_2$O$_2$ and HRP are added by 1% to the gel. The 300 µL gel is pipetted out to the bottom of each culture dish and left to set.

Then the activated thread scaffolds are taken out of the fridge and rinsed with sterile PBS 3 times. Thread samples were sterilized by soaking in ethanol 70% (v/v) for 1 h. The thread scaffolds are then laid on top of the gel in the culture dish before adding the NG108-15 (1.5×10$^5$) cells per dish with added 3 mL medium as shown in Figure 5.2. NG108-15 cells on top of thread scaffolds were then incubated at 37°C in a 5% CO$_2$ humidified incubator for 4 days.
Figure 6.2 Image of electrical stimulation set up for sf-rGO threads, sf-rMoS$_2$-threads and control cells in a 24-well plate.

After electrical stimulation, the cells were incubated in cell culture medium for another 24 h at 37°C in 5% CO$_2$ incubator. Live/Dead® cytotoxicity assay with Calcein AM and PI were carried out using similar procedure as described earlier. Non-stimulated NG108-15 cells/scaffolds served as control.

6.2.5. PROLIFERATION CULTURE AND ASSAY OF ELECTRICAL STIMULATED CELL-SCAFFOLDS

For the cell/scaffolds proliferation studies, the NG108-15 cells were cultured and then seeded onto the surface functionalized and sterilized thread scaffolds for a period of 5 days after being electrically stimulated as was mentioned in previous section. In brief, the sf-rGO thread and sf-rMoS$_2$ thread scaffolds were sterilized by soaking in 70% ethanol for 1 h, and then 4 replicates of each scaffold were placed on pre-coated wells with CMC TYR gel, in a 24-well plate tissue culture.

NG108-15 cell solution with the density of 5×10$^4$ cell/mm was placed on top of the scaffolds followed by adding 2 mL of culture medium. NG108-15 cells and thread scaffolds were cultured for 4 days, changing medium every 2 days. On day 5, half of the scaffolds were
electrically stimulated at 60 mV at 5 Hz for 5 min followed by 48 h of incubation at 37°C in CO₂ incubator.

The NG108-15 cells were trypsinized and collected at different time intervals, followed by calculating the number of cells proliferating within the scaffolds by quantifying the DNA content using a Quanti-iT PicoGreen ds-DNA assay (Life Technologies, Mulgrave, VIC, Australia).

Cell proliferation is calculated by measuring the double standard DNA (ds-DNA) content of the cells. Ds-DNA content in the cell lysates was calculated with the PicoGreen® Kit (Molecular Probes). PicoGreen® dye binds to ds-DNA and the fluorescence intensity produced is relative to the ds-DNA concentration in solution and finally to the cell number.

Here, non-stimulated cell/scaffold samples were compared with stimulated ones and ES-cells as control. In brief, the ES-cell/scaffolds were trypsinized after reculturing for 1, 3, 5, and 7 days. First, the cells are washed twice by cold DPBS, and then centrifuged for 5 min at 1500 rpm. The obtained cell pellet was collected for lysis using NP40 cell lysis buffer for 30 min on ice, followed by vortexing them every 10 min, using (Grant bio, PV-1 Vortex Mixer, Essex CM1 UK). Cell lysates collected by two freeze/thaw processes after centrifugation at 13000 rpm for 10 min at 4°C. Each 100 µL of clear lysate was aliquoted to a new 96-well plate and incubated for 5 min with 100 µL of PicoGreen® reagent. Fluorescence intensity was evaluated by a microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The DNA standard curve was obtained by cell lysates containing a known number of NG108-15 cells. The number of cells in each sample treatment was established by correlating the DNA standard curve. Non stimulated cells served as control in all experiments.
6.2.6. **Live cell imaging by voltage-sensitive dye “FluoVolt™ Membrane potential”**

6.2.6.1. **Differentiating cell culture**

NG108-15 cells (passage 23-35) were cultured in the medium mentioned earlier in a glass bottom chambers over sterilized sf-rGO thread and sf-rMoS₂ thread samples. Cells were cultured at a density of $5 \times 10^4$ cells per chamber adding 2 mL of culture medium and they were incubated for 4 days at 37°C in 5% humidified incubator to allow attachment and spreading, followed by change of medium every 2 days.

Differentiation was induced on day 4 by replacing culture medium with differentiation medium by adding 1 mL of sterile cAMP (Cyclic Monophosphate sodium, Sigma Aldrich, U.S.A.) with serum free medium to each chamber and cultured in 37°C in humidified atmosphere in 5% CO₂ for further 6 days.

On day 6 the cells were stained with voltage-sensitive dye, (FluoVolt™) to be able to carry out live cell imaging using a laser scanning confocal microscope.

6.2.7. **FluoVolt™ Membrane potential solution preparation**

In brief, to a 15 mL tube, 100µL of 100X PowerLoad™ Concentrate (Component B) and 10 µL of FluoVolt™ dye, 1000X (Component A) were mixed using vortex in order to prepare fresh FluoVolt™ Loading Solution. Then 10 mL of Physiological buffer sterile PBS was added to tube to mix. The medium from adherent cells were removed and cells were washed twice with sterile PBS. Then 2 mL of FluoVolt™ Loading Solution was added to cells, and incubated the cells at room temperature for 15-30 min. Then FluoVolt™ Loading Solution is removed; cells are washed twice in PBS. For optimal results, a sterile-filtered, 2
M Glucose Stock Solution was also prepared to support cell health in longer hour experiments, to study primary or differentiated neural cell types.

