Biomaterials to change astrocyte behaviour and morphology for brain repair

Francesca L Maclean

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Francesca Leigh Maclean

28 March 2017
Dedicated

to

Halcyon Lucas

* *

The matriarch of life-long learners
Acknowledgements

I owe my thanks to a plethora of people for their assistance, support, friendship, and love throughout my journey over the past three years.

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Ubuntu: I am, because you are.
Abstract

The incapacity of the central nervous system (CNS) to regenerate is a barrier to the effective treatment of neurodegenerative diseases and traumatic injuries. Of particular importance in treating traumatic injuries is the CNS’s inflammatory response, which is a complex response that does not effectively transition from the growth-inhibitory and protective phase to a growth supportive phase that would allow for tissue repair and remodelling. Therefore, the astrocyte response to injury presents a valuable therapeutic target as they perform both cytotrophic and cytotoxic functions, sometimes concomitantly, after injury. The chronic persistence of scar-forming astrocytes presents a significant barrier to regeneration and hence, functional recovery. As such, understanding how particular cues, and when they are presented, affect astrocyte behaviour is of interest in developing tissue engineering solutions for traumatic brain injury (TBI). Biomaterials present an attractive solution candidate as they can mimic the extracellular matrix of the brain, present biologically relevant cues, and facilitate cell growth. Through understanding how biomaterials and the cues they can present impact astrocyte behaviour and morphology, we seek to inform the future design of biomaterials to harness the cytotrophic aspects of astrocytes and their response to injury to improve reparative outcomes.

In this thesis, the development and biological evaluation of nanofibrous biomaterial systems functionalized with galactose moieties or the anti-inflammatory polysaccharide, fucoidan are described. Electrospun poly(ε-caprolactone) nanofibre scaffolds were
fabricated and functionalized with biologically relevant heparin (anti-inflammatory) and poly(L-lysine) (PLL), or the novel galactose-presenting poly(L-lysine)-lactobionic acid (PLL-LBA). The research reported here demonstrates the functionalization and materials characterization, as well as biological evaluation in vitro and in vivo to elucidate the impact of nanofibrous morphology and the galactose moieties on astrocytes in culture as well as after TBI. The galactose-presenting scaffold could maintain a reduced inflammatory profile of astrocytes in vitro and resulted in neuroprotection at 7 days post injury in mice. These findings were extended upon by transitioning to the Fmoc-capped self-assembled peptide (SAP) hydrogel, Fmoc-DIKVAV, which can effectively fill a brain lesion, whilst also providing bioactive cues on the surface of the nanofibres within the hydrogel. This system was co-assembled with fucoidan to present anti-inflammatory cues after TBI, where it was found that structural support and no additional functionalization was required to reduce the primary astrocyte scar by ~50% compared to the stab control 7 days post injury. The presentation of fucoidan on the fibrils of Fmoc-DIKVAV increased the organization of astrocytes within the primary scar and also altered the morphology of the astrocytes far away from the lesion site. This demonstrates the ability of fucoidan to alter the morphology, and potentially the phenotype of reactive astrocyte after injury. Finally, this SAP system was evaluated as a 3-dimensional (3D) cell culture environment to enhance the understanding of astrocyte behaviour in culture as well as after lipopolysaccharide (LPS) or interleukin-1α (IL-1α) stimulation. Fucoidan delivered via the hydrogel system significantly reduced the proliferation of LPS-stimulated astrocytes compared to soluble fucoidan or the control, and exposure to the hydrogel resulted in significant reorganization of astrocyte networks in vitro, which was
also observed *in vivo*. Thus, this SAP hydrogel is promising as a 3D biomimetic cell culture environment for future studies of astrocytes.

Here, we have engineered functionalized nanofibrous biomaterial scaffolds that can be used *in vitro* and *in vivo* to impact astrocyte behaviour and morphology after injury or stimulation. The results presented can be used to better inform the design of future tissue engineering strategies that can manipulate the inflammatory response to improve functional recovery outcomes.
List of publications


Table of Contents

Acknowledgements ................................................................................................................... i

Abstract.................................................................................................................................. vi

List of publications .................................................................................................................... ix

Nomenclature............................................................................................................................ xix

CHAPTER ONE

Introduction ............................................................................................................................... 23

1.1 Background ....................................................................................................................... 23

1.1.1 Astrocytes and biomaterials to treat traumatic brain injury ...................... 24

1.1.2 Nanofibrous biomaterials ......................................................................................... 25

1.2 Aims ................................................................................................................................... 26

1.2.1 Aim 1: Investigate in vitro and in vivo impact of morphological and biological cues delivered via electrospun nanofibre scaffolds on astrocyte behaviour and morphology ......................................................................................... 26

1.2.2 Aim 2: Investigate the ability of the co-assembled Fmoc-DIKVAV and fucoidan system to alter the astrocytic response after traumatic injury ...... 27

1.2.3 Aim 3: Investigate Fmoc-DIKVAV as a three-dimensional cell culture environment ......................................................................................................................... 27

1.3 Thesis Outline .................................................................................................................... 28
1.3.1 Literature Review .................................................................28
1.3.2 Materials and Methods ..........................................................29
1.3.3 Materials development and biological assessment .......................29
1.3.4 Conclusions and future perspectives .......................................30

CHAPTER TWO

Biomaterial systems to resolve astrocyte inflammation ..................31

Abstract ..........................................................................................32

2.1 Introduction ................................................................................33

2.2 The inflammatory response of the body .......................................34

2.3 The inflammation response of the CNS ......................................36
  2.3.1 Shifting perceptions of astrocytes in CNS inflammation ..........38

2.4 Third-generation biomaterial strategies to control the inflammatory response after TBI .................................................................39
  2.4.1 The evolution of biomaterial strategies .................................39
  2.4.2 Design criteria for brain repair and modifying inflammation ......40
  2.4.3 Biomaterials to provide physical support to the lesion site ........41
  2.4.4 Matching the modulus of the brain .......................................44
  2.4.5 Controlled degradation of biomaterial candidates ..................50
  2.4.6 Modifying the inflammatory response via temporally delivery of cues 52
2.5 Conclusion and future perspectives.................................................56

CHAPTER THREE

Materials and methods.............................................................................61

3.1 PCL scaffold fabrication, galactose functionalisation, and characterisation
62

3.1.1 2D PCL preparation...........................................................................62

3.1.2 3D nanofibre scaffold preparation .....................................................62

3.1.3 Conjugation of poly-L-lysine and lactobionic acid...............................62

3.1.4 Nuclear Magnetic Resonance...............................................................63

3.1.5 Layer-by-layer functionalisation (2D scaffolds).................................63

3.1.6 Layer-by-layer functionalisation 3D scaffolds....................................64

3.1.7 Scanning electron microscopy...........................................................64

3.1.8 Contact angle measurements............................................................65

3.1.9 Quartz crystal microbalance and dissipation (QCM-D)......................65

3.2 Primary astrocyte culture on PCL nanofibre scaffolds..........................66

3.2.1 Animals and chemicals .................................................................66

3.2.2 Primary culture................................................................................66

3.2.3 Imaging and analysis of immunocytochemical staining......................68

3.3 In vivo implantation of PCL nanofibre scaffolds....................................68

3.3.1 Animals..........................................................................................68
3.3.2 Scaffold insertion .................................................................68
3.3.3 Tissue preparation ...............................................................69
3.3.4 Immunohistochemistry .........................................................70
3.3.5 Cell counts ...........................................................................70
3.4 Fmoc-DIKVAV synthesis and characterisation .........................71
  3.4.1 Solid-Phase Peptide Synthesis .............................................71
  3.4.2 Fmoc-SAP gel formation .....................................................72
  3.4.3 Fourier Transform Infra-Red Spectroscopy .........................72
  3.4.4 Rheology ...........................................................................72
  3.4.5 Transmission electron microscopy ......................................73
3.5 In vivo implantation of Fmoc-DIKVAV/fucoidan and characterisation ....73
  3.5.1 Stab injury and SAP implantation .......................................73
  3.5.2 Tissue preparation .............................................................74
  3.5.3 Immunohistochemistry ........................................................74
  3.5.4 Cell counts ........................................................................75
  3.5.5 Analysis of astrocyte scar and reconstruction ......................75
3.6 Primary astrocyte culture with Fmoc-DIKVAV/fucoidan ..............76
  3.6.1 Gel formation .....................................................................76
  3.6.2 Primary astrocyte cell culture ..............................................77
  3.6.3 LPS and IL-1a stimulation and MTT assays .........................77
3.6.4 Gel exposure experiments.........................................................79
3.6.5 Three-dimensional cultures.....................................................79
3.6.6 Immunocytochemistry..............................................................80
3.6.7 Network analysis......................................................................80
3.6.8 Stab injury and hydrogel implantation........................................81
3.6.9 Tissue preparation.....................................................................81
3.6.10 Immunohistochemistry............................................................81
3.6.11 Gel incubation.........................................................................82
3.6.12 Rheological testing.................................................................82
3.7 Data analysis................................................................................82

CHAPTER FOUR

Galactose-functionalised PCL nanofibre scaffolds attenuate inflammatory action of astrocytes in vitro and in vivo.........................................................83

Abstract...........................................................................................84

4.1 Introduction..................................................................................86

4.2 Results and Discussion..............................................................89

4.2.1 Conjugation of poly(L-lysine) and lactobionic acid presents galactose moiety 89

4.2.2 Nanofibrous morphology maintained after layer-by-layer functionalisation.................................................................90
4.2.3 *In vitro* assessment of the effect of scaffold functionalisation on astrocyte protein expression ................................................................. 94

4.2.4 *In vivo* assessment of the effect of scaffold implantation after stab injury 100

4.3 Conclusion .............................................................................................. 104

4.4 Acknowledgements ................................................................................. 104

4.5 Supplementary Information ................................................................... 106

CHAPTER FIVE

Reducing astrocytic scarring after traumatic brain injury with a multi-faceted anti-inflammatory hydrogel system ......................................................... 108

Abstract ....................................................................................................... 109

5.1 Introduction ............................................................................................ 111

5.2 Results and Discussion ........................................................................ 114

5.2.1 Co-assembly with fucoidan maintains the Fmoc-DIKVAV nanostructure ................................................................. 114

5.2.2 Scar extension and astrocyte organisation are altered by Fmoc-DIKVAV hydrogel implants ......................................................... 117

5.2.3 Morphological reconstruction reinforces the complexity of the astrocytic response ................................................................. 122

5.3 Conclusions ............................................................................................ 125
5.4 Acknowledgements.................................................................126

5.5 Supplementary Figures...........................................................126

CHAPTER SIX

A commentary on the need for 3D-biologically relevant in vitro environments to investigate astrocytes and their role in central nervous system inflammation ................................................................. 128

6.1 Abstract.................................................................................129

6.2 Introduction............................................................................130

CHAPTER SEVEN

Reduced proliferation of stimulated astrocytes and networked growth in a three-dimensional culture system................................................. 137

Abstract.....................................................................................138

7.1 Introduction............................................................................139

7.2 Results and Discussion ...........................................................141

7.2.1 Anti-proliferative effect of multicomponent Fmoc-SAP system.....141

7.2.2 Quasi-3D and 3D culture environments result in astrocyte network formation........................................................................143

7.2.3 Three-dimensional cell culture environment facilitates astrocyte network formation over time, with similar networking observed in vivo.....146

7.3 Conclusion.............................................................................150
## Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>AHNAK</td>
<td>neuroblast differentiation-associated protein</td>
</tr>
<tr>
<td>AM</td>
<td>astrocyte medium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AQP4</td>
<td>aquaporin-4</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain-barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Ca</td>
<td>calcium</td>
</tr>
<tr>
<td>CDMEM</td>
<td>completed DMEM</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycans</td>
</tr>
<tr>
<td>D₂O</td>
<td>deuterium oxide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIKVAV</td>
<td>laminin inspired peptide, Asp-Ile-Lys-Val-Ala-Val</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>div</td>
<td>days in vitro</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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</table>
DMSO  dimethyl sulfoxide
EAAT2  excitatory amino acid transporter 2
ECM  extracellular matrix
ECS  extracellular space
EDC  1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
FBR  foreign body reaction
FBS  fetal bovine serum
FGF  fibroblast growth factor
FRGDF  fibronectin inspired peptide, Phe-Arg-Gly-Asp-Phe
Gal-1  galectin-1
GDNF  glial-derived neurotrophic factor
GFAP  glial fibrillary acid protein
HBSS  Hank’s balanced salt solution
HBtU  O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexa-fluoro-phosphate
HCl  hydrochloric acid
HOBt  hydroxybenzotriazole
HS  Haishen
HSP70  heat shock protein 70
IL-1α  interleukin-1α
K  potassium
LBA  lactobionic acid
LbL  layer-by-layer
LPS  lipopolysaccharide
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide metabolic assay</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NeuN</td>
<td>nuclear antigen marker for neurons</td>
</tr>
<tr>
<td>NO</td>
<td>nitrous oxide</td>
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<tr>
<td>NPC</td>
<td>neural progenitor cells</td>
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<tr>
<td>NSC</td>
<td>neural stem cells</td>
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<tr>
<td>PA</td>
<td>peptide amphiphiles</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
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<tr>
<td>PCL</td>
<td>poly(ε-caprolactone)</td>
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<tr>
<td>PDL</td>
<td>poly(D-lysine)</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene-glycol)</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PLL</td>
<td>poly(L-lysine)</td>
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<td>PLLA</td>
<td>poly(L-lactic acid)</td>
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<tr>
<td>QCMD</td>
<td>quartz crystal microbalance with dissipation monitoring</td>
</tr>
<tr>
<td>RADA16-1</td>
<td>RADARADARADARADA peptide</td>
</tr>
<tr>
<td>RHS</td>
<td>right-hand side</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>SAP</td>
<td>self-assembled peptide</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SM132</td>
<td>neurofilament H, neurite marker</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TCP</td>
<td>tissue culture plastic</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TES</td>
<td>triethylsilane</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>YIGSRF</td>
<td>laminin-inspired peptide, Tyr-Ile-Gly-Ser-Arg-Phe</td>
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CHAPTER ONE

Introduction

1.1 Background

A key barrier to functional repair and regeneration following traumatic brain injury is the inflammatory response of the central nervous system (CNS). Injury is followed by orchestrated molecular and cellular events that are complex and perform both cytotoxic and cytotrophic functions [1-6]. Cells involved in this process, in particular astrocytes, have previously been highlighted as therapeutic targets [6]. The development of strategies to maximise the beneficial cytotrophic and minimise the harmful cytotoxic outcomes of the inflammatory response would contribute to the development of treatments to achieve improved functional recovery after injury.

Implantable, tissue specific biomaterials may provide an alternative treatment strategy for altering the inflammatory response of astrocytes [7], as well as encourage subsequent repair and regeneration [8]. Tissue engineering constructs comprising of biomaterial scaffolds can, with careful optimisation, deliver the physical, chemical, and biological cues required to alter the astrocytic response post-injury. Once control over the persistent astrocytic response has been achieved to create a growth-supportive environment, biomaterials can then also be used to facilitate either endogenous infiltration or the growth of transplanted cells to reform cellular networks across the lesioned area for
functional repair. The biomimicry of biomaterials and the plethora of functionalisation techniques makes these constructs an exciting candidate for traumatic brain injury therapy.

1.1.1 Astrocytes and biomaterials to treat traumatic brain injury

Astrocyte complexity in the inflammatory response of the CNS after injury presents a significant challenge for regenerative medicine, and an opportunity for tissue engineers. Specifically, the duality of their behaviour contributes to such complexity: reactive astrocytes (those responding to injury) are essential to non-neuronal and activated inflammatory cell containment, lesion area contraction, neuroprotection and subsequent functional recovery [9, 10], however, their persistence can present long-term physical and chemical barriers to repair and regeneration [2]. This thesis will focus on a penetrative brain injury model (called traumatic brain injury from herein, TBI), where the skull and blood-brain-barrier (BBB) are broken, and a non-uniform-shaped tissue damage results. The infiltration of fibroblasts and blood cells, and the introduction of foreign material initiates the inflammatory process that will see the formation of a glial (or, astrocyte) scar around the lesion by reactive astrocytes (termed as such in contrast to physiologically ‘active’ astrocytes) [2]. Understanding and harnessing the protective functions of astrocytes after injury, whilst also minimising the cytotoxic functions – and then exerting said control at the optimum time point - is essential to the successful development of a TBI treatment. At present, we need to better understand what signals to present, and when they should be presented to improve functional outcomes after injury.
Biomaterials that mimic the extracellular matrix (ECM) of the brain chemically, mechanically, and morphologically are ideal candidates for delivering signals that can further this understanding. They can present biologically relevant cues, support the growth of cells and be further functionalised to exert specific control over cells. For example, modification of biomaterials to present growth factors or other biomolecules can be used to control the behaviour of cells [11, 12]. Specifically, three-dimensional (3D) biomaterial in vitro environments have successfully altered astrocyte morphology and biological activity [7, 13, 14]. Such influences can be leveraged to develop a biomaterial system that can alter astrocyte morphology and behaviour in vivo, contributing to improving functional outcomes after injury. Biomaterial systems of interest should be thoroughly interrogated in vitro, and would also ideally assist in the development of in vitro environments that better replicate in vivo tissue to help improve our understanding of astrocyte behaviour.