6.2.8. **Preparing Live Cell Imaging Solution**

First 2M Glucose Stock Solution is diluted 1:100 into live cell imaging solution (LCIS) for a final glucose concentration of 20 mM. To visualize the membrane staining of FluoVol
tm dye, standard FITC settings is used on confocal microscope. To confirm positive responses from the dye, NG108-15 cells were treated with 10 µM Valinomycin (a potassium ionophore) for 30 min, and an equal volume of isotonic potassium chloride (KCl) solution was added to depolarize the cells. The pH of the solution was kept at 7.4.

6.2.9. **Optical Imaging of Voltage-Sensitive Dyes Signals**

The electrical activity, be it either spontaneous or evoked by chemical or physical stimulation, spreads within the cellular compartments and from cells to cells via synaptic connection. This spread of excitation within cells and the tissue is always accompanied by the flow of ionic current through the extracellular fluid. Related to the current is an extracellular voltage gradient that varies in time and space according to the time course of the temporal activity as well as the spatial distribution and orientation of the cells.

The recordings may exhibit slow field potentials as well as fast spikes arising from action potentials. However, membrane polarization of the target neurons is primarily affected by the voltage gradient generated by the local current density and tissue resistance in the vicinity of the cells.

The charge injected with current pulses depends only on the amplitude and duration of the pulse, whereas in the case of controlled voltage pulses the charge additionally depends on the tissue resistance and the capacity of the electrode-tissue interface.
The current technologies for the recordings of neural activity are: a) intracellular recordings and electrical stimulation through patch clamp electrodes, b) extracellular recordings and electrical stimulation by microelectrode arrays (MEAs), c) optical imaging and electrical stimulation using voltage-sensitive dyes (VSDs)\[137\]. The extracellular recording and stimulation using MEAs’ remained poor, since the amplitudes of field potentials varies between 10 µV to 1 mV and immense amount of computational power is needed to collect data and interpret the recorded waveforms \[210, 211\]. Patch clamping of neurons did not seem appropriate in this study, to record neuronal network activities \[212, 213\].

In this study, optical imaging using VSDs \[214-218\] seemed suitable to record extracellular field potential to reflect the spike activity of large populations of individual neurons, imaging of neuronal activity by inspecting the fluorescent changes in the intracellular calcium concentration \[219-221\]. The signals collected from the voltage-sensitive dyes are represented as the change in fluorescence intensity \[222\].

NG108-15 cells/scaffolds plated in a 35-mm petri dish with $5 \times 10^5$ cells/mm with 2 mL of culture medium seeded on the rGO-thread and rMoS$_2$-thread scaffolds were stained with voltage-sensitive dye; FluoVolt™ Membrane Potential (Life Technologies, Carlsbad, CA, USA). FluoVolt™ solution was prepared as stated earlier. The voltage-sensitive dye was added to the culture medium and the cells were incubated at 37°C under an atmosphere of 5% CO$_2$ for 15-20 min.

Voltage changes were measured from the changes in the fluorescence intensity of the voltage sensitive dye, FluoVolt™. Cells grown on the thread scaffolds were superfused with the dye solution according to the protocol mentioned earlier. Confocal images were obtained through a 100X objective which needed glass chambers Slide (Lab-Tek®) with an optically clear polystyrene plate bottom. The clear base permits better microscopic viewing. Membrane
staining was visualized with a laser system by using standard fluorescein isothiocyanate filter setting (excitation, none; emission 520). Images were recorded with an exposure time of 10 ms at 93-100 frames per second with a confocal scanner (CSU-X1, Yokogawa Australia) and an EMCCD camera iXon 3897, iXon Ultra 897 (Andor Technology Ltd, Belfast, UK, and then analyzed by using the Andor iQ (ver.2.9.1), Andor) and CSU pro software.

6.2.10. Statistical Analysis

A two-tail Student’s t-test was carried out to analyze differences between two groups, using GraphPad Prism version 6.01 (GraphPad Prism software Inc. La Jolla, CA, USA). All experiments were performed three to four times. All values are stated as mean ± standard deviation($n = 3$). Non-parametric one-way ANOVA tests were also done. Differences between groups were considered significant at $p < 0.05$.

6.3. Results and Discussion

6.3.1. Optimal Intensity of Electrical Stimulation on NG108-15 Cell Line

Confocal images of immunostaining in Figure 6.3 show NG108-15 cells after electrical stimulation of at 20 V with durations of 5, 30 and 60 min. After two days of culture time and one more day after electrical stimulation, it was seen that almost 90% of the cells are viable. Cell growth has increased as the duration of electrical stimulation has prolonged. In all cases of electrical stimulation applied potential of 20 V made the medium boil. However, in the case of 5 min duration, electrical stimulation was over before reaching the point of boiling the medium.
Cell apoptosis assay with PI staining revealed that NG108-15 cells tolerate 60 mV of potential intensity well when compared to the non-stimulated cells. This result is similar to other study, when the potential intensity was increased to 300 mV/mm, 600 mV/mm or 1000 mV/mm, the Schwann cells showed approximately 96% increase in cell apoptosis [122]. These outcomes are consistent with the results from other studies as well, that potential more than 250 mV caused the medium to boil in other cases [196]. Therefore, electrical stimulation carried out at 60 mV was considered suitable for NG108-15 cells as it didn’t result in major apoptosis.

<table>
<thead>
<tr>
<th></th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 V, 5 min</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>20 V, 30 min</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>20 V, 60 min</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Control Cells</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 6.3** Confocal laser scanning microscope images of Calcein AM and PI on NG108-15 cells electrically stimulated at 20V with different duration. Analysis of electrical stimulation of NG108-15 cells at 20V potential at different duration of 5, 30 and 60 min are reported, 24 hours post electrical stimulation, bright field images (a), Viable cells are stained with Calcein AM showing in green (b), Dead cells floating are stained with propidium iodide (PI) (C), and finally overlay of all these fields (d). Scale bar represents 100µm
CHAPTER SIX

To compare the constant potential applied to the cell/scaffold, the constant potential of 100 mV corresponds to a current of almost 100 µAmp, considering the resistance of the scaffold is 1kΩ [24]. This value of current is validated based on former electrical stimulation studies, in vivo in rats employing current of 0.6 µAmp [123], 10-30 µAmp[125] and 400 µAmp[124].

Next the cultured NG108-15 neural cells were electrically stimulated at 60 mV for 5, 30 and 60 min to investigate the viability and spreading of the cells after 24 h post electrical stimulation. The results shown in Figure 6.4 indicate that as the duration of stimulation increases, the cells growth and accumulation is increased. However, the overgrowth of cells is not desirable as they accumulate densely and there is no space left for cells to differentiate. The electrically stimulated cells at 20 V for 5 min have spread and their viability is close to the control cells.

<table>
<thead>
<tr>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mV, 5 mins</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>60 mV, 30 mins</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>60 mV, 60 mins</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>Control</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
6.3.2. Proliferation of Nerve Cell/Scaffolds after Electrical Stimulation

The cells grown on sf-rGO and sf-rMoS2 thread scaffold were evaluated for their proliferation behaviour using PicoGreen® assay before and after electrical stimulation at 60mV for 5min.

Proliferation of the NG108-15 cells grown onto the thread scaffolds were assessed by determining the DNA content, considering the DNA is a cellular component that precisely relates to the number of the cells.

Figure 6.5 shows that cell number gradually increased over time for electrically stimulated sf-rGO thread scaffold ($p < 0.0013, n = 4$), and non-stimulated sf-rGO thread scaffold ($p < 0.0001, n = 4$), indicating overall the electrical stimulation did not introduce detrimental effect on cell proliferation.

On day 1 and day 2 the cell number of stimulated scaffolds was not significantly different ($p < 0.3424, n = 4$) from that of the non-stimulated cells, but after day 3, it was observed that the cell number in stimulated sf-rGO thread scaffold appeared to be higher than those from non-stimulated cell/scaffolds.

These results shown here are consistent with the findings of other studies in the literature [129]. Zhuang et al. [223] reported enhanced proliferation of MC3T3-E1 clonal osteoblastic cells after electrical stimulation at 2 mV/mm for 30min-24h. Bovine primary osteoblast was electrically stimulated at 6 V/mm for 5-18 days, the results showed significant increase in proliferation in non-confluent cultures [224]. Kim et al. [204] applied electrical stimulation
on rat calvarial osteoblast with 1.5 µA/cm² biphasic current for 6 h or 24 h/day. High proliferation was observed in continuously stimulated samples. In other studies, on human bone marrow MSCs they reported 57% increase in cell proliferation [205]. Shin et al electrically stimulated human cutaneous fibroblasts at 50mV/mm using a PPy/PPLA scaffold, cells adhered, spread and proliferate well [189].

**Figure 6.5** NG108-15 cell proliferation after electrical stimulation seeded inside the sf-rGO tissue scaffolds. Quant-iT PicoGreen® ds DNA assay was used to quantify the cell proliferation by the DNA content. The data represent mean ± standard error (n = 4).

**Figure 6.6** shows that the NG108-15 cells proliferated gradually over 5 day interval in both the electrically stimulated (p < 0.0002, n = 4), and non-stimulated (p < 0.0190, n = 4) scaffolds. It is seen that the non-stimulated cell/scaffolds proliferated slightly slower from day 3 (p < 0.3424, n = 4). However, the stimulated cell/scaffolds were noted to maintain their proliferation behaviour throughout the whole experiment time.
Figure 6.6 NG108-15 cells proliferation after electrical stimulation seeded inside the sf-rMoS₂ tissue scaffolds.