1.1.2 Nanofibrous biomaterials

Although many biomaterials have properties such as controlled degradation and tailored biomolecule release, this thesis has focussed on the use of nanofibrous biomaterials. As the brain’s ECM is fibrous, nanofibrous biomaterials are better suited to mimicking the brain ECM compared to biomaterials such as alginate or poly(ethylene glycol) (PEG). In this thesis the ability of poly(ε-caprolactone) (PCL) electrospun nanofibre scaffolds and the self-assembled peptide hydrogel, Fmoc-DIKVAV to alter astrocyte morphology and behaviour in vitro and in vivo is investigated. Furthermore, modification of these materials by presenting a galactose moiety in a novel polymer attached to the surface of the electrospun scaffold, or by co-assembling an anti-inflammatory polysaccharide
fucoidan with Fmoc-DIKVAV provides further opportunity to examine pathways for influencing astrocyte morphology and behaviour. The biomaterial systems have been investigated for use in vitro and in vivo to gain a better understanding of the astrocytic response and to inform future biomaterial design for traumatic brain injury treatments.

1.2 Aims

We hypothesised that functionalised electrospun nanofibres and Fmoc-DIKVAV can be used to induce a morphological and behavioural change in astrocytes that can be used in vitro and in vivo to better understand their function in models of trauma. To demonstrate this, our aims were targeted towards materials development and the assessment of biological impact in vitro and in vivo. Specifically, the aims were:

**Aim 1:** Investigate in vitro and in vivo impact of morphological and biological cues delivered via electrospun nanofibre scaffolds on astrocyte behaviour and morphology.

**Aim 2:** Investigate the ability of the co-assembled Fmoc-DIKVAV and fucoidan system to alter the astrocytic response after traumatic injury.

**Aim 3:** Investigate Fmoc-DIKVAV as a three-dimensional cell culture environment.

1.2.1 **Aim 1:** Investigate in vitro and in vivo impact of morphological and biological cues delivered via electrospun nanofibre scaffolds on astrocyte behaviour and morphology

This aim focused on the functionalisation of electrospun PCL nanofibres and subsequent in vitro and in vivo testing. This included the synthesis of a novel polymer presenting a galactose moiety (PLL-LBA), and the functionalisation of nanofibres using the layer-by-layer method. The ability for this biomaterial system to maintain a reduced
inflammatory profile of astrocytes *in vitro*, and the effect on neuronal survival after traumatic injury and implantation was assessed.

1.2.2 Aim 2: Investigate the ability of the co-assembled Fmoc-DIKVAV and fucoidan system to alter the astrocytic response after traumatic injury

Following on from the outcomes of Aim 1, this aim focused on Fmoc-DIKVAV, an injectable hydrogel can be better as a tissue engineering construct for traumatic injuries *in vivo*. Such a tissue engineering treatment strategy for traumatic brain injury will need to be able to support cell infiltration and growth, but first the astrocytic response to injury needs to be altered. Thus, this aim assessed the co-assembled Fmoc-DIKVAV + fucoidan system’s ability to alter the astrocytic scarring and the morphology of astrocytes after traumatic injury *in vivo*.

1.2.3 Aim 3: Investigate Fmoc-DIKVAV as a three-dimensional cell culture environment

This aim utilised Fmoc-DIKVAV, the material assessed *in vivo* in Aim 2, as a 3D cell culture environment. Its impact on astrocyte morphology *in vitro* was explored, which is vital for further investigation of this material in improving current cell culture environments and as an *in vivo* strategy. This involved analysis of astrocyte morphology formed in multiple culture set-ups, as well as an evaluation of the impact of the co-assembled system from Aim 2 on the metabolic activity and morphology of LPS- and IL-1α-stimulated astrocytes. This allowed us to better understand this system’s effect on reactive astrocytes.
1.3 Thesis Outline

This thesis focuses on the development and evaluation of biomaterials to alter the morphology and behaviour of astrocytes for traumatic brain injury. The following chapters are either published articles, submitted manuscripts or manuscripts in preparation. They can be divided into five sections, as outlined here. The thesis begins with a literature review to present the past and current research on biomaterials and the associated design criteria for traumatic injury – with a particular focus on addressing the inflammatory response of the brain. Then, materials development and biological assessment of the astrocytic response \textit{in vitro} and \textit{in vivo} is presented. The thesis concludes by presenting future directions for the development of biomaterials to treat traumatic injury.

1.3.1 Literature Review

Chapter 2 provides a review of the past and current literature on biomaterials for treating traumatic injury. In particular, the complex inflammatory response of the brain is presented along with the associated biomaterials design criteria and potential candidates for a successful treatment strategy to address such an inflammatory response. The current gaps in the research field and the complexity of astrocytic behaviour is highlighted in this review, establishing a necessary context for the research presented in this thesis.
1.3.2 Materials and Methods

Chapter 3 presents the materials and methods, including fabrication, functionalisation, and materials characterisation of electrospun PCL nanofibres and Fmoc-DIKVAV, as well as the surgical and cell culture protocols used for biological assessment.

1.3.3 Materials development and biological assessment

1.3.3.1 Nanofibrous scaffold influence in vitro and in vivo

Chapter 4 presents the development and subsequent functionalisation of nanofibrous scaffolds with a galactose-presenting novel polymer. This biomaterial system was evaluated in vitro and in vivo after traumatic injury (Appendices A, B, C includes further research conducted on the use of nanofibre scaffolds for inflammation research to which I contributed).

1.3.3.2 Co-assembled Fmoc-DIKVAV and fucoidan in vivo implantation

Chapter 5 presents the co-assembly of Fmoc-DIKVAV with fucoidan and assesses the impact of implantation after traumatic injury on astrocyte scar formation, organisation, and morphology. Fmoc-DIKVAV was selected for this research to address the geometric limitations of nanofibre scaffolds in filling an odd-shaped lesion (as highlighted in Chapters 2 and 6), ensuring this work will have relevance as a future treatment strategy.

1.3.3.3 Three-dimensional culture environment of Fmoc-DIKVAV

Chapter 6 highlights the importance of the development of 3D environments for treating CNS inflammation, whilst Chapter 7 presents the in vitro investigation of Fmoc-DIKVAV as a 3D culture system for astrocytes. The morphology and cytoskeletal
organisation of astrocytes is assessed, as is the impact of the Fmoc-DIKVAV and fucoidan on LPS- and IL-1α-stimulated astrocytes.

1.3.4 Conclusions and future perspectives

Chapter 8 provides concluding remarks on the research presented in this thesis and its contributions and impact on the research field of biomaterials for traumatic brain injury. Perspectives for the future directions of using Fmoc-DIKVAV as a treatment strategy for traumatic injury are also presented.
This chapter provides an overview of inflammation and the use of biomaterials to treat traumatic brain injuries. The inflammatory response of the body, and specifically how the process works in the central nervous system is discussed. Key perspectives of the astrocytic response are presented, consistent with the shifting paradigm regarding their contribution to repair and regeneration, rather than solely cytotoxic contributions, post-injury. Design criteria of biomaterial strategies to treat traumatic brain injury are discussed, and those with implications on the inflammatory process are discussed and potential biomaterial candidates are investigated. Future perspectives of the field are then presented, with this review setting the context for the following research chapters presented in this thesis.
Abstract

The inflammatory response within the central nervous system is a tightly regulated sequence of events that can exhibit both cytotoxic and cytotrophic effects. This concomitant duality in function is the foundation of the complexity of this response, and currently, delineation of the two behaviours has not been achieved. This presents many challenges for tissue engineers when designing treatments to repair and regenerate damaged tissue after brain insult, such as in traumatic brain injury (TBI) or stroke, where permanent dysfunction is often observed. The changing perspective of brain inflammation from a toxic and growth inhibitory mechanism to one that is also protective and reparative significantly alters the approach used to overcome the ubiquitous barrier to regeneration. Thus, the use of biomaterials to provide the necessary material properties for neural tissue engineering constructs, and temporally alter the inflammatory response of the brain is an exciting approach for neural regeneration. Understanding, evaluating, and designing strategies to manipulate the inflammatory response to optimise repair and regeneration after injury is an innovative approach, with the potential to drastically improve brain repair.
2.1 Introduction

Repair of neural tissue is a complex, and as yet unmet, challenge providing significant opportunity to tissue engineers. Such repair after the loss of neurons or axons from injury or disease in the adult brain is limited, and not because of insufficient neurogenic potential, but rather nascent cells lack cues and signals that can guide survival, differentiation, and reconnection [15]. Many of these cues are provided by the extracellular matrix (ECM), astrocytes, microglia, and a range of cytokines. When the blood-brain-barrier (BBB) is ruptured by penetrating brain trauma, there is an ingress of blood and fibroblasts, which along with other participants in inflammation (including macrophages, circulating white blood cells, and systemic cytokines). This complicates the usually inflammatory response found in the brain following injury or cell death with an intact BBB. The present challenge for tissue engineers is that the delivery of many engineering constructs designed to assist repair also cause a penetrating brain injury that must also be considered when assessing the value and contribution of the constructs under consideration. For this reason, the penetrating brain wound is a standard model where the control is a stab wound to the brain, and can be used to assess the influence of engineering constructs on this inflammatory response.

The inflammatory response of the brain is complicated, and unlike many other organs in the body, such as the skin or liver, does not transition to a reparative phase where functional repair and regeneration is observed. Here, we review the application of third generation biomaterials for neural tissue engineering applications, with a particular focus on the need for temporal attenuation of the inflammatory response after penetrating brain injury. Initially, the inflammatory response of non-CNS organs is
compared and contrasted with that of the brain, to provide a holistic view of the inflammatory cascade and its associated events within the body. The complexity of CNS inflammation, the design criteria of biomaterials to assist in its resolution, and thus improve outcomes of treatment strategies are discussed, and finally, perspectives on the future direction of the field are proposed.

2.2 The inflammatory response of the body

Inflammation is a tightly regulated response to tissue injury or infection, as well as a key component of tissue-homeostasis. It was initially characterized by Celsus in A.D. 25, using four cardinal signs: pain, swelling, redness, and heat. After injury, the body’s initial response is to minimize tissue damage and maintain the integrity of the undamaged surrounding tissue, through haemostasis, inflammation, proliferation, and, finally, remodelling.

The inflammatory response is dependent on the type of injury and important factors including whether there is vascular damage, and if foreign material has been introduced. Vascular damage elicits a hemostatic response directed at minimising blood loss from the organism. Blood loss is prevented by vasospasm with slowed microcirculation, and coagulation is initiated when tissue factor is exposed to blood [16]. Blood entry into the extracellular space (ECS) is reduced by an increased ECM pressure, however, this can compromise cell viability and capillary function, including an increase in capillary permeability due to leaking endothelium. The subsequent oedema causes the classic sign of swelling in inflammation. However, the extravasation of blood into the ECS also triggers an inflammatory response in its own right, and this is a significant factor in
penetrating brain injury. When foreign body debris is present after injury, such as blood-borne molecules or proteins, a foreign-body response (FBR) is initiated, in which macrophages release chemotactic factors to recruit neutrophils to the site of injury, and consequently, debris is phagocytosed [17]. Increased blood flow, at the body’s core temperature, to the injury site corresponds to the heat indicator. The cytokines induce a response in local nerve cells, and the contributory acidic local environment causes the pain associated with inflammation.

These inflammatory actions, whilst crucial to tissue repair, can spread to the adjacent healthy tissue and cause secondary cell damage and death unless contained. This is achieved by the recruitment of fibroblasts to the injury site to form a scar around the damaged tissue which effectively demarcates the healthy, non-affected tissue and contains the inflammatory response. This initial protective, defensive phase begins to resolve itself approximately 3-5 days after injury, where cell proliferation and tissue remodelling can occur, with neovascularization causing the redness associated with inflammation. This inflammation response is common to many injuries within the body, and can be resolved effectively in the so-called ‘frontline’ organs such as the skin or liver, which are continually subjected to traumatic, infectious, thermal, and toxic injury. These organs have the vast capacity for repair processes including: the formation of blood clots; cell death and inflammation; cell proliferation for new tissue formation, including (neo)vascularisation and reconstruction of tissue through both stem cell regeneration and cytotrophic transformation; and tissue remodelling [2, 18]. However, as injury to these organs is expected, they effectively transition from each phase in the inflammatory response to eventual repair. Contrastingly, in the brain, the transition from the initial
protective inflammatory phases to the reparative phases where new tissue is constructed and remodelled is not as successful. The reasons behind this insufficient transition of phases are at present not well understood, and presents valuable therapeutic target.

2.3 The inflammation response of the CNS

The CNS is generally considered immune-privileged, although the absolute use of this terminology has been criticized [19] - here we use the term to describe the unique immunological properties of the brain and spinal cord [20, 21]. The unique defensive repertoire of the CNS includes mechanisms that aim to first minimize the likelihood of injury or infection, and then respond to injury stimuli. Opportunity for trauma is reduced as the brain is surrounded with bone, and floats in cerebrospinal fluid (CSF), so these front-line defences of the brain differ to that of the skin, which is constantly exposed to trauma. The selectivity of the blood-brain-barrier (BBB), a sophisticated mechanism by which brain endothelial cells and astrocytes form physical, transport, and metabolic barriers as reviewed in [22], protects the brain from non-CNS organisms, pathogens and toxins. As previously mentioned, the inflammatory response of the brain is highly complex and involves an organized cascade of cellular and biological events. Disruption of the BBB as a consequence of injury results in a rapid response consisting of coagulation, platelet aggregation and clot formation, which are followed by a sequence of synchronous cellular responses. Such responses from include the infiltration of inflammatory cells, phagocytosis of cellular debris, and sub-acute death of parenchymal cells. Additionally, when the meninges are penetrated, meningeal fibroblasts form a fibrotic scar within the lesion core, along with matrix molecules including laminin, fibronectin and collagen that form scaffolds to support macrophage and leukocyte
infiltration [2, 23]. Microglia and NG2-oligodendrocyte precursor cells (NG2-OPC) immediately migrate to the site of tissue damage and BBB leakage. However, astrocytes remain in situ, and undergo proliferation limited to the lesion penumbra [10, 24], experiencing significant hypertrophy and increase their expression of intermediate filament proteins including glial fibrillary acid protein (GFAP), nestin and vimentin in response to injury [4]. This phenotype of astrocytes is referred to as ‘reactive’, as compared to the physiologically active astrocyte phenotype in healthy tissue. These reactive astrocytes can entangle their filamentous processes to form a physical barrier (termed either the astrocyte or glial scar) around the lesion core that inhibits immediate axon regeneration after injury, but also restricts the migration of inflammatory cells into viable tissue preventing secondary degeneration [25].

In acute injuries that are not complicated with infection or further tissue damage, the lesion core, astrocyte scar and the peri-lesion perimeter undergo tissue formation remodelling for an extended period of time [2]. Conversely, if the inflammatory response is more severe and persistent after chronic insult, tissue remodelling and consequent functional recovery is severely inhibited. A persistent inflammatory response, particularly the astrocyte scar around the lesion core, is an extreme response to injury, and the subsequent chemical and physical barriers result in long-term inhibition of neural repair and regeneration.

The interconnected mechanisms of cells involved in the CNS inflammatory response increases the complexity of developing tissue engineering approaches for repair, and thus is currently a problematic aspect crucial to regeneration. Although we recognise the importance of microglia, macrophages and other infiltrating inflammatory cells after
injury, a prevalent barrier to regeneration is the persistent astrocyte scar, and represents the focus of this review. There may be interplay between the long-term reactive behaviour of astocytes and microglia, however, this is yet to be thoroughly investigated within the literature. As such, this review will focus on biomaterials that can be used to overcome the barrier to regeneration after injury due to the astrocytic response (specifically, penetrative traumatic brain injury). We anticipate that successful biomaterial treatments could be easily adapted to target other inflammatory cell types (i.e. microglia/macrophages), and this would be an ambitious future direction of the field of tissue engineering to facilitate repair and functional recovery after penetrative traumatic brain injury.

2.3.1 Shifting perceptions of astocytes in CNS inflammation

In recent years there has been a shift in the perception that astocytes are either good (“quiescent”) or bad (“reactive”), with the recognition that astocytes, whilst they may be reactive after injury, serve essential functions including maintenance of the BBB, supporting neuronal survival, and preventing excessive inflammatory cell infiltration, and hence should not be thought of in such a binary sense [2, 10, 26]. As has previously been reported [2], the term “quiescent” perpetuates a flawed understanding of astocytes. Under physiological conditions, astocytes are dynamic and active; maintaining fluid, pH and ion homeostasis through Aquaporin 4 (AQP4) water channels and transporters for K⁺, whilst also modulating synaptic transmission through the uptake and release of neurotransmitters, and quite clearly, are noAt merely resting or inactive [27]. The many different roles and functions astocytes execute give rise to varying phenotypes which have not yet been completely characterised, and hence reiterates their complexity. Given
such dynamism, we consider physiological astrocytes as “active”, and when responding to injury, they will be referred to as “reactive”. The reason for our terminology is that we believe that astrocyte phenotype is multifaceted and temporally dependent. In the acute phase after injury, reactive astrocytes prevent growth through the lesion by presenting physical and biochemical barriers. However, this behaviour is ultimately cytотrophic as it prevents axons from growing into an environment that would not support their continued growth and survival, and would result in an increased number of dystrophic axons, or even axonal death. The truly cytotoxic behaviour of astrocytes is the chronic persistence of the astrocyte scar. Here we will discuss the design criteria of a TBI treatment and evaluate the suitability of particular biomaterials to satisfy these criteria, highlighting the knowledge gaps that need to be addressed for the advancement of the field.

2.4 Third-generation biomaterial strategies to control the inflammatory response after TBI

2.4.1 The evolution of biomaterial strategies

Biomaterials have long been used in medical treatments. Initially the focus was on providing mechanical and structural support in age-related ailments such as hip and dental replacements because first-generation, ‘bio-inert’, biomaterials could not replace more complex living tissues [28]. Interest then switched to second-generation biomaterials that might exert control on the surrounding environment, such as ceramic hydroxylapatite serving as a bioactive fixation agent for orthopedic applications [29, 30]. Although so-called inert materials do in some way interact with and alter their milieu,
the distinction between first- and second-generation materials could be considered to be the degree of influence over the surrounding environment, and the extent to which that influence can be controlled. These first- and second-generation biomaterials were limited by implant failure rates and lifetimes requiring revision surgery, in addition to the inability of synthetic materials to respond to changes in the physiological environment and stimuli [31]. Third-generation biomaterials are focused on providing instructive cues on the molecular level, respond to the physiological environment and potentially be resorbed, leaving nothing but newly regenerated host tissue. As such, this class of biomaterials has generated optimism for TBI/neural tissue engineering applications to influence and instruct host tissue to achieve repair and regeneration.

2.4.2 Design criteria for brain repair and modifying inflammation

If third-generation biomaterials are to have a place in the treatment of TBI, then targeted manipulation of the inflammatory response by implanted biomaterials will be important. Here, we discuss the overarching strategy for achieving functional recovery after injury, and then identify those approaches that modify the inflammatory response.

A designed biomaterial for the treatment of traumatic brain injury should therefore consider one or more of the following design criteria. It should be noted that the satisfaction of all criteria in one material is still to be achieved, and will most likely be the outcome of significant collaboration and materials development.

1. The material should physically support the tissue surrounding the injury.
2. The material should match the biophysical environment of the brain (water content, pH etc.).
3. The material should approach the elastic modulus of brain tissue.
4. The material should allow cell infiltration through and across the lesion site.

5. The material should mimic the three-dimensional (3D) nanofibrous architecture of the brain’s extra-cellular matrix.

6. The material should provide temporal biological cues to the surrounding tissue based on the stages of the inflammatory response.

Many of these criteria are relevant to attenuating the inflammatory response after injury. Physical support (criteria 1) is crucial as further tissue collapse after the initial swelling post-injury can exacerbate the existing inflammatory response. Matching the modulus of the brain (criteria 3) will also avoid the development of a FBR from microtrauma that arises from modulus mismatch, such as in the case of electrodes [32, 33]. Finally, the temporal delivery of biological cues (criteria 6) is of great importance in an inflammatory context. It is important to allow the inflammatory astrocytic response to exist in the short-term, however, the persistence of this reaction needs to be attenuated, which could be achieved through temporally controlled biological intervention, after which, cell infiltration can be supported (criteria 4). Here we present a review of candidate biomaterials.

2.4.3 Biomaterials to provide physical support to the lesion site

A penetrative TBI from a gun-shot or stab injury, results in an odd-shaped lesion site in the brain. This primary injury causes severe tissue damage, followed by swelling which can necessitate a craniotomy to reduce the swelling. Once the swelling subsides, structural support to the damaged area is necessary to avoid tissue collapse. Therefore, any treatment strategy for TBI must offer physical support to the lesion site. There are many biomaterial candidates that would satisfy the criteria of physical support, including
electrospun nanofibre scaffolds and hydrogels. These materials can be injected (nanofibre scaffolds with a plunger [34], and hydrogels through a needle, particularly if they are shear-thinning [35]), so can be implanted in a minimally invasive fashion.

Electrospun nanofibre scaffolds are an interesting biomaterial candidate as they can be easily fabricated from biocompatible and biodegradable materials, and also mimic the nanofibrous architecture of the ECM, possess a high surface area-to-volume ratio, and can be functionalized to provide instructive cues to cells through the immobilization of growth factors and other biologically relevant cues [11, 36-39]. The 3D architecture of nanofibres has been previously shown to shift the biological profile of astrocytes to that of a more cytotoxic nature in vitro [7], which could have potential value in vivo, altering astrocytic phenotype after injury. Randomly aligned poly(ε-caprolactone) (PCL) scaffolds significantly reduced microglia and astrocyte numbers after implantation into the caudate putamen of male Wistar rats compared to the wire control [34]. After 60 days, this scaffold also supported the infiltration of neurites, compared to neurite growth which was directed around the scaffold when nanofibres were partially aligned.

Nanofibre scaffolds have also been investigated for their ability to deliver biological cues in vivo. For example, when primary cortical neural cells were transplanted adjacent to PCL scaffolds with glial-cell derived neurotrophic factor (GDNF) immobilized to the surface, their survival, proliferation and neurite outgrowth was enhanced [40]. These studies demonstrate the utility of nanofibre scaffolds in attenuating the inflammatory response of the brain, as well as facilitating cell growth. However, a shortcoming of electrospun nanofibres, as previously highlighted [26, 41], is the geometrical constraint associated with possessing a sheet-like structure on the macroscale. These sheets can be
rolled upon themselves to form an injectable cylinder, however, this structure cannot form intimate contact with surrounding tissue in an odd-shaped lesion, as is commonly found in TBI (Figure 2-1), although they would be of interest for grafting over damaged nerves or in spinal cord injuries [42]. Therefore, to satisfy the criteria of providing physical support to the lesion, injectable hydrogels are an ideal candidate, as they can flow to fill the lesion site and prevent further tissue collapse after injury.

![Figure 2-1: Arrows showing the failure of an implanted nanofibre scaffold in forming intimate contact with neurites. Adapted from [34], reproduced with permission from Elsevier.](image)

It is worth noting, however, that nanofibre scaffolds do have significant promise for in vitro investigation of astrocytes and their inflammatory behaviour. Given their capacity to provide a 3D nanofibrous environment, present biological cues [11, 34, 42], and alter astrocyte phenotype and behaviour [7, 13, 43], electrospun nanofibrous scaffolds can be used to better understand astrocytes and their complex behaviour in vitro. Additionally, since in vitro use of hydrogels as 3D environments can present challenges (for instance
with handling, degradation and imaging), nanofibre scaffolds can facilitate the shift of
*in vitro* investigation from 2D to 3D environments.

2.4.4 Matching the modulus of the brain

The elastic modulus of the brain varies from approximately 0.1-2 kPa, and also varies
with white and grey matter [44, 45]. It is vital that any biomaterial system selected for
TBI treatment can approach the modulus of brain tissue to minimize microtraumas
associated with mismatch, and given the varying nature of the brain’s modulus, a system
which can be mechanically tuned is also ideal. Modulus mismatch can be the downfall
of many medical devices, due to the mechanosensitivity of microglia and astrocytes. Not
only does the implantation procedure cause an inflammatory response (as it is essentially
another form of penetrating injury), but modulus mismatch initiates a FBR, which can
be a result of constant microshearing, so microglia and astrocytes become reactive and
seal off the implant. This is an important consideration, as the formation of the astrocyte
scar as part of the FBR prevents the implant from having contact with host tissue to
enable regeneration. This was clearly demonstrated when polyacrylamide (PAA) gels,
at either 0.1 kPa (compliant) or 30 kPa (stiff) were implanted into Sprague-Dawley rat
brains, Figure 2-2 [46]. There was an increased astrocyte and microglia response when
the stiff (30 kPa) hydrogel is implanted, highlighting the importance of the consideration
of the modulus of biomaterials when designing a treatment strategy for TBI.
Given their ability to fill a void (compared to nanofibre scaffolds), hydrogels will be primarily considered when assessing biomaterials to match the modulus of brain tissue, as they can also satisfy the previous criteria of providing physical support [47, 48]. Many hydrogel systems not only match the modulus of the brain tissue, but have tunable mechanical properties, making them ideal tissue engineering constructs for TBI. Mechanical properties can be tuned through various routes, including cross-linker or precursor concentration, and fibre density. Previously, mechanical properties have been of great interest for researchers in controlling the differentiation, proliferation and morphology of stem cells [45, 49], which has led to a large number of hydrogels being investigated for their tunable mechanical properties.

A commonly used hydrogel for tissue engineering applications extracted from brown algae, alginate, has a modulus which is dependent on molecular weight and cross-linking. Alginate consists of linear co-polymers comprising blocks of α-L-guluronate (G)
and (1,4)-linked β-D-mannuronate (M) residues, with the G-blocks being key determinants of mechanical properties through co-polymer length and molecular weight [50]. Ionic cross-linking of alginate is a common gelation method, with the divalent calcium ion being a popular ion choice. Calcium ion cross-linking can be controlled through the use of low-solubility solutions which result in a slower release of Ca\(^{2+}\) and thus a more controlled gelation and consequently, more uniform mechanical properties. To match the modulus of brain tissue, the alginate and Ca\(^{2+}\) concentration can be optimized within the gelation process [51]. Ionic cross-linking limits the stability of the hydrogel over longer time periods, which could be advantageous for brain repair if the release of the divalent ions (Ca\(^{2+}\)), and thus hydrogel dissolution, could be controlled. Alternatively, covalent cross-linking can offer a greater degree of control over the mechanical properties of alginate, however, these cross-linking reactions can have unreacted and cytotoxic reagents, so further work is required to remove these cytotoxic agents from the covalently cross-linked alginate gels.

Hyaluronic acid, a polysaccharide abundant in the native ECM, has been extensively investigated for its tunable mechanical properties that are dependent on molecular weight, chemical crosslinking, and ultra-violet (UV) cross-linking. These determining variables can also be combined to optimize hydrogel formation and modulus. For example, chemical cross-linking of methacrylated hyaluronic acid with a dithiol cross-linker (via a Michael addition mechanism), coupled with UV cross-linking resulted in a consistent peak modulus of the hydrogel (~100 kPa). However, without UV cross-linking, hydrogel modulus increased over two orders of magnitude with increasing cross-linker concentration, reaching ~ 100 kPa with 100% crosslinker consumption [52].
Modification of hyaluronic acid with photo-crosslinked methacrylate groups can yield a range of moduli, matching that of a neonatal brain to that of an adult spinal cord, demonstrating the utility of this system’s tunable modulus [53].

A synthetic hydrogel with significant potential for enhanced functionality is poly(ethylene glycol) (PEG). The various ways to crosslink PEG hydrogels have been extensively reviewed elsewhere [54]. Increased cross-linking of star-PEG/heparin hydrogels increased the associated storage modulus, accompanied by a reduction in hydrogel hydration, demonstrating the impact of modulus on water uptake (a key characteristic of hydrogels for tissue engineering) [55]. Additionally, modification of PEG hydrogels with biological moieties such as the cell adhesion peptide sequence, RGD, can also affect the modulus. PEG hydrogels cross-linked with varying concentrations of PEG-dithiol (PEG-SH) showed an increase in modulus when the sequence RGD was attached to the hydrogel [56]. These relationships between modulus and other material properties demonstrate the need for the optimisation of cross-links, water uptake, biological functionalization, and modulus is required when designing a tissue engineering construct for brain repair.

All of these hydrogels present desirable mechanical properties for use in treating traumatic brain injury, however, they are unable to mimic the nanofibrous structure of the ECM (criteria 6), and as such, the remainder of the review will focus on the use of nanofibrous hydrogels, or composite materials which can satisfy the same criteria. Such nanofibrous hydrogels are a more recently developed class of hydrogels, self-assembled peptide (SAP) hydrogels. They form fibrils within a hydrogel, and can provide a variety of biological functionality either through the peptide sequence or additional
functionalization. The modulus of these SAPs is dependent on the peptide sequence and concentration, as well as the gelation mechanism. The formation of the nanofibres determine the bulk physical properties of the hydrogels: for example, peptide amphiphiles (PAs) such as C_{16}A_{4}G_{3}S(P)KGE-COOH (termed PA-1) can form a hydrogel at concentrations as dilute as 0.5 wt%, forming nanofibres 6-7 nm in diameter [57]. PAs consist of a short hydrophobic alkyl chain, attached to a short hydrophilic (relative to the alkyl chain) peptide sequence, which self-assemble to form a hydrogel with high-aspect-ratio nanofibres [58]. This self-assembly is driven by the thermodynamic incompatibility between the different regions of the chain, and triggered by charge neutralization via the addition of metal ions [59]. These PAs can aggregate to form different morphologies including spherical micelles, cylinders, and even bilayer vesicles [60]. Gelation and initial solution pH have been shown to be influencers of PA modulus. PA-1’s modulus reaches a plateau when gelled with Ca^{2+} concentrations of 20-30 mM, due to a saturation of metal ion-PA interactions, whilst the initial pH of the solution influences the gel formation, reaching a maximum modulus of ~1000 Pa above pH 9. This is a result of deprotonation, which creates further negative charges that interact with Ca^{2+}, stabilizing interfibre bonds and increasing the density of fibre crosslinks, and thus, modulus [57]. RADA16-1 is another synthetic amphiphilic peptide, however it has a comparably low modulus, which has been noted as a limitation of its use [61, 62]. Modification with the addition of a peptide motif inspired by spider silk, GPGGY, tripled the modulus compared to unmodified RADA16-1, however it still remained low (~20 Pa). RADA16-1 is also limited by its low pH, which resulted in increased inflammation when transplanted into a spinal cord injury without at least 7 days ‘pre-
treatment’ where the pH was neutralized using culture medium [63]. These issues highlight the need for further development of the RADA16-1 system before it is as competitive as other SAP systems for treating traumatic brain injury.

Similar modulus dependence on gelation mechanism as seen in PA-1 is also observed in the Fmoc-capped self-assembled peptide hydrogels. Short bioactive peptide sequences are attached to the aromatic Fmoc-group, which undergoes self-assembly in response to a pH-switch. The morphology and modulus of Fmoc-FRGDF can be manipulated via the final ionic strength and the rate of pH change during gelation, with an increased ionic strength associated with faster gel formations and thus increased stiffness of the hydrogel [64]. Additionally, using glucono-δ-lactone to replace hydrochloric acid in the pH switch decreased the gelation rate, resulting in a decreased modulus compared to hydrogels formed using hydrochloric acid. The addition of D-residues in Fmoc-IKVAV, Fmoc-DIKVAV, Fmoc-DDIKVAV, and Fmoc-DDDIKVAV was associated with increasing stiffness [65], demonstrating that the peptide sequence, ionic strength, peptide concentration, and the rate of gel formation can be used to tune the moduli of Fmoc-SAPs to match that of brain tissue.

Matching and ideally tuning the moduli of these nanofibrous hydrogels is vital in the development of a biomaterial system to treat traumatic injury, and here we have presented many nanofibrous hydrogels which can do so. Therefore, these materials should be the focus of future research effort to further develop their functionality and eventual deployment as a TBI treatment.
2.4.5 Controlled degradation of biomaterial candidates

Mechanical properties of material candidates are a crucial design consideration to avoid a FBR, and since temporal control of the surrounding environment is required, a material that can change in a timely manner to facilitate cell infiltration, or deliver a repertoire of signals is also ideal. The relationship between moduli and hydrogel degradation is an important consideration for these material systems to achieve functional repair and regeneration at the lesion site.