The results obtained here are consistent with the findings of other studies in the literature regarding cell proliferations after electrical stimulation [129]. Zhuang et al. [223] reported enhanced proliferation of MC3T3-E1 clonal osteoblastic cells after electrical stimulation at 2 mV/mm for 30 min-24 h. Bovine primary osteoblast was electrically stimulated at 6 V/mm for 5-18 days, the results showed significant increase in proliferation in non-confluent cultures [224]. Kim et al. [204] applied electrical stimulation on rat calvarial osteoblast with 1.5 µA/cm² biphasic current for 6 h or 24 h /day. High proliferation was observed in continuously stimulated samples. In other studies, on human bone marrow MSCs, they reported 57% increase in cell proliferation [205]. Shin et al.[189] electrically stimulated human cutaneous fibroblasts at 50 mV/mm using a PPy/PPLA scaffold, cells adhered, spread and proliferated well.

Electrical stimulation of thread scaffolds supported cell proliferation as the culture continued to day 5 by 12%-70% in sf-rGO scaffold and 35%-50% in sf-rMoS₂ scaffold. As a result, from the comparison of these two tissue scaffolds, it is noted that sf-rGO thread scaffolds
have slightly better support for stimulated neural cells proliferation when compared to the sf-
rMoS$_2$ thread scaffolds.

6.3.3. **Cell morphology after electrical stimulation of cell-scaffold**

The communication between neural cells and electrically conductive biomaterials begins when negatively charged surface of biomaterials (rGO particles and nanoparticles of rMoS$_2$) absorbs positively charged matrix proteins. The morphology of cells grown on the non-functionalized thread scaffolds (rGO and rMoS$_2$) as well as sf-rGO and sf-rMoS$_2$ thread scaffolds was observed by cytoplasm staining, using Calcein AM for 15-20 min, and stained with propidium iodide (PI) for 2-5 min in the dark at room temperature, the actin filaments were observed by laser scanning confocal microscope.

Fluorescence micrographs of NG108-15 attached to the thread scaffolds 24 h post electrical stimulation are shown in **Figures 6.7 and 6.8**.

Adhesion and spreading are seen on all sample scaffolds as well as inside the filament’s layers of thread fibers and it is obvious that more cells are attached to the sf-rGO thread and sf-rMoS$_2$ thread than non-functionalized rGO and rMoS$_2$ thread.

**Figure 6.7** and **Figure 6.8** (a-d) shows that NG108-15 cells were well attached to the sf-rGO and sf-rMoS$_2$ thread scaffolds and were viable right through culture. The control cells, with no electrical stimulation applied, showed lesser level of spreading and proliferating along the thread scaffolds. The stimulated cell/scaffolds with 60 mV for 5 min demonstrated good viability and healthy morphology of the NG108-15 cells when compared to the non-stimulated cell/scaffolds (**Figures 6.7-8**). The cells on both thread scaffolds show better
proliferation after electrical stimulation indicating that these scaffolds support cells for nerve tissue engineering.

In Figures 6.7-8, the cell/scaffolds stained with PI are shown to observe unhealthy and dead cells on the scaffolds before and after electrical stimulation. As it is observed, in both scaffolds, electrical stimulation has greatly decreased the number of unhealthy and dead cells within the scaffolds. Most filaments of the thread scaffolds were covered by viable cells (stained green). The sf-rGO thread scaffolds showed more cell growth than those of sf-rMoS₂ thread scaffolds.
<table>
<thead>
<tr>
<th></th>
<th>(a) Non ES-sf rGO scaffold</th>
<th>(b) ES-sf-rGO scaffold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merged</td>
<td><img src="merged.png" alt="Merged Image" /></td>
<td><img src="merged.png" alt="Merged Image" /></td>
</tr>
<tr>
<td>Calcein AM</td>
<td><img src="calcein.png" alt="Calcein AM Image" /></td>
<td><img src="calcein.png" alt="Calcein AM Image" /></td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td><img src="propidium.png" alt="Propidium Iodide Image" /></td>
<td><img src="propidium.png" alt="Propidium Iodide Image" /></td>
</tr>
</tbody>
</table>

**Figure 6.7** Confocal laser scanning microscope images of Calcein AM and PI on NG108-15 cells seeded onto the thread scaffolds, electrically stimulated at 60mV for 5 min at 5 Hz frequency. a) non-stimulated Cell/rGO thread scaffold, b) electrically stimulated cell/rGO thread scaffold. Scale bars represent 100 µm in all images.
6.3.4. Electrical Activity of Neurons Grown on Conductive Thread Scaffolds

The electrical activities of neurons on the scaffolds were studied using optical imaging to assess if the sf-rGO and sf-rMoS$_2$ thread scaffold can promote cell electrical activity. A
voltage-sensitive dye (FluoVolt) that detects changes in the extracellular voltage of nerve cells cultured on scaffold was employed in this study for visualization of electrical activity in cells.

In this research, a significant advance in the application of confocal laser scanning microscopy has been used to analyze fast membrane potential activities with voltage-sensitive dyes (VSDs). Optical imaging of the cells using VSDs aids detecting sharp spike signals developed from the rapid influx of sodium ions $\text{Na}^+$ during the upstroke phase of the action potential (AP). Recordings of extracellular field potential produced by action potentials using VSDs is mainly used for analyzing neuronal network activity as well as the detection of neuronal spikes [137].