There are a variety of mechanisms that can be used to control the degradation of a biomaterial. Some are inherent to the material itself: for example, PCL and poly(L-lactic acid) nanofibres are degradable via the hydrolysis of the ester linkages, whilst other mechanisms can be included within the material to impart controllable degradation. Other mechanisms are an addition to the biomaterial system to overcome a natural degradation limitation. For example, although alginate has been extensively investigated for both in vitro and in vivo use, it is not enzymatically degraded by mammals. With previous research demonstrating enhanced vascularization and tissue integration of cells transplanted within readily degradable materials, mechanisms to encourage this degradation have been investigated [66, 67]. Encapsulation of alginate-lyase within PLGA microspheres (which are then embedded within the alginate hydrogel) enabled a tunable enzymatic degradation of the alginate hydrogels, and facilitated a greater rate of proliferation when neural progenitor cells (NPCs) were also encapsulated [68]. This controlled degradation mechanism via microspheres could be introduced within the fibrous hydrogel systems to aid in degradation.
Proteolytic degradation can also be employed to achieve cell-mediated degradation. For example, a peptide sequence can be included in self-assembled peptides or PEG crosslinks that is sensitive to matrix metalloproteinases [69-72]. However, such control over material degradation may be limited in some cases. For example, Fmoc-DIKVAV is an ideal biomaterial candidate for use in the brain, given its biologically-relevant IKVAV sequence presented on the surface of the nanofibres, and that it is a gel at physiological pH – to further alter this system to control the degradation could interfere with the self-assembly mechanism, for example, and as such compromise the system’s desirable properties. To the best of the author’s knowledge, Fmoc-SAPs have not been investigated with such a degradation mechanism, and nor have they been comprehensively characterized for their degradation in vivo, highlighting potential avenues for future investigation. Where degradation cannot easily be controlled, without compromising material properties, it may be of interest to develop a composite material – where a valuable system such as Fmoc-DIKVAV is combined with another hydrogel which can provide a high degree of control over degradation – for example, PEG, or even another SAP (Fmoc- or PA-based, containing an enzymatically degraded peptide sequence). Such a composite, as visualized in Figure 2-3 would satisfy key tissue engineering criteria to treat TBI. This composite system has been preliminarily investigated using RADA16 functionalised with RGD which were chemically crosslinked to PEG to form a hydrogel with modulated mechanical properties and a nanofibrous structure, demonstrating the achievable nature of such a composite [73]. These degradation mechanisms could be optimized to achieve temporal delivery of cues to modify the inflammatory response and facilitate cell infiltration. This could
potentially be combined with the delivery mechanisms discussed in the next section to achieve a temporal delivery profile as desired.

As previously established the inflammatory astrocytic response after injury is complex, and necessary for repair. The cytotoxic effects of this response are here considered to be the persistent nature of the astrocyte scar around the lesion site. Thus, when designing a biomaterial construct to resolve this inflammation, temporal control of this response is required. After a stab injury, astrocyte numbers have been found to peak at 7 days (although their particular phenotype/sub-phenotype of reactivity is unknown) [74]. Within the first two weeks post-injury, astrocytes proliferate and form the scar, with scar remodelling occurring from 3 weeks onwards, where it can remain persistent in severe injuries [2]. Therefore, from weeks 3-4 post injury is the ideal time-point at which to

2.4.6 Modifying the inflammatory response via temporally delivery of cues

As previously established the inflammatory astrocytic response after injury is complex, and necessary for repair. The cytotoxic effects of this response are here considered to be the persistent nature of the astrocyte scar around the lesion site. Thus, when designing a biomaterial construct to resolve this inflammation, temporal control of this response is required. After a stab injury, astrocyte numbers have been found to peak at 7 days (although their particular phenotype/sub-phenotype of reactivity is unknown) [74]. Within the first two weeks post-injury, astrocytes proliferate and form the scar, with scar remodelling occurring from 3 weeks onwards, where it can remain persistent in severe injuries [2]. Therefore, from weeks 3-4 post injury is the ideal time-point at which to
begin the delivery of therapeutic agents to resolve the inflammatory process and provide a growth-supportive environment.

Mechanisms which can be employed to achieve temporal delivery of therapeutic agents, other than material degradation as discussed above, include diffusion and enzymatic-triggered delivery. One avenue for delivery is the incorporation of molecules via co-assembly or mixing within the hydrogel construct, which has been achieved with the anti-inflammatory fucoidan [12] as well as brain-derived neurotrophic factor (BDNF) and GDNF [75] within various Fmoc-SAP systems. A delayed release of BDNF was achieved via modification with chitosan, demonstrating that either the delivery mechanism or the therapeutic agent itself can be modified to alter the delivery time. Incorporating drug delivery vehicles within a hydrogel construct would provide two diffusive barriers to release – first, out of the delivery vehicle, and then from within the hydrogel to the surrounding tissue. Examples of such delivery vehicles include microsphere and nanoparticles: nanoparticles containing flavopiridol have reduced cavitation, and pro-inflammatory cytokine expression after spinal cord injury [76]. However, it was found that these nanoparticles only had sustained release of 3 days in vitro, and so, including them within a hydrogel would delay the release of such a therapeutic agent to a more desirable time-point. A similar delivery mechanism can be achieved using electrospun nanofibres, where the therapeutic agent is included within the electrospinning emulsion, and the subsequent nanofibres are cut into short nanofibres and included within a hydrogel. The diffusion from these fibres is visualized in Figure 2-4, and when combined within a hydrogel, mitigates a burst release of drug and delays the subsequent release [77].
Another mechanism which can be combined to provide temporal release and degradation is enzymatic-triggered release of therapeutic agents from the hydrogel itself. This has been elegantly demonstrated with a proangiogenic peptide included within the cross-links of PEG, and which was released upon exposure to host matrix metalloproteinases (MMPs) [70]. This resulted in the bulk degradation of the material and significant angiogenesis at the site of hydrogel implantation, demonstrating the therapeutic utility of this system. To modulate the inflammatory response, this enzymatically-responsive release could be used to deliver anti-inflammatory therapeutic agents if the enzyme-responsive linker could be tailored to enzymes that would be present at 3-4 weeks post injury.

Long term delivery of growth factors can be achieved through covalent immobilization on scaffolds, such as nanofibres [11, 42], which can then be incorporated into a hydrogel as short fibres, to satisfy the previous criteria discussed. This would provide sustained and long term delivery of biological cues which would be of use when encouraging regeneration after scar resolution, however, in itself does not provide the ideal level of
temporal control. Therefore, these short nanofibres would be valuable when incorporated as one of many components in a multifaceted system.

There are a variety of delivery mechanisms which can impart temporal control over therapeutic agent release, however, considerable investigation into what additional cues are the most effective in resolving the persisting scar needs to first be completed. What would be of interest are therapeutic agents that can impact on astrocyte morphology, which is linked with astrocyte phenotype. The family of Rho-GTPases regulate the assembly of cytoskeletal processes which determine cell shape and contractility, which could be valuable for changing astrocyte morphology/phenotype, the mechanotransductive signals RhoA and CdC42 are activated when neural stem cells (NSCs) respond to ECM stiffness (which we have previously discussed as a key consideration for a TBI treatment) [78]. In particular, Rho Kinase have been highlighted as a valuable therapeutic target for reactive astrocytes (its suitability for mediating astrocyte reactivity (after stroke) is comprehensively reviewed in [79]) as its inhibition has resulted in reduced lysophosphatidic-induced stress fibres and focal adhesions [80], as well as decreased F-actin but increased G-actin in mature primary astrocytes grown on nanofibre scaffolds, indicating a more cytotrophic phenotype of astrocytes [43]. Contrastingly, when delivered with fibroblast growth factor (FGF), the sulfated polysaccharide extracted from sea cucumber, Haishen (HS), modulated astrocyte morphological transformation, increased cell proliferation and was thus proposed as an adjuvant to induce astrocyte reactivity. These two examples demonstrate that changes in astrocyte morphology can induce both physiologically active and reactive astrocytes, and thus therapeutic agents need to be selected carefully. Astrocyte activity has also been
modulated by the presentation of poly(D-lysine) (PDL) grafted to the thermally gelling hydrogel xyloglucan. After 60 days, the percentage area of astrocytes (GFAP⁺) and neurites (SMI32⁺) were both highest when the greatest amount of PDL was grafted to the xyloglucan hydrogel [74]. The increase of both astrocytes and neurite infiltration with the PDL presentation, and possibly an alteration in astrocyte phenotype from reactive to growth-supportive, suggests that saccharides would be a valuable avenue of investigation for the attenuation of inflammation after injury. Thus, we propose that the inclusion of anti-inflammatory saccharides to alter astrocyte phenotype (and morphology) is a valuable avenue of investigation for the development of systems to resolve the astrocyte scar after TBI. This includes the presentation of galactose moieties such as lactobionic acid, for example, or the anti-inflammatory sulfated polysaccharide fucoidan. Fucoidan has induced apoptosis and the decreased expression of pro-inflammatory cytokines in cancer cells when delivered via Fmoc-FRGDF [12], whilst the neutral polysaccharide PPQN has suppressed nitrous oxide (NO) production and pro-inflammatory cytokine secretions in lipopolysaccharide (LPS) stimulated macrophages [81]. These results highlight the potential for the inclusion of anti-inflammatory saccharides, which can then be included via a temporal delivery mechanism within the biomaterial constructs we have discussed here to develop an effective TBI treatment.

2.5 Conclusion and future perspectives

Here, we have discussed the complexity of inflammation within the central nervous system, and have specifically focused on evaluating current biomaterial systems for developing a treatment for TBI which can also impact on the inflammatory response.
Given the complexity, as well as the incomplete knowledge and characterization of the astrocyte response, tissue engineering solutions to TBI face many challenges. As such, we have presented tissue engineering design criteria for a solution to be used to TBI, and explored the criteria that are of particular importance in addressing the inflammatory response. It is essential that any biomaterial system can fill an odd-shaped lesion void, forming intimate contact with the surrounding tissue to prevent further tissue collapse. Thus, hydrogel systems were explored over nanofibrous scaffolds, as they can easily fill a void. In particular, hydrogels with modulus matching that of brain tissue, and ideally with a tunable modulus, are of particular interest to facilitate neural growth and mitigate a foreign body response.

There are many systems possessing tunable moduli, dependent on gelation mechanism and cross-linker, precursor, or fibre density. However, many of these systems do not satisfy a key criteria for a TBI tissue engineering construct, which is to possess a nanofibrous architecture mimicking that of the brain. Thus, self-assembled peptides are of particular interest in developing a biomaterial treatment strategy for TBI. The modulus of these materials can be tuned, and their degradation can also be tuned via degradable peptide links or through the development of a composite hydrogel system with another material component with tunable degradation. The comprehensive characterization of material degradation, and possibly the addition of controllable degradation mechanisms are necessary for the development of a successful, potentially resorbable biomaterial TBI treatment. Finally, we briefly discussed potential mechanisms to impart temporal delivery of therapeutic agents to resolve the persistent astrocyte scar to maximize their reparative functions after injury. Enzymatic-trigger or
diffusive release, as well as long-term immobilization of therapeutic factors are potential delivery options, however, further research into the ideal factors is required.

To enhance the success of tissue engineering constructs for TBI, we suggest that the investigation of composite material systems is the most promising avenue of investigation. Combining well-characterised hydrogel systems with tunable moduli and novel self-assembled peptide hydrogels, as well as temporal delivery mechanisms will satisfy the tissue engineering criteria presented here for TBI treatment. By designing biomaterial systems that can satisfy the criteria for an in vivo treatment strategy, their in vitro investigation will be more valuable than current in vitro work conducted in 2D environments that fail to replicate ECM. During the development of a biomaterial system to treat TBI, the impact on astrocyte inflammation should be continually tested in vitro parallel to material development. We and others have previously discussed the importance of developing 3D cell culture environments to study neuroinflammation [26, 82, 83], and similarly, we need to develop biomaterial systems that can be an effective treatment in vivo, and which we can test first in vitro. It would be of value to combine these biomaterial systems with bioreactors to better mimic the dynamic in vivo environment and thus improve the accuracy of the in vitro models used [41]. Paired with this in vitro investigation is the subsequent analysis of astrocyte response, particularly since the in vitro environment is significantly more simplified than its in vivo counterpart.

We urge the field to develop characterization techniques that can be used in vitro and in vivo, but also ones that reflect an understanding of the complexity of the astrocyte inflammatory response. Although characterizing inflammation in the body by the presence and number of astrocytes can give an indication of the existence and possibly
the severity of inflammation, it is unhelpfully simplistic in understanding the implications and potential therapeutic targets of such inflammation. To fully understand inflammation, cells involved should be characterized by their temporal and spatial phenotypes, not just their numbers or presence, as previously expressed in [84]. Adding to the complexity of understanding astrocytes and their role in inflammation, the possibility of different sub-types of reactive astrocytes needs to be recognised. Molecular and genomic analysis can provide insight into the different phenotypes and subsequent sub-types of astrocytes after injury. For example, genomic analysis of reactive astrocytes in either a stroke (middle cerebral artery occlusion, MCAO) or systemic endotoxin injection (LPS) model of neuroinflammation revealed that the gene expression profile is dependent on the injury stimuli [85]. Although a core set of genes were expressed amongst reactive astrocytes in both models (GFAP, vimentin, Lcn2, Serpina3n), nestin was inducted sevenfold in the MCAO, whilst no induction was observed in the LPS model. The expression of nestin was restricted to astrocytes near the lesion core, whilst reactive astrocytes expressing GFAP were found in more distal regions, as well as near the lesion core. Differences in gene expression and the localisation of well-established markers for reactive astrocytes clearly demonstrates the heterogeneity of reactive astrocytes [85]. Thus, there is an imperative for the scientific community to thoroughly characterize the phenotype of astrocytes after injury, using genomic and molecular analytical tools, as well as the incorporation of morphological and scar dimension analysis. The comprehensive characterization of reactive astrocyte phenotypes and sub-types can elucidate potential therapeutic targets to control the astrocyte response after injury and promote functional regeneration.
Developing biomaterial systems that can address inflammation after TBI provides the necessary foundation on which regenerative strategies can be developed to revolutionise functional recovery outcomes after TBI. Although there are many avenues of investigation required before an effective TBI treatment can be employed in the clinic, using tissue engineering is an exciting approach, and will require the productive and innovative collaboration between biologists, tissue engineers, and clinicians to prove successful.
CHAPTER THREE

Materials and methods

The following chapter provides a detailed description of the materials and experimental methods used throughout this thesis, ordered by the appearance of each research chapter that follows. All material fabrication or synthesis, characterization, as well as the biological work in Section 3.6 was conducted in the Laboratory of Advanced Biomaterials (LAB) at the Australian National University (ANU). *In vitro* and *in vivo* work presented in Section 3.2, 3.3, and 3.5 was conducted at the Florey Institute for Neuroscience and Mental Health, and the scar analysis and astrocyte reconstruction in Section 3.5.5 was conducted at the Hunter Medical Research Institute. Scanning electron microscopy and confocal microscopy from Section 3.1 and 3.5 were conducted at the Centre of Advanced Microscopy, and the fluorescent microscopy from Section 3.6 was conducted at the Imaging & Cytometry Facility in the John Curtin School of Medical Research, both at the ANU.
3.1 PCL scaffold fabrication, galactose functionalisation, and characterisation

3.1.1 2D PCL preparation

2D PCL (Sigma-Aldrich, St Louis, MO, USA, molecular weight = 70,000 – 90,000) substrate was compression molded at 80 °C and quenched in an ice bath.

3.1.2 3D nanofibre scaffold preparation

Poly(ε-caprolactone) (PCL) was obtained from Sigma-Aldrich (St Louis, MO, USA, molecular weight = 70,000 – 90,000). Polymer solutions of 13% (w/v) were made with 3:1 chloroform (Chem Supply Pty Ltd, Australia) and dichloromethane (Sigma Aldrich, MO, USA), magnetically stirred at room temperature for two hours.

The 13% PCL solution was placed in a plastic syringe with an 18 gauge needle for electrospinning at 16 kV and electrospun at 1.25 mL/hr, with a working distance of 10 cm from the collector (a mandrel rotating at 5 ms⁻¹). The collected scaffold was then stored in a desiccator.

3.1.3 Conjugation of poly-L-lysine and lactobionic acid

50 mg of poly-L-lysine hydrobromide (PLL, Sigma Aldrich, MO, USA, molecular weight = 30,000 - 70,000), 40 mg of lactobionic acid (LBA, Tokyo Chemical Industry, Tokyo, Japan) and 3 mL of dimethyl sulphoxide (DMSO, Ajax Finechem, Australia) were combined and stirred on heat (75 °C) for two hours. 40 mL of diethyl ether (Merck, MA, USA) was added and left to stir at 100 °C until the PLL-LBA had precipitated out. 40 mL of de-ionised water was then added and left to stir for 5 minutes. The solution was dialysed in cellulose membranes (MWCO: 12 – 14,000, Spectra/Por, USA) against
water and then lyophilised for four days (Christ Alpha 1-2 LDplus Freeze Dryer, John Morris). This conjugated polymer is denoted as “PLL-LBA”.

3.1.4 Nuclear Magnetic Resonance

10 mg of PLL-LBA was dissolved in 0.6 mL deuterium oxide (D₂O, Sigma Aldrich, MO, USA) and transferred into a NMR tube (NE-UL5-8”, New Era Enterprises, USA), which was placed in the autosampler of the NMR machine (Varian MR-400).

3.1.5 Layer-by-layer functionalisation (2D scaffolds)

2D PCL samples were submerged in 70% ethanol (Merck,, Australia) for 10 minutes followed by three 5 minute phosphate-buffered saline (PBS, pH 7.4) washes. Samples were soaked in 20 mg/mL polyethyleneimine (PEI, 50% in H₂O; Sigma Aldrich, USA) in PBS buffer (pH 7.4) for two hours, then rinsed in PBS. Samples were then soaked in 5 mg/mL heparin sodium salt from porcine intestinal mucosa (Hep, Grade I-A, ≥180 USP units/mg Sigma Aldrich, USA) in PBS for 15 minutes, followed by a 10 minute PBS buffer wash. They were then soaked in 1 mg/mL poly-L-lysine hydrobromide (PLL; Sigma Aldrich, USA; molecular weight = 30,000 - 70,000), or PLL-LBA, in PBS for 15 minutes, followed by a 10 minute PBS wash. The deposition cycle was repeated for 5 bilayers. Samples were crosslinked with 2% 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Thermo Scientific, Japan) in PBS overnight, followed by three 10 minute PBS wash. Excess liquid was removed from the samples using kimwipes, dried overnight and subsequently stored in a desiccator.
3.1.6 Layer-by-layer functionalisation 3D scaffolds

6.5 × 2.5 cm² pieces of PCL nanofibre scaffolds were submerged in 70% ethanol (Merck, Australia) for 10 minutes, followed by three 5 minute PBS washes. Samples were soaked in 20 mg/mL polyethylenimine (PEI, 50% in H₂O; Sigma Aldrich, USA) in PBS buffer (pH 7.4) for two hours, then rinsed in PBS. The samples were then secured to glass slides that were inserted into the StratoSequence 6 (nanoStrata Inc., USA) sample holder. The samples were then submerged in 5 mg/mL heparin (heparin sodium salt from porcine intestinal mucosa, Grade I-A, ≥180 USP units/mg; Sigma Aldrich, USA) in PBS for 15 minutes, followed by three PBS washes (5, 10 and 5 minutes). Samples were then submerged in 1 mg/mL poly-L-lysine hydrobromide (PLL; Sigma Aldrich, USA; molecular weight = 30,000 - 70,000), or PLL-LBA, in PBS for 15 minutes, followed by three PBS washes (5, 10 and 5 minutes). This cycle was repeated for 4 bilayers. Samples were crosslinked with 2% 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Thermo Scientific, Japan) in PBS overnight, followed by three 10 minute PBS wash. Excess liquid was removed from the samples using kimwipes, samples were then hung on clips in an enclosed chamber overnight to dry, then stored in a desiccator.

Samples with heparin and poly-L-lysine layers are denoted are “PCL- Hep+PLL”, and samples with heparin and PLL-LBA are denoted as “PCL- Hep+PLL-LBA”.

3.1.7 Scanning electron microscopy

Samples were sputter coated with platinum at 20 mA for one minute. All scanning electron microscopy (SEM) images were taken under 3 kV with a working distance of 3.5 mm on a Zeiss UltraPlus FESEM, using a 20 µm aperture. The average diameters of
the fibres were determined using Image J (NIH) to measure a total of 20 fibres across four different samples. Results are expressed as mean ± SEM.

3.1.8 Contact angle measurements

Contact angle measurements were performed with a KSV CAM200 contact angle goniometer (KSV Instruments) at room temperature, using the sessile drop method with deionised water. A 1.2 mm diameter needle was used to introduce a 6 μL water drop onto the sample surface by gravity. The measurements were taken over three replicate samples and averaged.

3.1.9 Quartz crystal microbalance and dissipation (QCM-D)

Gold coated quartz crystals were cleaned by UV/ozone treatment for 20 minutes followed by washing in piranha solution consisting of deionised water: ammonia: H2O2 (5:1:1) before being rinsed thoroughly in deionised water. The crystals were then activated in 5 mM methyl 3-mercaptopropionate (Sigma-Aldrich, St Louis, MO, USA) in absolute ethanol for 8 hours in preparation for coating with a thick coating of PCL. The alkane-gold crystal was then attached to a house built spin-coater with a drop of 0.05 g/mL PCL and tetrachloroethane (Sigma-Aldrich, St Louis, MO, USA) solution being placed on the centre of the crystal, and spun for 1 minutes at 4500 rpm to obtain a PCL thin film on the crystal. SEM was used to optimise the process and confirm complete coating with the absence of pin holes in the thin film (data note shown). The LbL deposition process was then conducted and monitored using QCM-D (Q-Sense E4, Sweden). Initially 0.5 X PBS buffer (pH 7.4) was pumped through the analysis chamber (30 minutes) for stabilisation. 500 μL of each polyelectrolytes and the subsequent PBS washes between electrolyte deposition, were periodically (times replication the LbL
conditions described above) injected into the chamber over 54 seconds. Recordings were taken at 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> overtone (15, 25 and 35 MHz respectively). Modelling of frequency differences (Q-Sense software), which qualitatively indicates the mass deposited, was conducted using the viscoelastic model.

3.2 Primary astrocyte culture on PCL nanofibre scaffolds

3.2.1 Animals and chemicals

C57/BL6 mice were obtained from the Florey Institute of Neuroscience and Mental Health, Australia. All experiments received ethical approval from the Florey Neuroscience Institutes Animal Experimentation Ethics Committee (Ethics Approval Number 07-061). Experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code for the Care and Use of Animals for Experimental Purposes in Australia. All chemicals used to prepare biological solutions, buffers and media were purchased from BDH Laboratories (Australia).

3.2.2 Primary culture

Secondary astrocytic cultures were established from forebrain of postnatal d1.5 mice as previously described in [86]. Briefly, forebrains were dissected in ice-cold solution (HBSS, Hanks balanced salt solution: 137 mM NaCl, 5.37 mM KCl, 4.1 mM NaHCO<sub>3</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 0.13 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM HEPES, 1 mM sodium pyruvate, 13 mM D(+)-glucose, 0.01 g/L phenol red), containing 3 mg/ml bovine serum albumin (BSA) and 1.2 mM MgSO<sub>4</sub>, pH7.4). Cells were dissociated, centrifuged, and the pellet resuspended in astrocytic
medium (AM: DMEM, Dulbecco’s modified eagle medium, 10% FBS, 100 U/ml penicillin/streptomycin, 0.25% (v/v) Fungizone™), preheated to 36.5°C at a volume of 5 ml per brain and plated at 10 ml per 75 cm² flask. Cells were maintained in a humidified incubator supplied with 5% CO₂ at 36.5°C and complete medium changes were carried out twice weekly.

After 10 days in vitro (div), when a confluent layer had formed, the cells were shaken overnight (180 rpm) and rinsed in fresh medium to remove non-astrocytic cells. Astrocytes were subsequently detached using 5 mM EDTA (10 min at 37°C) and seeded in 96-well plates at 8 x 10³ cells/well with the PCL samples (see below; PCL, PCL-Hep+PLL, PCL-Hep+PLL-LBA) in 96 well plates, or in 24-well plates with coverslips (2D sample, 2 x 10⁴ cells/well). Plates were incubated overnight and a full medium change was performed 24 hours later to remove adherent cells. AM was subsequently changed every 3 div, with each assay was performed on subcultured astrocytes at 14 div and 22 div.

Scaffolds were washed in 80% ethanol for 10 minutes, followed by three PBS washes. Glass inserts (1 mm x 7 mm x 4 mm; to ensure scaffolds did not float) were washed in 80% ethanol and left to dry in a sterile biosafety cabinet for 30 minutes. Scaffolds were then placed in the well plates, with the inserts on top. 5 minutes UV exposure was utilised as the final sterilisation before cell seeding.

Immunocytochemistry for GFAP, F-actin and G-actin has been described previously [86], as has the procedure for AHNAK [43].
3.2.3 Imaging and analysis of immunocytochemical staining

Photomicrographs of astrocytes in each culture environment were captured to use for analysis. Images were taken at 100X and 200X using an Olympus Camedia C-5050 Zoom digital camera through an Olympus IX71 inverted microscope. Two independent experiments produced at least 12 images from triplicate wells for each protein. Background threshold was set and the area (integrated density of fluorescence) above this was measured using ImageJ (NIH). Image data was analysed using a repeated measures two-way ANOVA and the appropriate Bonferroni post-hoc tests on Prism (v.6.0; GraphPad, USA), and comparisons of the area above threshold were made. Values are expressed as mean ± SEM.

3.3 In vivo implantation of PCL nanofibre scaffolds

3.3.1 Animals

C57/BL6 mice were obtained from the Florey Institute of Neuroscience and Mental Health, Australia. All experiments received ethical approval from the Florey Neuroscience Institutes Animal Experimentation Ethics Committee (Ethics Approval Number 12-051). Experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code for the Care and Use of Animals for Experimental Purposes in Australia.

3.3.2 Scaffold insertion

Scaffold implantation follows a similar procedure as reported in [34]. Briefly, C57 BL/6 mice (male and female) were anaesthetised with 1-2% Isoflurane and placed in a
stereotaxic frame whereby a small hole was drilled into the skull (from bregma: AP +0.5 mm, L -2.0 mm). Three types of scaffolds were tested; control PCL, PCL-Hep+PLL, and PCL- Hep+PLL-LBA, as well as a control stab. Scaffolds were sterilised with 80% ethanol and rolled into small capsule sizes and inserted into a 21G guide needle. The guide needle was attached to a Hamilton needle which was lowered through the hole in the skull 3.0 mm deep. The guide needle was then raised and the plunger with the scaffold was descended another 0.5 mm deep to ensure scaffold insertion into the caudate putamen.

Each type of scaffold was inserted into six mice; three of which were killed at 7 days post-surgery, and the other three at 21 days post-surgery. Two mice also received a needle injury without the scaffold, representing a stab wound, and one killed at 7 days and one killed at 21 days. In total 20 mice were used.

3.3.3 Tissue preparation

Mice were perfused 7 days and 21 days post-surgery with warmed (37 °C) 0.1 M PBS, followed by 35 mL of chilled 4% PFA (Sigma Aldrich, Australia) in 0.1 M PBS and 0.2% picric acid (4°C; pH 7.4). The brains were removed and post-fixed for one hour, then left over two nights at 4°C in a 30% sucrose PBS solution. The brain tissues were then frozen and cut serially with a cryostat. The striatum was cut at 20 μm thick in a 1:10 series on to slides double coated with 0.1% chrome alum (Ajax Chemicals, Australia) and 1% gelatine (Sigma Aldrich, Australia). Slides were individually heated by a glass hot rod to ensure tissue adhesion; and no loss of scaffold material throughout staining process later.
3.3.4 Immunohistochemistry

Anti-GFAP immunohistochemistry was performed by incubating one series of sections through the striatum, at 1:500 rabbit x GFAP antibody (Dako, Australia) over two nights at room temperature (in PBS, 0.3% Triton X-100 and 1.0% normal donkey serum). After several washes and 6% goat serum block for 15 minutes, slides were incubated for a further 3 hours at room temperature in secondary antibody donkey, anti-rabbit Alexa Fluor 594 at 1:100, (Millipore, Australia). Sections were coverslipped using Dako fluorescence mounting medium (Dako, Australia).

NeuN immunohistochemistry was performed by incubating one series of sections through the striatum, at 1:600 mouse x NeuN antibody (Millipore, Australia) over two nights at room temperature (in PBS, 0.3% Triton X-100 and 1.0% normal goat serum). After several washes and 6% goat serum block for 15 minutes, slides were incubated for a further 3 hours at room temperature in secondary antibody goat anti-mouse Alexa Fluor 488 at 1:100, (Millipore, Australia). Sections were coverslipped using Dako fluorescence mounting medium (Dako, Australia).

3.3.5 Cell counts

GFAP labelled astrocytes were counted ‘close’ to scaffold (at the scaffold-parenchyma interface) and ‘far’ away from scaffold (600 µm away from scaffold). The Steroinvestigator program (Micro-BrightField, VT, USA) was used to derive counts at pre-determined fractionator intervals of x=80 µm, y=80 µm, and a counting frame of x=70 µm, y=70 µm. Counts were made using the 60x oil lens. Total markers counted were then divided by the total area (µm²) to achieve a final density of cells/astrocytes per area (µm²). The same method was used to count the NeuN cell numbers.
3.4 Fmoc-DIKAVAV synthesis and characterisation

3.4.1 Solid-Phase Peptide Synthesis

Fmoc-DIKAVAV was synthesized using solid phase peptide synthesis, at a molar scale of 0.4 mmol in a custom built reaction vessel. Fmoc protected amino acids, \( O \)-benzotriazole-\( N,N,N',N' \)-tetramethyl-uronium-hexa-fluoro-phosphate (HBtU) and hydroxybenzotriazole (HOBT), and Wang based resins were purchased from GL Biochem (China), and all other chemicals were bought from Sigma Aldrich. Dimethylformamide (DMF) was dried for a minimum of 2 hours with 4 A molecular sieves before use.

Amino acids were added to the resin amino acid through stepwise deprotection of the N-terminal Fmoc protecting group using 20 \( \text{v/v}\% \) piperidine in DMF, and a subsequent coupling of the next Fmoc-amino acid in the sequence in a solution of HBtU, HOBT, and \( N,N \)-diisopropylethylamine (DIPEA) in DMF. Each deprotection/coupling step was verified using a Kaiser test to detect free amines. Deprotection and coupling is repeated until the DIKVAV sequence was synthesized, with the final Fmoc group not removed. The final resin and Fmoc-protected peptide was washed with ethanol and dried under a constant vacuum for two days. A solution of trifluoroacetic acid (TFA), 2.5\% triethylsilane (TES) and 2.5\% deionised water was prepared to cleave the resin-peptide bond. The resin protected peptide was left to stand in the cleavage solution for two hours, and following cleavage, the peptide solution was filtered through glass wool. Excess TFA was evaporated using nitrogen until 5 mL peptide solution remained. The peptide solution was precipitated in a solution of 10 mM hydrochloric acid (HCl),
washed twice in HCl and then five times in cold diethyl ether. The peptide was collected and dried under constant vacuum for seven days, then ground into a fine powder.

3.4.2 Fmoc-SAP gel formation

10 or 5 mg of Fmoc-DIKVAV (and 2.5 or 5 mg/mL fucoidan if applicable) was dissolved in 400 μL of deionized water, then 30-40 μL of 0.5 M sodium hydroxide (NaOH, Bacto Australia). 0.1 M hydrochloric acid (HCl, Merck) was then added dropwise to slowly reduce the pH, with continuous vortexing, until physiological pH was reached, measured using a microprobe pH meter. PBS was then added to make the gel up to 20 mg/mL, and the gel was exposed to ultraviolet (UV) light for 20 minutes.

Note Fmoc-DIKVAV (95-99% desalted) used in Chapter 6 was manufactured by Pepmic, China. The gel formation procedure was the same as above.

3.4.3 Fourier Transform Infra-Red Spectroscopy

Fourier transform infrared spectroscopy was performed using Alpha Platinum Attenuated Total Reflectance FTIR (Bruker Optics). 30 μL of Fmoc-SAP gel was placed on the single reflection diamond and spread over the crystal using the pressure applicator. A baseline absorbance of water was subtracted from the total absorbance of the Fmoc-SAPs.

3.4.4 Rheology

A Kinexus Pro+ Rheometer (Malvern) was used to determine the viscoelastic properties of the Fmoc-SAP gels, with a 20 mm smooth flat plate with a solvent trap was used. The Fmoc-SAP gel was placed on the plate with a gap size of 0.2 mm. A shear strain of 0.4% was used with a frequency of 0.1 – 100 Hz.
3.4.5 Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on a HITACHI HA7100 TEM at 100 kV. Formvar coated copper grids were glow discharged for 30 seconds at 15 mA. Negative stains with 0.75% uranyl formate (UF) were used to image the Fmoc-SAP gels, with the procedure fully described in [35].

3.5 In vivo implantation of Fmoc-DIKVAV/fucoidan and characterisation

3.5.1 Stab injury and SAP implantation

C57 BL/6 mice (male and female) were anaesthetised with 1-2% Isoflurane and placed in a stereotaxic frame whereby a small hole was drilled into the skull. A 21G needle was attached to a Hamilton needle which was lowered into the RHS striatum (co-ordinates AP+0.5 mm, L-2.0 mm, deep -3.0 mm from bregma) to create the stab injury.

Hydrogels were exposed to UV for 20 minutes prior to implantation, and were drawn up into a Hamilton syringe, to which a fine glass capillary was attached. 3 μL of SAP (Fmoc-DIKVAV, Fmoc-DIKVAV + 2 mg/mL fucoidan, or, Fmoc-DIKVAV + 5 mg/mL fucoidan) was injected into the stab injury, in increments of 0.5 μL/0.5 mm (moving upwards from AP+0.5 mm, L-2.0 mm, deep -3.0 mm from bregma).