**Figure 6.9** Electrical response of neuron cells on sf-rGO thread scaffolds. Field potentials are calculated by FluoVolt™ membrane potential and laser confocal microscopic live cell imaging. Field potential traces from cells on sf-rGO thread scaffold are shown and the electrical activity of neuronal network formed on the tissue scaffold is confirmed. X-axis represents Time in msec and Y-axis or transmembrane voltage is presented as fluorescent intensity.
The field potential (FP) waveforms were recorded at 5 Hz frequency using VSDs through confocal live imaging. Frequency is critical for recording accurate field potential waveforms, it is confirmed that field potential duration at basal conditions correlates with action potential duration, as obtained by live cell imaging using voltage-sensitive dye [135].

The first sharp deflection in the field potential waveform is created by sodium ion (Na\(^+\)) channel activation during membrane depolarization. The second peak of positive deflection happens with the activation of potassium ion (K\(^+\)) channel during membrane repolarization. This time interval is regarded as field potential duration. The negative phase between the first and second peaks of the FP waveform is associated with the plateau phase of the action potential waveform, implying that the amplitude of the negative plateau potential corresponds to the influx of calcium ions (Ca\(^{2+}\)) [135].

**Electrical activity of Neurons on sf-rMoS\(_2\) Thread scaffold**

![Electrical activity of Neurons on sf-rMoS\(_2\) Thread scaffold](image)

**Figure 6.10** Electrical response of neuron cells on sf-rMoS\(_2\) thread scaffolds. Field potentials are calculated by FluoVolt™ membrane potential and laser confocal microscopic live cell imaging. Field potential traces from cells on sf-rMoS\(_2\) thread scaffold are shown and the electrical activity of neuronal network formed on the tissue scaffold is confirmed. X-axis
represents Time in milli seconds (ms) and Y-axis or transmembrane voltage is presented as fluorescent intensity.

Filed potential waveforms’ interpretation is challenging, since filed potential signals are collected from the communication between the electrodes and cell membrane, and not direct measurements of membrane current or action potential. Nonetheless, this study revealed that the cells grown on sf-rGO and sf-rMoS2 thread scaffold involved in a healthy electrical activity when are electrically stimulated.

**Figure 6.9** and **Figure 6.10** demonstrate FP waveform recorded while an electrical stimulation was applied. The difference in fluorescence intensity measured before the start of AP and soon after the AP extends to the plateau is considered as the AP amplitude.

These results indicated that on the application of electrical stimulation, both thread scaffolds supported electrical activity of neurons grown on them, as both FP waveforms (sf-rGO and sf-rMoS2) in **Figure 6.9** and **Figure 6.10** revealed changes in the FP waveforms indicating cells electrical behavior. However, it is noted that the optical recording of FP waveform of sf-rGO thread scaffold represented different FP waveform compared to that of sf-rMoS2 thread scaffold. This could be explained according to literature that follows. The effect of different materials or drugs on neurons results in specific electrical activity parameters, including the fluorescence intensity duration. Asakura *et al*. have reported the application of FP waveforms to evaluate the effect of different drugs on QT prolongation and proarrhythmic. They have used MEA based assays in drug screening, since different drugs have different effects on the FP and AP waveforms. [135].

Zong *et al*. reported functional studies of primary cardiomyocytes on the scaffolds using optical imaging of the electrical activity of the cells stained with voltage sensitive dyes [195]. The optical imaging approach to record electrical activity in cultured cells using voltage-sensitive dyes has been studied according to the literature [225-227].
Therefore, it can be seen that rGO’s FP waveform after initial sharp spike recorded has taken longer in the plateau phase of the FP waveform before reaching for the second spike. Nonetheless, in the case of rMoS2 FP waveforms, within the same frame of time as rGO FP waveforms, the graph has revealed several changes in the fluorescence intensity interpreting to FP waveforms and AP waveforms.

These results confirmed functionality of these conductive surface engineered thread scaffolds when seeded with neurons.

6.4. CONCLUSION

In this study, the optimal conditions for electrical stimulation of NG108-15 cells grown on the engineered conductive rGO and rMoS2 thread scaffolds were attained. The surface functionalized rGO and rMoS2 thread scaffolds demonstrated good biocompatibility after electrical stimulation as shown in Live/Dead assay and confocal images. Electrical stimulation enhanced proliferation rate of NG108-15 cells grown onto rGO and rMoS2 thread scaffolds confirmed by PicoGreen assay with sf-rMoS2 thread scaffolds showing better proliferation behavior. Confocal microscopy of VSDs stained intracellular scaffolds showed that sf-rGO thread scaffolds have slightly better support for cell adhesion. Confocal live imaging with the aid of VSDs further confirmed that both thread scaffolds support electrical activity and functionality of the neurons grown on them. In summary, sf-rGO thread scaffolds and sf-rMoS2 thread scaffolds provide both flexibility and functionality for neurons to achieve structural and functional fit for nerve tissue constructs.
CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 GENERAL DISCUSSION

Peripheral nerve injury is a common worldwide medical condition that enormously affects the patients’ quality of life. Even though the peripheral nerve system has a better capability for axonal regeneration after damage compared to the central nervous system, impulsive peripheral nerve repair is almost always imperfect with weak functionality. Once peripheral nerve injury causes a considerable nerve gap and nerve stump suturing is impossible to bridge the gap, implant of a graft is considered to connect the nerve stumps [13].

Tissue engineering aids surgeons to repair or replace damaged or malfunctioned body part with tissue engineered implant [142]. Nerve tissue scaffolds combine nerve cells, biomolecules and a scaffold to support the repair and regeneration of tissues.

This study tried to develop two novel nerve tissue scaffolds with appropriate conductivity, biocompatibility and functionality. These nerve tissue scaffolds have the potential to serve as new nerve tissue scaffold for reconstruction of peripheral nerve injury.

The first biomaterial considered for the novel nerve tissue conduit is graphene oxide, to be reduced and coated onto the common cotton thread to make it to a conductive scaffold. Electrochemically reduced graphene oxide coated thread showed better conductivity when compared to the metal iodide reduced graphene oxide coated thread. The electrical conductivity value of both reduced graphene oxides are in consistent with previous studies [46, 81, 84, 154, 228]. ER-GO 2% coated thread showed better permeability compared to the FeI2-GO coated thread due to the amount of graphene oxide and metal iodide concentration on the thread. This is the first study that report the use of graphene coated thread as a nerve
CHAPTER SEVEN

tissue scaffold, there is no report on permeability of ER-GO and FeI2-GO coated thread to be compared.

Next the focus is on the biocompatibility of these coated threads towards PC12 cells, the results confirmed that all rGO coated threads showed good biocompatibility. FeI2-rGO coated thread demonstrated better viability in first 5 days of experiments but on day 7 ER-GO coated thread revealed consistent viability towards PC12 cells. The good biocompatibility shown by rGO coated threads from both reduction methods are in consistent with the previous research showing biocompatibility of graphene and reduced graphene [50] also a vast review on biocompatibility of graphene towards mammalian cells and bacteria [229].

A novel tissue scaffold is developed by employing common cotton thread as a natural, simple and cost effective scaffold and rendered conductive through reduced graphene oxide coating. The cotton thread is treated with CPAM first to enhance the adsorption of biomaterials investigated for this study [72]. The major objective of the first project is achieved by appropriate conductivity of rGO coated thread, good permeability and finally its biocompatibility.

The development of this novel 3D thread scaffold introduces new opportunities to use common cotton thread as nerve tissue scaffolds in the tissue engineering field. The results from its permeability, conductivity and biocompatibility towards nerve cells have proven that the threads’ flexible interconnected porous structure, have great potential as three dimensional cell culture structures. This tissue scaffold can be used for peripheral nerve injuries, wound healing or nerve surgery suture.

Inspiring by the successful development of rGO-thread scaffolds, a new emergent material in the field of biomedical application, molybdenum disulphide is considered as the second biomaterial for the first time to be coated onto cotton thread in this study. Molybdenum
disulphide made conductive through lithium ion intercalation followed by a phase transition 2H to 1T, while coated onto the cotton thread. The results from the XPS characterization of rMoS$_2$-thread are in consistent with the previous study of rMoS$_2$ nanoflakes [99].

The results of electrical conductivity proved excellent conductivity when compared to the rGO-thread’s conductivity. The electrical conductivity of rMoS$_2$-thread has an improvement of five orders of magnitude when compared to that of rGO-thread. Since all cell experiments are carried out in hydrated conditions (cell medium), conductivity of rMoS$_2$-thread is reported also in wet conditions, which reveals an improvement of its conductivity by 3 orders of magnitude when compared to its dry state and rGO-thread’s conductivity.

The wicking rate of rMoS$_2$-thread is one of the important properties of tissue scaffolds. Current tissue scaffold system has limited permeability due to lack of vascular system. The use of cotton thread aids in mimicking the vascular system to promote permeability, in terms of moving nutrients, removing waste and other biomolecules. The permeability of rMoS$_2$-thread is calculated and reported to be slightly better than that of rGO-thread.

The rMoS$_2$-thread showed good biocompatibility towards PC12 cells. Since the success in developing two compatible tissue scaffolds, the biocompatibility of rGO-thread and rMoS$_2$-thread is investigated towards another adherent cell line. Cell adhesion to the fibres of tissue scaffold is crucial to the success of scaffold. In order to improve cell adhesion, another cell line, adherent hybrid cell NG108-15, is selected to be employed for seeding onto these nerve tissue scaffolds to be investigated in all experiment. Thread scaffold (rMoS$_2$) showed even better biocompatibility to NG108-15 cells after lithium ion intercalation. These results are in consistent with the few studies that have been done recently on biocompatibility of MoS$_2$ nanoflakes or nanosheets concerning biomedical research [32, 107-111, 193, 230-232].
Two conductive, biocompatible tissue scaffolds with two of the most important 2D materials (rGO, rMoS$_2$) developed in Chapter 3 and 4 could be used as a promising tissue scaffold model to study different cell types and tissue intended for tissue engineering applications. For the next phase of the experiments rGO 2% is selected because showed better permeability, biocompatibility when compared to the other methods of reduction as well as 5% concentration of graphene oxide.