Each group was n=4, and there was an additional group which received only a stab injury (stab control).
3.5.2 Tissue preparation

7 days and 21 days post-surgery mice were perfused with warmed (37 °C) 0.1 M PBS, followed by 35 mL of chilled 4% PFA (Sigma Aldrich, Australia) in 0.1 M PBS and 0.2% picric acid (4°C; pH 7.4). The brains were removed and post-fixed for one hour, left for two nights at 4°C in a 30% sucrose PBS solution. They were then frozen and cut serially with a cryostat at 20 μm thick in a 1:10 series on to slides double coated with 0.1% chrome alum (Ajax Chemicals, Australia) and 1% gelatine (Sigma Aldrich, Australia). Slides were individually heated by a glass hot rod to ensure tissue adhesion; and no loss of scaffold material throughout staining process later.

3.5.3 Immunohistochemistry

Immunohistochemistry was performed by incubating tissue slides with either anti-GFAP (rabbit anti-glial fibrillary acidic protein, 1:500, Dako, Australia) and anti-OX42 (mouse anti-CD11b (clone OX-42), 1:100, Bio-Rad, Australia) or, anti-GFAP (rabbit anti-GFAP, 1:500, Dako, Australia) and anti-SMI32 (mouse anti-neurofilament H non-phosphorylated, 1:300, Convance, Australia) overnight at room temperature (in PBS, 0.3% Triton X-100 and 1.0% normal donkey serum). After two five minute PBS washes, and a 6% goat serum block for 20 minutes, slides were incubated for a further four hours at room temperature in secondary antibodies (donkey anti-rabbit Alexa Fluor 594 and donkey anti-mouse Alexa-Fluor 488, Jackson Immuno Research Laboratories, USA, both at 1:100). The secondary antibodies were removed after four hours and replaced with a hoechst solutions (Hoechst 33342, 1:1000, Invitrogen, Australia) for five minutes, followed by three five minute PBS washes. Slides were then washed in de-ionised water,
excess water removed and coverslipped using fluorescent mounting medium (Dako, Australia).

3.5.4 Cell counts

GFAP labelled astrocytes were counted ‘close’ to the SAP implant (at the SAP-parenchyma interface) and ‘far’ away from scaffold (600 µm away from scaffold). The Steroinvestigator program (Micro-BrightField, VT, USA) was used to derive counts at pre-determined fractionator intervals of x=80 µm, y=80 µm, and a counting frame of x=70 µm, y=70 µm, with the counting area 70 µm (x) by 1000 µm (y). Counts were made using the 63X oil lens. Total markers counted were then divided by the total area (µm²) to achieve a final density of cells per area (µm²).

3.5.5 Analysis of astrocyte scar and reconstruction

The hemisphere area, lesion/implant area, primary astrocyte scar and, local astrocytic disturbance were analysed using Image J (NIH). Images were taken at 25X on Leica SP8, and cells labelled for GFAP. The primary astrocytic scar and local astrocytic disturbance distances were measured perpendicular to the needle tract.

Glia Reconstruct was used to analyse individual astrocytes labelled with GFAP, as previously described in [87], with some alterations. Astrocytes within the primary scar could not be reconstructed due to their dense, fibrous network. Therefore, astrocytes were analysed at a minimum distance of 600 µm from the edge of the lesion or implant to enable accurate reconstructions. Maximum intensity projections of z-stacks (7-10 µm) at 25X were converted to black and white and astrocytes were digitally reconstructed using algorithms executed in Matlab (v2013b, The MathsWorks, Inc.). Size filtering was
utilised to discern cellular bodies, which revealed information such as cell and process area, and, cell perimeter. Specifically, immunolabeled astrocytes (GFAP⁺) were segmented from the background using the multi-level Otsu’s thresholding method which calculates thresholds to minimize the interclass pixel intensity variance between the various classes. The classes used in this analysis were soma, processes, and background. Morphological parameters including cell area and perimeter were automatically calculated using the Matlab image-processing toolbox function “regionprops”. The total number of pixels is given as the area, and the perimeter is length of the boundary of the region. For each animal (n=4 in each experimental group), three separate images were chosen for reconstruction.

3.6 Primary astrocyte culture with Fmoc-DIKVAV/fucoidan

3.6.1 Gel formation

5mg Fmoc-DIKVAV (99% desalted, Pepmic, China) (and 2 or 5 mg/mL fucoidan if applicable) was dissolved in 400 μL deionized water, to which 30 μL 0.5 M sodium hydroxide (Bacto, Australia) was added. 95 μL 0.1 M hydrochloric acid (Merck, Australia) was then added dropwise to slowly reduce the pH with continuous vortexing. Once physiological pH was reached (measured with a microprobe pH meter), hydrogels were exposed to UV for two hours, after which the final volume was reached using Dulbecco’s Modified Eagle’s Medium High Glucose (DMEM, HyClone, United Kingdom) ready for immediate use in culture.
3.6.2 Primary astrocyte cell culture

Primary astrocyte cell culture was conducted as described in [88]. Briefly, postnatal day 1.5-2 Swiss mice pups were sacrificed and their forebrains were dissected in cold DMEM High Glucose (HyClone). Tissue was dissociated and centrifuged for 10 minutes at 1000 rpm, and resuspended in CDMEM 10:10:1 (complete DMEM, 10% fetal bovine serum (Gibco), 10% horse serum (Gibco), 1% penicillin/streptomycin (Hyclone) at 37°C, with 15 mL per forebrain. Cells were plated on poly-d-lysine (PDL) coated T75 flasks, with 15mL cell suspension per flask. Cells were maintained at 37°C and 5% CO2 for two weeks.

At 14 div, with a confluent cell layer, media was changed and left to equilibrate in the incubator for 2-3 hours, after which the flask lid was sealed with parafilm and the flask was placed on an orbital shaker (230 rpm at 37°C) overnight. Media was then removed, washed once with PBS (1X) to remove non-astrocytic cells. Astrocytes were detached from the flask using 3 mL trypsin (4 minutes at 37°C, HyClone), added to 15 mL CDMEM 5:5:1 (5% fetal bovine serum (Gibco), 5% horse serum (Gibco), 1% penicillin/streptomycin (Hyclone) and centrifuged for 10 minutes at 1000 rpm. The resultant cell pellet was resuspended in 3 mL CDMEM 5:5:1 and cell counts were performed with 10 μL cell suspension, 10 μL PBS, and 80 μL trypan blue.

Cells were seeded as per details in the following sections.

3.6.3 LPS and IL-1α stimulation and MTT assays

Cells were seeded at 3,000 cells per well in a 96-well plate and were maintained for two weeks prior to toxin/cytokine exposure with media changes every 5-7 days.
Lipopolysaccharide (LPS, Sigma Aldrich) solutions were prepared using a serial dilution method to achieve concentrations of 25, 50, 100, 150, and 200 ng/mL in CDMEM 5:5:1. Cell media was replaced with 100 μL of the relevant LPS solutions and placed in the incubator for 4 hours. After LPS exposure, 100 μL of either CDMEM 5:5:1, Fmoc-DIKVAV, or Fmoc-DIKVAV + 2 mg/mL solutions were added to the wells. Hydrogel solutions were prepared at 1:6 with CDMEM 5:5:1 (to yield a mixture of 20 μL hydrogel and 100 μL for each well), to avoid a hydrogel layer forming over the cells and interfering with the subsequent assay. Plates were incubated for a further 3 days before the MTT assay.

Interleukin-1α (IL-1α, R&D Systems) solutions were prepared using a serial dilution method to achieve concentrations of 25, 50, 100, 150, and 200 ng/mL in CDMEM 5:5:1. Cell media was replaced with 100 μL of the relevant IL-1α solutions and placed in the incubator for 3 days. After IL-1α exposure, 100 μL of either CDMEM 5:5:1, Fmoc-DIKVAV, or Fmoc-DIKVAV + 2 mg/mL solutions were added to the wells (formulation as described above). Plates were incubated for a further 3 days before the MTT assay.

A Vybrant MTT cell proliferation assay (Life Technologies) was performed as per the manufacturer's instructions. Briefly, a mixture of 100 μL CDMEM 5:5:1 and 10 μL MTT dye was added to each well (with 2 additional negative control wells), covered with foil and incubated for 4 hours, after which the solution was replaced with 100 μL sterile dimethyl sulfoxide (DMSO, Sigma Aldrich) and incubated for 10 minutes, covered in foil. The absorbance was measured using Tecan Infinite M200 Pro plate
reader and Tecan iControl at 570 nm, with 60 seconds orbital shaking prior to reading, at 9-16 reads per well.

Values were averaged and normalized, and plotted as mean ± SEM.

3.6.4 Gel exposure experiments

To investigate the effect of Fmoc-DIKVAV on cell morphology and organization when on top of astrocytes, cells were seeded at 10,000 cells/well in 48 well plates and maintained for 2-3 weeks prior to hydrogel exposure to ensure there was a confluent layer to observe changes. Hydrogels were prepared as described above, and diluted at 10, 20, 40, 60, and 80% in CDME 5:5:1. Media was removed from cells, with 150-200 μL hydrogel dilutions added to each well.

Cells were maintained for 7 days with the gel (“Gel on”), then removed for 7 days and replaced with media (“Gel on-off”), and then replaced again with hydrogel (“Gel on-off-on”). For the “Gel on” group, hydrogel solutions were replenished every 7 days.

3.6.5 Three-dimensional cultures

To grow astrocytes in a three-dimensional (3D), Fmoc-DIKVAV hydrogels were plated on top of PDL-coated TCP. 200-400 μL of hydrogel was added to each well, and left under UV in a Biosafety Cabinet II for 2 hours, with the well plate lids off, and then incubated overnight to equilibrate. Cells were then seeded on top of the hydrogels the next day (400,000 cells/well for a 24 well plate), and maintained for 14 or 24 days.
3.6.6 Immunocytochemistry

Upon the completion of the culture period, cells were fixed with 4% paraformaldehyde for 15 minutes at 37°C, followed by three PBS (1X) washes. Plates were then sealed with parafilm and stored in the fridge until immunocytochemistry.

Cells were incubated with primary antibodies for GFAP (1:500, abcam), and AQP-4 (1:200, abcam) in 5% donkey serum (Merck) and 0.3% Triton-X (Sigma Aldrich) at room temperature overnight, followed by 3x 10 minute PBS washes and a 30 minute 10% donkey block (Merck). Secondary antibodies with Alexa Fluor-488 or -594 (1:250, abcam) in 2% donkey serum (Merck) and 0.3% Triton-X (Sigma Aldrich) were then added for 1.5 hours, followed by a 5 minute Hoechst (1:1000, Life Technologies) incubation. Where applicable, F-actin (1:250, Life Technologies) incubation was performed after the secondary antibody attachment for 45 minutes at 4°C. Cells were then visualised with an Olympus IX 71 fluorescence microscope.

3.6.7 Network analysis

The resultant astrocyte networks were analysed using ImageJ (NIH, v1.48). The total area of network was measured by F-actin+ staining, with any pore areas within the network being subtracted. Cell density (cells/μm²) was calculated by:

\[
\frac{\text{number of cells in network}}{\text{F-actin + area of network}}
\]

Astrocyte network length was calculated by measuring the length of all connecting processes, which met at network ‘nodes’.

80
3.6.8 Stab injury and hydrogel implantation

C57 BL/6 mice (male and female) were anaesthetised with 1-2% Isoflurane. Once in a stereotaxic frame, a small hold was drilled into the skull, followed by the lowering of a 21G needle attached to a Hamilton needle into the RHS striatum (co-ordinates AP+0.5 mm, L-2.0 mm, deep -3.0 mm from bregma) to create the stab injury. Hydrogels were exposed to UV for 20 minutes prior to implantation, and were drawn up into a Hamilton syringe, which was attached to a fine glass capillary. 3 μL of hydrogel was injected into the stab injury, in increments of 0.5 μL/0.5 mm, moving upwards.

3.6.9 Tissue preparation

Mice were perfused 21 days post-implantation with warmed (37°C) 0.1 M PBS, followed by 35 mL of chilled 4% PFA (Sigma Aldrich) in 0.1 M PBS and 0.2% picric acid (4°C; pH 7.4). Brains were post-fixed for one hour, left for two nights at 4°C in a 30% sucrose PBS solution. Frozen brains were cryosectioned at 20 μm thick in a 1:10 series on to slides double coated with 0.1% chrome alum (Ajax Chemicals) and 1% gelatine (Sigma Aldrich).

3.6.10 Immunohistochemistry

Tissue slides were incubated with anti-GFAP (rabbit anti-glial fibrillary acidic protein, 1:500, Dako) overnight at room temperature (in PBS, 0.3% Triton X-100 and 1.0% normal donkey serum). After two five minute PBS washes, and a 6% goat serum block for 20 minutes, slides were incubated for a further four hours at room temperature in the secondary antibody donkey anti-rabbit Alexa Fluor 594 (Jackson Immuno Research Laboratories, 1:100). Secondary antibody attachment was followed by a hoechst solution (Hoechst 33342, 1:1000, Invitrogen) for five minutes, then three five minute
PBS washes. Slides were then washed in de-ionised water, excess water removed and coverslipped using fluorescent mounting medium (Dako). Tissue was then visualised using a Zeiss Confocal LSM 780.

3.6.11 Gel incubation

400 μL/well of Fmoc-DIKVAV hydrogel was transferred into 48-well plates and 200 μL of PBS was added on top of the hydrogel. Hydrogels were incubated at 37°C and 5% CO2 incubation for 21 or 60 days and PBS was replenished every 3-4 days.

3.6.12 Rheological testing

The viscoelastic properties of the control and incubated hydrogels were determined using a Kinexus Pro+ Rheometer (Malvern), with a 20 mm smooth flat plate with a solvent trap. The PBS supernatant was removed from each well, after which the Fmoc-DIKVAV gel was placed on the plate and left at room temperature for 2 minutes. A 0.2 mm loading gap was used with a shear strain of 0.4% and a frequency of 0.1 – 100 Hz.

3.7 Data analysis

All values presented are mean ± SEM. Data were subjected to unpaired student t-tests (Chapter 4), and 2-way ANOVA or unpaired t-tests (Chapter 5 and 7) using Prism v6.0 (GraphPad, USA).
CHAPTER FOUR

Galactose-functionalised PCL nanofibre scaffolds attenuate inflammatory action of astrocytes

\textit{in vitro} and \textit{in vivo}

This chapter describes the novel synthesis of the galactose moiety-presenting PLL polymer, PLL-LBA, and the subsequent functionalization of polymer nanofibres using the layer-by-layer method. \textit{In vitro} and \textit{in vivo} investigation of the astrocyte response to the nanofibre scaffolds is presented. This work was concurrent to the investigation of PCL nanofibre scaffolds as 3D cell culture environments for astrocytes (Appendices A and B), and as an inflammatory mediator with tethered interleukin-10 for peripheral nerve injury (Appendix C). Although nanofibre scaffolds are limited for \textit{in vivo} use as highlighted in Chapter 2, they are suitable for \textit{in vitro} investigation, and this work provides a foundation upon which research with nanofibrous hydrogels can extend (see Chapter 5).


**Publication relevant to this chapter:**

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*Please see Appendix A for published article.*

**Abstract**

Astrocytes represent an attractive therapeutic target for the treatment of traumatic brain injury in the glial scar, which inhibits functional repair and recovery if persistent. Many biomaterial systems have been investigated for neural tissue engineering applications, including electrospun nanofibres, which are a favourable biomaterial as they can mimic the fibrous architecture of the extracellular matrix, and are conveniently modified to present biologically relevant cues to aid in regeneration. Here, we synthesised a novel galactose-presenting polymer, poly(L-lysine)-lactobonic acid (PLL-LBA), for use in layer-by-layer (LbL) functionalisation of poly(ε-caprolactone) (PCL) nanofibres, to covalently attach galactose moieties to the nanofibre scaffold surface. We have assessed the use of this novel biomaterial system in vitro and in vivo, and have shown, for the first time, the ability of galactose to maintain an attenuated inflammatory profile of astrocytes in culture, and to increase the survival of neurons after traumatic injury, as compared to control PCL nanofibres. This study highlights the importance of galactose in controlling the astrocytic response, and provides a promising biomaterial system to deliver the
essential morphological and biological cues to achieve functional repair after traumatic brain injury.
4.1 Introduction

When the brain or spinal cord experiences traumatic injury, an essential inflammatory response is initiated. This response is a highly orchestrated series of events which includes microglial (and macrophage) recruitment to the lesion to digest cellular debris [42, 89, 90] and activation of astrocytes [2]. Although this inflammatory response is an integral part of repair, regeneration and reconstruction of tissue following injury, unchecked it may also compromise recovery as extensive astrogliosis prevents the transition to a regenerative phase. As such, astrocytes and their inflammatory behaviour present a promising therapeutic target for achieving functional regeneration after injury.