The research in Chapter 5 regarded surface functionalization of the newly developed thread scaffolds in order to enhance cell adhesion. Both thread scaffolds (rGO2%, rMoS$_2$) surfaces was activated by CDI followed by conjugation with gelatin. The amount of amine content on the surface of thread scaffolds is evaluated by Acid Orange II assay after functionalization. The condensation reaction between the amine of gelatin and imidazole ester in CDI was confirmed successful from the assay results. Cell-scaffold communication is facilitated with arginine-glycine-aspartate (RGD) within the gelatine [233, 234].

The permeability of surface functionalized thread scaffolds is investigated to confirm the wicking properties of threads. The permeability assay proved that surface functionalization has decreased the wicking speed of liquid through the scaffolds. To overcome this problem, plasma treatment is carried out on surface functionalized thread scaffolds.

The second permeability assay on treated and untreated sample scaffolds proved that plasma treatment improved the wicking rate in surface functionalized thread scaffolds [64]. The results showed that rMoS$_2$-thread scaffold has better permeability when compared to rGO-thread scaffold.

NG108-15 cells are seeded onto the surface functionalized thread scaffolds for 4 days. Then fluorescent Live/Dead assay using Calcein AM and PI staining to investigate viability and attachment of cells on the scaffolds using confocal microscopy. The result revealed that
thread scaffolds supported good cell adhesion and viability in both scaffolds. These results are in consistent with the previous studies on cell adhesion [235].

Next, the biocompatibility of surface functionalized thread scaffolds to NG108-15 cells is investigated towards NG108-15 cells using alamarBlue® assay. The cells are seeded onto scaffolds for 7 days. The results indicate that the cells are viable and proliferating well over 7 days of culture because of improved cell-scaffold interaction enhanced by RGD peptide. The results confirm that surface functionalized rGO and rMoS₂ thread scaffolds are biocompatible to NG108-15 cells. These results are in consistent with the similar studies from the literature [235]. Given the novelty of surface functionalized thread scaffold as a nerve tissue scaffold, comparison with other tissue scaffolds is not possible.

Thread substrate as a tissue scaffold, with its natural characteristic advantages of cost effective and availability, creates an appealing substrate for tissue engineering in repair and replacement of tissues in peripheral nerve injuries, and can be employed in other tissue engineering applications.

In Chapter 6, the study has focused on creating a functional nerve tissue scaffolds to replace the damaged or malfunctioning peripheral nerve tissue. NG108-15 cells survived after electrical stimulation with 60 mV potential at 5 Hz for 5min, as cells' good viability was confirmed by Calcein AM staining. This suggested that this electrical stimulation setting is suitable for stimulation of NG108-15 cells. These results are in consistent with the similar studies on other cell lines with the same electrical stimulation setting [24, 120].

After establishing the proper electrical stimulation setting suitable for NG108-15 cells, they were seeded onto the sf-rGO and sf-rMoS₂ thread scaffolds for 4 days to improve cell adhesion. Following electrical stimulation of cell/scaffolds on day 4, they are incubated for 24 h before being stained with Calcein AM to assess the cells’ viability.
CHAPTER SEVEN

To investigate the effect of electrical stimulation on the proliferation rate of NG108-15 cells, the PicoGreen® assay was undertaken for five consecutive days. The deoxyribonucleic acid (DNA) content was measured to determine the ability of electrically stimulated cell/scaffolds to proliferate. The results reveal that the electrically stimulated nerve cells remained viable and proliferated well when compared to the non-stimulated cells imbedded in both thread scaffolds. This confirms that rGO-thread and rMoS₂-thread scaffolds are both suitable for nerve tissue engineering applications.

The thread scaffolds represent the fibrillar construction of the extracellular matrix (ECM) that offer cellular guidance, viability and functionality. Functionality of engineered scaffolds is verified by live cell imaging using voltage-sensitive dyes. Electrical activity of NG108-15 cells is investigated using optical live cell imaging. Cell behavior on both thread scaffolds were investigated and compared to determine and compare functionality of the scaffolds for nerve tissue regeneration.

Extracellular field potential generated by action potential is recorded, while the cell/scaffolds are electrically stimulated.

Functional studies of NG108-15 cells on the thread scaffolds proved that both scaffolds show great electrical response. However, NG108-15 cells demonstrated faster electrical response on rMoS₂-thread scaffolds compared to rGO-thread scaffolds. Different materials or drugs generate different response curve of field potential [135]. The concentration of the substance used also affects the field potential curves [135]. Due to the novelty of electrical stimulation of nerve cells on conductive thread scaffolds, comparisons with other tissue scaffolds are currently difficult given the lack of similar studies.
CHAPTER SEVEN

These experimental evidences from electrical activity of nerve cells on 3D thread scaffolds (rGO and rMoS₂) confirm the functionality of both thread scaffolds for peripheral nerve injuries; wound healing or other applications in tissue engineering.

7.2. GENERAL CONCLUSION

This research has added to the knowledge of conductive textile, thread microfluidics, tissue scaffold, nerve tissue engineering for peripheral nerve injuries, wound healing or tissue implant.