Activated astrocytes proliferate in the injury’s penumbra where they hypertrophy and increase the production of intermediate filaments such as glial fibrillary acid protein (GFAP) [10]. This represents a switch from normally active, cytotrophic astrocytes to a defensive, reactive phenotype [2, 3, 25, 27] that is responsible for inhibiting growth by secreting pro-inflammatory molecules such as chondroitin sulfate proteoglycans (CSPGs) [91] and inflammatory cytokines [92] required for the removal of damaged tissue debris. These reactive astrocytes demarcate the area of injury by forming a fibrous mesh (sometimes called a glial scar) that preserves the integrity of the surrounding tissue by preventing secondary degeneration of adjacent spared tissue by pro-inflammatory molecules [9], swelling and impaired nutrient and oxygen delivery. Once tissue integrity is established and debris is removed, there is then a switch by astrocytes to the cytotrophic phenotype leading to repair and reconstruction. While the pro-inflammatory phenotype is essential for the initial stages of repair of damaged CNS tissue, development of treatment strategies that control or minimise the duration of this
inflammatory stage and enhance the transition to a cytotrophic phenotype requires a thorough understanding of phenotypes and behaviours of astrocyte at various stages post-injury.

Astrocyte phenotyping is primarily based on morphology. Active astrocytes have several fine processes that interweave around neuronal cell bodies and their processes, whilst reactive astrocytes are characterised by hypertrophic processes, as well as an increase in glial fibrillary acidic protein (GFAP) expression \[93\]. However molecular markers such as GFAP, galectin-1 (Gal-1), neuroblast differentiation-associated protein (AHNAK), and filamentous actin (F-actin) may also point to astrocyte phenotype \[43\]. GFAP is upregulated in the inflammatory phenotype of astrocytes and we have shown that minor astrocyte processes express AHNAK and co-localise with GFAP to provide a more holistic view of the astrocytic ‘arbour’ \[43\]. Gal-1 is a signalling protein that has been implicated in various inflammatory pathways, and is upregulated in reactive astrocytes (hypertrophic and GFAP-positive) after CNS injury \[94\]. Stress fibre formation and actin cytoskeletal changes are commonly observed in astrocytes after injury, typically in their inflammatory mode \[95\]. These four markers can be used to augment morphology in identifying the astrocyte phenotype.

Current 2D cell culture systems do not properly recapitulate the \textit{in vivo} environment, and hence, may not accurately reflect \textit{in vivo} cell morphology and behaviour \[7, 13, 26\]. Consequently, we established a 3D culture system with electrospun scaffolds to mimic the fibrous structure of the extracellular matrix (ECM), whilst also presenting biologically relevant cues, to study common inflammatory markers in astrocytes. Electrospun nanofibres provide a high surface area-to-volume ratio, high porosity and a
fibril size which are similar to the ECM and are thus attractive materials to use in tissue engineering [8, 41, 96]. More information about the electrospinning process can be found in the following reviews [36, 37, 97]. We have previously shown that primary astrocytes cultured on 3D poly(ε-caprolactone) (PCL) electrospun nanofibre scaffolds infiltrate in 3D through the scaffold, reduce GFAP expression and upregulate the expression of BDNF and the L-glutamate transporter SLC1A2 [7]. Astrocytes cultured on 3D PCL nanofibers in the presence of Rho Kinase (ROCK) inhibitors, develop a cytotoxic phenotype [7, 43]. Astrocytes have also been successfully maintained in biofunctionalised 3D culture systems, with increased L-glutamate uptake when cultured on poly(L-lactic acid) nanofibres coated with fibronectin [98], and reduced cytoskeletal stress when cultured on polyether based polyurethane nanofibres coated with polyornithine and laminin [13]. These studies demonstrate the benefits of biofunctionalising a 3D scaffold to provide the mechanical and biochemical cues necessary to recapitulate the 3D in vivo environment, and thus a desirable culture environment to investigate astrocyte biology.

As PCL nanofiber scaffolds induce a cytotoxic phenotype in astrocytes [7], are biocompatible and biodegradable [8] we functionalised these nanofibres using the layer-by-layer (LbL) method [99], with biologically relevant polyelectrolytes – heparin (anti-inflammatory), poly-L-lysine (cell adhesion) and a novel polymer, poly-L-lysine conjugated with lactobionic acid (PLL-LBA), which presents galactose moieties that are of interest. Xyloglucan (containing galactose moieties) attenuates the inflammatory response after implantation, with astrocyte numbers decreasing to almost homeostatic levels after 60 days [74]. It is also understood that galectins, which are implicated in
inflammation [100], bind to galactose [101], and influence the astrocytic cytoskeleton via the Rho/ROCK pathway (i.e. are anti-inflammatory) enhancing BDNF expression [102].

Here we investigated the impact of functionalised PCL nanofibres on the morphology and hallmark proteins associated with inflammatory astrocytes in vitro, and on neuronal survival in vivo, presenting unique data to demonstrate the potential of such a novel biomaterial system to influence astrocyte behaviour for central nervous system inflammation attenuation.

4.2 Results and Discussion

4.2.1 Conjugation of poly(L-lysine) and lactobionic acid presents galactose moiety

The conjugation mechanism of PLL and LBA allowed the presentation of the galactose moiety of the δ lactone form of LBA (Figure 4-1). The attachment was confirmed using NMR, and the percentage of LBA attachment to PLL was 30-40% (NMR spectra shown in Figure S 4-1). This synthesis procedure is an easy, reproducible method of presenting multiple biologically relevant moieties in one molecule, which can then be used in a multi-faceted system to provide physical and biochemical signals in vitro and in vivo.
PCL nanofibre scaffolds retain a nanofibrous morphology, and scaffold porosity following functionalisation with heparin, PLL and PLL-LBA, whilst enhancing the biochemical profile of PCL nanofibre scaffolds. SEM images of electronspun nanofibres are shown in Figure 4-2, before (A-B) and after (C-F) LbL functionalisation. Nanofibrous morphology is similar in each group, regardless of whether functionalisation was with Hep+PLL (C-D) or Hep+PLL-LBA (E-F). Changes from the observed smooth fibre surface (A-B) to the ‘rippled’ fibre surface after functionalisation (C-F) indicate the presence of the covalently bonded ‘layers’ as a result of the LbL process. This was observed in SEM images of both the Hep+PLL and Hep+PLL-LBA functionalised nanofibres, implying that the inclusion of the LBA moiety on PLL does not affect the functionalising of PCL nanofibres by PLL-LBA, using the LbL method.
Figure 4-2: A-F) SEM images showing A-B) unfunctionalised PCL nanofibres, C-D) PCL nanofibres functionalised with Hep+PLL Lbl, and E-F) PCL nanofibres functionalised with Hep+PLL-LBA. Scale bar A-C, E = 1 μm, D, F = 200 nm. G) Mean fibre diameter of scaffolds before and after Lbl functionalisation. Values are the mean ± SEM of 20 observations. No statistical difference between control groups and corresponding functionalised group.
The conclusion that functionalisation was similar in Hep+PLL and Hep+PLL-LBA groups was also supported by the finding that average fibre diameter did not change following LbL functionalisation (Figure 4-2 G). As functionalised scaffolds maintained the morphology, scaffold porosity and average fibre diameter of control scaffolds, we contend that the presentation of chemical cues due to functionalisation will be the mechanisms for any observed differences in cells cultured on the functionalised scaffolds.

Quartz crystal microbalance and dissipation (QCM-D) and water contact angle were then used to test this conclusion. A small increase was found in the change of dissipation after the first heparin and PLL depositions (Figure 4-3), which is consistent with previous QCM-D finding that used QCM-D to characterise LbL functionalisation [99]. The $\Delta D$ was larger after the second heparin adsorption, and increased further after each layer deposition, regardless of whether PLL or PLL-LBA were used as the polycation. These data confirm consistent mass deposition after each layer during the functionalisation process, up to four bilayers.

![Figure 4-3](image)

Figure 4-3: Change in dissipation throughout the layer deposition process in A) Hep+PLL and B) Hep+PLL-LBA.
Water contact angle measurements of layer-deposition on PEI-activated 2D PCL samples show clearly alternating contact angles for the heparin and PLL or PLL-LBA polyelectrolyte multi-layers after the first four terminating layers (Figure 4-4). The alternating contact angles for the heparin (~60°) and PLL or PLL-LBA (~55°) terminating layers demonstrate the hydrophobic and hydrophilic properties of the respective layers, and also confirm the presence of distinctly different layers throughout the LbL process. There is a trend for a lower contact angle for the PLL-LBA layers compared to PLL, which is consistent with the presence of hydroxyl groups from the LBA. These hydroxyl groups result in greater hydrophilicity for the PLL-LBA layers, but do not result in a significant difference in hydrophilicity compared to the PLL layers.

![Graph showing water contact angles for different layers](image)

**Figure 4-4:** Contact angle measurements for each layer deposited in Hep+PLL and Hep+PLL-LBA, starting with unmodified PCL, then the PEI aminolysation, followed by alternating heparin (H) and PLL or PLL-LBA (P) layers. Values are the mean ± SEM of 3 observations.

Using the LbL method, PCL nanofibres have been effectively functionalised with heparin and PLL or PLL-LBA, confirmed by SEM, QCM-D and water contact angle analysis. The LbL approach demonstrated here adds to a range of strategies from our team for the
presentation of molecular entities to regulate cellular architecture and biology [75, 99, 103, 104] and is recognized to have diverse applications in biomedicine [105, 106]. Importantly, this simple, reproducible technique has allowed for the presentation of multiple biologically relevant cues to potentially influence the behaviour of astrocytes in vitro and in vivo, with implications in controlling the CNS inflammatory response.

4.2.3 In vitro assessment of the effect of scaffold functionalisation on astrocyte protein expression

Primary murine astrocytes were grown on 2D PCL, PCL nanofibres (3D PCL), PCL functionalised with Hep+PLL (PCL-Hep+PLL) and PCL functionalised with Hep+PLL-LBA (PCL-Hep+PLL-LBA) for 4 or 12 div (days in vitro, equivalent to 14 and 22 div as secondary astrocytes were generated and plated at 10 div). Protein expression by astrocytes sub-cultured on the PCL samples was quantified using immunolabelling and morphometric analysis.

Astrocyte phenotype has previously been considered binary, being either healthy (good) or reactive (bad). On the basis of gene profiling, the notation of A1 and A2 phenotypes to describe reactive astrocytes promoting neuroinflammation and healing respectively (respectively) [85, 107] have been proposed, a concept paralleling the M1 pro-inflammatory and M2 immunosuppressive notations for microglia (and macrophages) [108]. An alternate perspective is that reactive astrocytes exist on a spectrum of trophic to toxic phenotypes, dependent on the time-point after injury, and its severity. The activation of astrocytes, with cell numbers peaking at 7-10 days post injury, inhibits immediate growth through physical and biochemical barriers, resulting in dystrophic axons [3, 8]. This should not, however, be interpreted as being exclusively cytotoxic
behaviour. Reactive astrocytes possess cytotrophic functions, as their selective ablation results in failed blood-brain barrier (BBB) repair, prolonged infiltration of macrophages, local neuronal death as well as significant functional deterioration [10]. This spectrum of responses demonstrates the importance of reactive astrocytes and the need to understand their complex role within the CNS after injury. We hypothesise that optimal recovery will occur when the existing and currently persistent reactive astrocytosis is, at an appropriate time-point, abated to allow repair and regeneration, with endogenous astrocytes performing their normal cytotrophic and supportive functions.

4.2.3.1 3D morphology of nanofibres provides a superior culture environment

All 3D culture conditions provided the same morphological cues (as seen in Figure 4-2), effectively mimicking the nanofibrous morphology of the native ECM, and thus providing a cell culture environment more representative of the in vivo environment compared to the current 2D environments used to study cell behaviour. At 4 div, expressions of GFAP, AHNAK, Gal-1, and F-actin by astrocytes on all three nanofibrous scaffolds (PCL, PCL-Hep+PLL, PCL-Hep+PLL-LBA) was similar regardless of functionalisation, but lower than by astrocytes cultured on 2D PCL (Figure 4-5 A-D). This attenuation of expression of proteins typically associated with an inflammatory astrocytic phenotype implies that 3D morphological cues provided by the nanofibrous scaffolds, induce a cytotrophic phenotype within a short culture period. We would expect that this would be associated with upregulated BDNF and EAAT2 expression [7].
4.2.3.2 Galactose moiety maintains an attenuated astrocyte inflammatory phenotype at 12 div

The reduction in expression of protein associated with an inflammatory profile did not persist when astrocytes were cultured on scaffolds that provided 3D morphological cues (Figure 4-5 A-D). By 12 div of astrocytes being cultured on 2D PCL, 3D PCL and PCL-Hep+PLL, the expression of all of these four proteins was increased. Expression of these proteins by astrocytes cultured on the 2D PCL and 3D PCL was similar, indicating that at 12 div, the 3D morphology of PCL alone did not control astrocyte protein expression. Similarly at 12 div, presenting heparin or PLL through 3D PCL or PCL-Hep+PLL, did not maintain its effect on the morphology or inflammatory profile of cultured astrocytes, as the expression levels of all four proteins was greater than their respective levels at 4 div of culture. This evidence implies that the initial switch to a cytotrophic profile at 4 div was not maintained and, by 12 div, there was a return to an inflammatory profile. We propose that such a shift to an inflammatory profile can be attributed to the insufficiencies of a static, one-cell type culture environment lacking the cell-cell and cell-ECM dynamics found in vivo, to maintain sensitive primary astrocytes. In future, the use of bioreactors and mixed cultures would create a more dynamic culture environment that would elucidate valuable information to understand astrocyte behaviour in vitro, and thus improve subsequent translation of strategies in vivo [109].
Figure 4-5: A-D) Morphometric analysis of primary astrocyte expression of A) GFAP, B) AHNAK, C) F-actin, and D) Gal-1 cultured on 2D PCL, 3D PCL, PCL-Hep+PLL, and PCL-Hep+PLL-LBA. Values are expressed in pixels and represent mean ± SEM from 2 independent experiments producing at least 12 images from triplicate wells for each biomarker. * denotes significant difference to PCL-Hep+PLL-LBA at the same time point, ^ denotes significant difference between time points of the same culture environment, p < 0.05. E) AHNAK (red)/GFAP (green) and Gal-1 (red)/GFAP (green) staining of astrocytes grown on 2D PCL, 3D PCL, PCL-Hep+PLL and PCL-Hep+PLL-LBA at 12 div. Scale bar = 50 μm.
In contrast at 12 div GFAP, AHNAK, Gal-1 or F-actin expression of astrocytes cultured on PCL-Hep+PLL-LBA remained at 4 div levels and was significantly lower than under all other culture conditions. These observations imply the attenuation of the inflammatory profile was maintained when astrocytes were cultured in the presence of the galactose moiety in PLL-LBA. This difference is most likely a direct result of the covalent presentation of galactose moieties on the surface of the PCL nanofibers, as 3D morphological cues such as presentation of heparin or PLL by PCL-Hep+PLL-LBA did not maintain reduced levels of GFAP, AHNAK, F-actin, and Gal-1.

As Gal-1 expression was also reduced in the presence of galactose moieties, it raises the question of its role in identifying any influence of the galactose moiety on the expression of the other proteins (GFAP, AHNAK, F-actin) and indeed Gal-1 itself. Following ischemia, Gal-1 upregulation is linked to attenuating both astrogliosis and the production of inflammatory molecules (e.g. interleukin-1β), and increased BDNF production by astrocytes [102, 110]. This finding implies that the presence of the galactose moiety downregulating Gal-1 expression may also lead to lower expression of other “trophic” influences from astrocytes such as BDNF. The Gal-1 expression of astrocytes from 4 to 12 div did not increase when cultured on PCL-Hep+PLL-LBA, indicating the culture environment presenting LBA on the nanofibres did not induce the anti-inflammatory action of astrocytes associated with Gal-1 (which would thus result in an increased expression). Additionally, the effect of a longer culture period increasing Gal-1 expression (as seen with 2D, 3D, and PCL-Hep+PLL culture conditions) was not observed when LBA was presented on the surface of the nanofibres, further
demonstrating the ability of the galactose moiety to influence aspects of the inflammatory response of astrocytes, specifically, via the action of Gal-1.