The first part of this thesis introduced a simple, cost effective and flexible 3D substrate as common cotton thread. Thread as a natural fiber has a 3D structure with voids and pores suitable for purpose of cell seeding. Graphene oxide was successfully synthesized. The adsorption of graphene oxide particles was enhanced with CPAM treated threads. Graphene oxide was reduced on the treated thread with two different simple, green and cost effective methods; electrochemical reduction and metal iodide treatment. Electrochemical reduction of graphene oxide is fast, simple and effective compared to other methods of reduction. Conductivity of the coated threads was calculated with two-point probe method. Both methods of reduction proved to have made the graphene oxide coated thread properly conductive. The graphene oxide coated threads’ permeability facilitates the exchange of neuritis, removing waste and other biomolecules which mimics the vascular system. Most of tissue scaffolds suffer from the lack of vascular system. Finally, graphene coated thread demonstrated good biocompatibility towards nerve cells. The development of this 3D conductive thread scaffold initiates new opportunities to using simple thread as a substrate in field of tissue engineering.

The second part of the thesis introduced emergent 2D materials with excellent properties new to the biomedical field, molybdenum disulphide to be employed as a biomaterial. This part
CHAPTER SEVEN

was concerned with investigating the biocompatibility of molybdenum disulphide coated thread towards nerve cells. Molybdenum disulphide nanoparticles were coated onto CPAM treated cotton thread and made conductive through lithium ion intercalation. The calculated data revealed great conductivity of molybdenum disulphide especially in the hydrated conditions. This new developed conductive thread scaffold also showed great permeability. The proven good biocompatibility of molybdenum disulphide coated thread makes it a promising 3D cell culture system to be used as a novel nerve tissue scaffold.

The third part of the thesis has focused on surface engineering both novel thread scaffolds to enhance cell adhesion and cell-scaffolds interactions. The thread scaffolds were treated with CDI conjugated with gelatin to modify surface chemistry of the threads to facilitate attachment of cells to obtain fast regeneration. The physiochemistry assay proved the amination of the thread scaffolds to be successful. The NG108-15 cells adhere and proliferate well on the surface functionalized thread scaffolds. This makes them great culture substrate for cell seeding. The permeability of the surface functionalized thread scaffolds was improved by plasma treatment. Both thread scaffolds showed great biocompatibility to nerve cells after surface functionalization.

The forth part of this thesis is concerned with the functionality of these new developed nerve tissue scaffolds. A comprehensive study investigated proper electrical stimulation setting was for NG108-15 cells. Frequency proved to be an important factor when recording electrical activity of cells using voltage-sensitive dyes. Electrical stimulation enhanced NG108-15 cell survival and growth on the scaffolds. Proliferation studies confirmed significant effect of electrical stimulation on proliferation behavior of cells through the conductive thread scaffolds. Functional studies of NG108-15 cell on each scaffolds (rGO, rMoS$_2$) confirmed the proper response of cell/scaffolds to electrical activity of cells. This confirms that rGO-thread
scaffold and rMoS$_2$-thread scaffolds are both highly promising tissue-engineered scaffolds in the field of nerve tissue regeneration.

7.3 Future Perspectives

Given the success of development of rGO conductive tissue scaffold and rMoS$_2$ conductive tissue scaffolds, surface functionalization of tissue scaffolds and electrical stimulation of these two conductive tissue scaffolds, other tissue engineering applications are achievable.

- The developed rGO-thread or rMoS$_2$ thread scaffolds developed in Chapter 3 and 4 can be employed as suture. Thread scaffolds seeded with the appropriate cell type can be developed to ocular, neural or cardiovascular surgery suture.

- Another potential application of the cell-laden conductive cotton thread is creating in vitro disease models and neural drug testing platforms.

- Silk has been employed as a tissue scaffold or biomedical suture material for a long time [236]. However, some biocompatibility issues have concerned use of silkworm silk. Also preparation process of silk compared to cotton thread is a complicated and time consuming process. Therefore, the treated cotton thread can be a promising alternative to most of silk application in tissue engineering and biomedical suturing.

- One of the most significant properties of rMoS$_2$-thread developed in Chapter 4 is the conductivity. Lithium ion intercalation as a simple and effective approach was used to make molybdenum disulphide conductive. Conductive rMoS$_2$-thread can be employed as a promising alternative to application of conductive textile or development of advanced textiles [237]. Also, lithium ion intercalation of graphene oxide could be
CHAPTER SEVEN

considered to investigate how this method affects further improvement of reduced graphene oxide’s conductivity.

- In tissue engineering, a scaffold needs a proper architecture for cell adhesion, direct proliferation and differentiation into a functional tissue or organ. The successful surface functionalization of thread scaffolds in Chapter 5, enhanced cell adhesion as an important scaffold property. Hence, thread structures can be seeded with appropriate cell type in order to develop a desired tissue or graft.

- The electrical stimulation of cells and recording the electrical activity of neurons was developed in Chapter 6. The live cell imaging using voltage-sensitive dye is considerably faster to record the field potential generated by action potential of excitable cells. These established approaches can be used for cardiac tissue engineering [238]. Also thread has a natural unidirectional–oriented fibrous that is suitable for pericardial suture [239] and peripheral nerve repair.
Bibliography

REFERENCES


Bibliography


Bibliography

127. Kim, H.I. and J.R. Lince, Direct visualization of sliding-indu

175
Bibliography