Increased GFAP expression by astrocytes and hypertrophy are the most commonly used markers of reactive astrogliosis after injury or disease [27, 111]. Although GFAP expression of astrocytes was reduced on the three 3D nanofibre scaffolds at 4 div, it significantly increased between 4 and 12 div for all culture conditions, with the exception of PCL-Hep+PLL-LBA, where expression remained constant from 4 to 12 div. Immunolabelling of GFAP with AHNAK or Gal-1 (Figure 4-5 E) at 12 div confirmed a reduction in GFAP, AHNAK and Gal-1 expression. Astrocytes grew in tight colonies on PCL-Hep+PLL-LBA, in contrast to the widely spread astrocytes observed on 2D PCL, 3D PCL control, and PCL-Hep+PLL (individual staining of AHNAK, Gal-1, F-actin and GFAP shown in Supplementary Figure 1). These colonies in culture could indicate the localised interaction of astrocytes with galactose moieties on the surface of the nanofibres, inhibiting process elongation and migration. Similarly, F-actin staining also increased for all culture conditions at 12 div with the exception of PCL-Hep+PLL-LBA, with astrocytes also forming tighter colonies compared to all other culture conditions (data not shown). Patterns of GFAP, AHNAK, and F-actin immunostaining, in concert with reduced Gal-1 expression, were indicative of decreased stellation, with less spindly processes suggesting decreased migration and process outgrowth, although importantly L-glutamate transporter activity was not compromised relative to other 3D cultures at 12 div (data not shown). These observations were similar to our earlier findings in astrocytes exposed to the Rho Kinase (ROCK) inhibitor, Fasudil [86], and demonstrate the persistent effect of the covalent attachment of the galactose moiety,
LBA on PCL-Hep+PLL-LBA. Therefore, we propose that the galactose moiety may act as a ROCK inhibitor, acting through Gal-1, as similar changes in morphology and expression have been observed in many other studies [14, 86]. In vivo assessment of the effect of scaffold implantation after stab injury

The influence of the various 3D nanofibrous scaffolds on the in vivo response of astrocytes to traumatic (stab) brain injury was assessed next. As functionalising the PCL nanofibres with heparin and PLL did not affect astrocyte protein expression in vitro, and astrocyte numbers are fewer in the presence of PCL nanofibre scaffolds than with a stab injury alone [34], we did not use a stab wound as a control or PCL-Hep+PLL scaffold. Rather, the effect of un-functionalised PCL nanofibre scaffold was compared with galactose presentation on PCL nanofibres.

Although the astrocyte response was the focus of the in vitro studies, in vivo studies provide the opportunity to also examine the effect on the surrounding neuronal population. Hence, both neurons (NeuN+) and astrocytes (GFAP+) were counted.

4.2.3.3. Galactose functionalisation promotes long distance neuronal survival 7 days after injury

Seven days after injury and subsequent scaffold implantation, GFAP+ astrocytes and neurons (NeuN+) at the edge of galactose presenting scaffolds (PCL-Hep+PLL-LBA) were significantly greater than adjacent to PCL scaffolds (Figure 4-6 A and B).

At a distance (600 μm) from the two scaffolds, astrocyte numbers were similar but the number of neurons both close to and at a distance from the PCL-Hep+PLL-LBA scaffolds was significantly greater than was the case for PCL scaffolds. An increased
presence of neurons both close to, and away from the scaffold, and the associated increased number of astrocytes suggests attenuation of the typically growth prohibitive actions of astrocytes observed in the acute time period after injury, and notably at the 7 day time point investigated here. Such attenuation is attributed to the galactose moieties present on PCL-Hep+PLL-LBA, and highlights that greater astrocyte numbers should not be directly correlated with cytotoxic behaviour, and reinforces the need for a holistic view of astrocyte behaviour after injury. We propose that future studies should compare the protein expression profile of astrocytes under the two conditions.

After injury, astrocytes inhibit growth through a physical barrier formed around the lesion, intertwining their hypertrophic processes, as well as through the secretion of inhibitory molecules such as some CSPGs [10, 27]. Additionally, as neurons die after traumatic injury [112, 113], an increased neuronal survival rate when galactose is presented on the surface of PCL nanofibres is extremely promising. Astrocytes at the scaffold surface would interact with the galactose moieties, which we have shown maintain a non-inflammatory profile of astrocytes in vitro. Given the complex cell-cell signalling that occurs in vivo, it is expected that the attenuation of the inflammatory

Figure 4-6: Cell counts per area for A) GFAP+ (astrocytes) and B) NeuN+ (neurons), at 7 days and 21 days after scaffold implantation, either close or away from the implanted PCL or PCL-Hep+PLL-LBA scaffold. Values are the mean ± SEM of 3 observations. * p < 0.05, *** p < 0.001 and **** p < 0.0001
profile of astrocytes, as demonstrated *in vitro*, would subsequently influence neuronal survival after traumatic injury *in vivo*. The exact mechanism of such protection or rescue needs to be investigated further to gain a more complete understanding of the action of galactose-interacting astrocytes on neuronal survival. However, data presented here is a promising proof-of-concept strategy to promote neuronal survival within the acute phase post-injury.

4.2.3.4 ‘Homeostatic’ astrocyte and neuron numbers reached at 21 days

The initial difference in astrocyte numbers observed close to the scaffold at 7 days was not present at 21 days, with no significant difference between astrocyte numbers close to the PCL or PCL-Hep+PLL-LBA scaffold. However, there was a significant increase in astrocyte numbers close to the PCL control from 7 to 21 days, with similar counts to those of PCL-Hep+PLL-LBA. We have previously shown that astrocyte numbers peak at ~10 days post injury [34, 74], so it is reasonable to conclude that astrocytes had not reached peak numbers at seven days, explaining the increased numbers from 7 to 21 days. It is also logical to expect greater astrocyte numbers at the interface of the implanted scaffold, compared to distant from the lesioned area, as significant trauma to the surrounding tissue has occurred, as reflected by the lower neuronal populations. Although more comprehensive analysis of the astrocytic response to these implanted scaffolds is required, these initial findings could suggest that reactive astrocytes (which we did not investigate *in vitro*), interact more favourably with the galactose moieties than with the control nanofibres, hence, result in higher astrocyte numbers at 7 days, and this is sustained through to 21 days. The greater action of the galactose moieties observed *in
*vivo* (within 7 days) is in agreement with the *in vitro* data which showed a significant effect at 12 *div*.

Increased cell numbers from 7 to 21 days were also observed within the neuronal population within both groups: there was a significant increase in neuronal numbers close to and far away from the PCL scaffold, as well as close to the PCL-Hep+PLL-LBA scaffold. There was no significant difference between neuron numbers close to or far away from either of the scaffolds – all of which are similar to the neuron counts far away from the PCL-Hep+PLL-LBA scaffold at 7 days. This evidence demonstrates that the presence of the galactose moieties presented on the surface of PCL nanofibre scaffolds had a significant effect on astrocyte and neuron counts at seven days, but had no significant effect at a longer time point viz. 21 days *in vivo*. As both astrocyte and neuron numbers are similar in each group at 21 days, our findings suggest a potential ‘homeostatic’ level of cell numbers, within an inflammatory context, has been reached by 21 days.

Although the *in vivo* data presented here demonstrate that covalently attached galactose moieties on PCL nanofibres have a significant effect on astrocytic and neuronal population numbers after traumatic injury, the differing changes in astrocyte numbers close to each of the scaffolds from the 7 to 21 day time points (an increase for PCL, no change for PCL-Hep+PLL-LBA), and the associated effect on neuronal survival cannot be elucidated from only cell numbers. Further work to characterise the biological profile of these astrocytes and their morphology (together with their phenotype), and investigate the exact mechanism through which the neuronal population is protected would provide a greater understanding of this promising TBI treatment strategy, and better inform
future development of this system to achieve long-acting biological influence on astrocytes after traumatic injury.

4.3 Conclusion

We demonstrated a covalent functionalisation of PCL nanofibres to present galactose moieties which attenuates the inflammatory profile of astrocytes in vitro, and promotes neuron survival after traumatic brain injury in vivo. Galactose functionalisation of PCL nanofibres maintained an attenuated inflammatory profile of astrocytes at a longer culture period, with the same scaffold resulting in increased astrocyte numbers close to the scaffold after implantation into a TBI, accompanied by increased neuron numbers at 7 days post-injury. Although further biological investigation is required to fully understand the mechanisms behind such neuronal protection, this study presents a promising proof-of-concept for the application of the layer-by-layer functionalisation method to present covalently attached galactose for to influence astrocyte behaviour, with implications for the development of future traumatic brain injury treatment strategies.

4.4 Acknowledgements

This research was supported by funding from an NHMRC project grant (APP1020332). FLM was supported by an Australian Postgraduate Award; MKH and PMB were supported by NHMRC Research Fellowships APP1020401 and APP1019833, respectively; DRN was supported by a NHMRC Career Development Fellowship (APP1050684). Access to the facilities of the Australian National University Centre for Advanced Microscopy (CAM) with funding through the Australian Microscopy and
Microanalysis Research Facility (AMMRF) is gratefully acknowledged. The Florey Institute of Neuroscience and Mental Health receives infrastructure support from the Victorian State Government (Australia).
Figure S 4-1: NMR spectra for novel polymer PLL-LBA
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Figure S 4-2: AHNAK, Gal-1, and F-actin (red) and GFAP (green) staining of astrocytes grown on 2D PCL, 3D PCL, PCL-Hep+PLL and PCL-Hep+PLL-LA at 4 and 12 div. Scale bar = 100 μm
Chapter 5 extends on the use of saccharides presented in the previous chapter. The nanofibrous self-assembled peptide hydrogel, Fmoc-DIKVAV is used to address the limitations of nanofibrous scaffolds for \textit{in vivo} treatment strategies for traumatic brain injury. This hydrogel system was co-assembled with the anti-inflammatory saccharide, fucoidan, and the material properties as well as impact on scar formation is investigated. This chapter presents a foundation of \textit{in vivo} analysis which can be used to further the field’s understanding of the astrocytic response to saccharides after injury.
Publication relevant to this chapter:

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Please see Appendix B for published article.

Abstract

Traumatic brain injury results in devastating long term functional damage due to the growth-inhibition of the inflammatory response, and in particular, the complex response of astrocytes. Sustained, non-steroidal anti-inflammatory approaches that can attenuate this response are of interest to improve therapeutic outcomes, particularly when combined with a tissue engineering construct that recapitulates the physiological microenvironment to facilitate functional repair. Here, we present a multifaceted, therapeutic extracellular-matrix mimic consisting of a co-assembled scaffold with a laminin-inspired self-assembled peptide hydrogel, Fmoc-DIKVAV, and the anti-inflammatory macromolecule, fucoidan. At 7 days post injury, our novel multicomponent hydrogel system presenting biologically relevant nanofibres and the anti-inflammatory fucoidan attenuated the primary glial scar to half that of a stab (control) injury. Further, the presentation of fucoidan increased the organisation of astrocytes within the glial scar, whilst also significantly changing the morphology of astrocytes distal from the administered hydrogel and further into the parenchyma. This
demonstrates the anti-inflammatory fucoidan, present on the surface of the Fmoc-DIKVAV nanofibres, causes a change in astrocyte phenotype post injury attenuating ‘reactive’ astrocytosis. For the first time, we present a multicomponent tissue engineering construct to promote a growth-permissive environment \textit{in vivo}, and thus, increase the potential for repair and regeneration after traumatic brain injury.
5.1 Introduction

Unlike many ‘frontline’ organs, such as the liver or skin, the central nervous system’s (CNS) response to traumatic injury is unable to repair damaged tissue, rather, it limits the damage through a sophisticated inflammatory response [2, 4, 24, 114]. Although the CNS undergoes repair phases similar to other organs (inflammation, cell proliferation, and tissue remodelling), its ability to resolve traumatic injury is severely impeded by the growth inhibitory environment created by the inflammatory response, which is orchestrated by many cells, including astrocytes [2].

The cellular and molecular mechanisms of the CNS, and more specifically, the brain, post-injury have been comprehensively reviewed elsewhere [2-5, 25]. Of particular interest is the impact of the astrocyte response on the ability of the CNS to regenerate. After injury, astrocytes become reactive, characterised by an increase in intermediate filament protein expression (including glial fibrillary acidic protein (GFAP), and vimentin), hypertrophy, and proliferation near the injury site. Reactive astrocytes are essential for inflammatory cell containment and preventing secondary degeneration through the demarcation of the lesion site through the formation of what is commonly known as the glial scar, a mesh-like structure of interwoven astrocyte processes [10, 114]. Astrocytes forming the glial scar, as well as pericytes, fibroblast and inflammatory cells, produce growth-inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) which are key to inhibiting growth immediately after injury [91]. The physical and chemical barriers created post-injury are essential for arresting secondary degeneration, but this same behaviour of astrocytes also led to the perception that they impede regeneration after injury. Astrocytes have a complex and crucial role in the repair and
reconstruction process that follows injury [1, 9, 10, 24, 114] and are thus likely therapeutic targets to facilitate regeneration after injury, as their behaviour can be manipulated to yield a growth-permissive or –supportive environment. Consequently, mechanisms that control astrocyte behaviour warrant further investigation.

While astrocytes initially inhibit post-injury regeneration, repair can be compromised when this inhibition persists. Regenerating axons stall at the border of mature lesions and form dystrophic endbulbs; contrary to initial opinion [115], the regenerative ability of these endbulbs is retained [15, 116]. This implies that altering the astrocyte phenotype after injury may provide a growth-permissive and stimulatory environment and present additional stimulatory cues which encourage axonal entry into the enervated regions. Thus, a treatment system that attenuates the inhibitory response of astrocytes, whilst concurrently providing a microenvironment to facilitate axons to traverse a lesion should be of therapeutic value. Such a multifaceted system could then be optimised to temporally control astrocyte phenotype, which would allow the important inflammatory action that occurs in the acute phase, whilst harnessing growth-permissive and supportive astrocyte action within the chronic phase after injury. However, the current challenge is to first develop a treatment system which can provide biological, chemical, and physical cues to the injury site to change the morphology and thus phenotype of astrocytes.

Biomaterial scaffolds are an ideal candidate to deliver biological cues to direct astrocytes toward a growth-permissive or –supportive phenotype to allow axonal infiltration into and the injury, whilst also providing a biologically relevant microenvironment that supports cell adhesion, growth and differentiation. Various scaffolds [40, 41, 117, 118],
selected for (one or more) properties that mimic the extra-cellular matrix (ECM), and, increasingly, provide specific biochemical cues \textit{in vivo} which can be controlled spatially and temporally to facilitate cell growth, differentiation and proliferation have been examined as treatment strategies for neural regeneration Here, we investigate Fluorenylmethyloxy carbonyl-capped self-assembled peptide hydrogels (Fmoc-SAPs) because their nanofibrous structure can also provide biochemical cues, whilst their shear thinning and void-filling properties allow for effective microinjection into a range of tissues [65, 119]. Fmoc-DIKVAV, a laminin-inspired peptide sequence that self-assembles \textit{via} \(\pi-\pi\) stacking of the aromatic groups and \(\beta\) sheet interactions [65] is of particular interest for neural applications. This self-assembly process allows for high-density presentation of the biologically relevant IKVAV sequence, which promotes neurite outgrowth when presented on a biomaterial scaffold [120], and is of biological relevance for neural tissue engineering contexts. The modulus of this material system can match that of the brain [65], and together these characteristics make Fmoc-DIKVAV an ideal materials candidate to alter the astrocytic response, and ultimately, facilitate repair and regeneration after injury. We have previously shown that the supramolecular assembly of Fmoc-SAPs with bioactive macromolecules provides a novel functionalization strategy to improve the capacity of the scaffolds to control cells. Of interest is the anti-inflammatory sulphonated polysaccharide, fucoidan, which has been shown to inhibit the production of excessive nitrous oxide (NO) and prostaglandin E\(_2\), and attenuated the expression of pro-inflammatory cytokines in lipopolysaccharide-induced BV2 microglial cells [121]. We have previously shown that fucoidan distributes itself within an Fmoc-FRGDF supramolecular scaffold to downregulate inflammatory
cytokine expression in cancer cells, thus, combining fucoidan and Fmoc-DIKVAV is a promising anti-inflammatory material construct for traumatic brain injury (TBI)[12].

In particular, we have shown that the anti-inflammatory polysulphonated polysaccharide, fucoidan, distributes itself within an Fmoc-FRGDF supramolecular scaffold to downregulate inflammatory cytokine expression in cancer cells [12].

Here, we report on the development and delivery of a growth-supportive physical and biochemical environment enabled by the Fmoc-DIKVAV scaffold, functionalised with the anti-inflammatory properties of fucoidan, as a multi-faceted biomaterial system with the potential to attenuate the inflammatory astrocytic response after traumatic brain injury and improve therapeutic outcomes.

### 5.2 Results and Discussion

#### 5.2.1 Co-assembly with fucoidan maintains the Fmoc-DIKVAV nanostructure

We have previously demonstrated the co-assembly of the fibronectin based sequence, Fmoc-FRGDF with fucoidan and demonstrated that the nanofibrillar architecture is maintained [18]. In order to confirm that the material properties which make Fmoc-DIKVAV scaffolds an attractive tissue engineering material choice for neural applications were also unaffected, we carried out a series of characterisation steps. Transmission electron microscopy (TEM) (Figure 5-1 A-C) showed the nanofibrous architecture of the Fmoc-DIKVAV sequence upon self-assembly (A) is maintained with the addition of 2 or 5 mg/mL fucoidan (B-C) to yield a co-assembled Fmoc-DIKVAV/fucoidan SAP system. Indicative measurements of the nano-bundle width from the TEM images (Figure 5-1 D) highlighted an increase in bundling with the
addition of fucoidan. This was attributed to the charged sulfate groups on the polysaccharide interacting with the charged groups on DIKVAV presented on the surface of the nanofibrils (particularly, the positively charged lysine) and subsequently promoting the closer bundling more of the self-assembled nanofibres.

Figure 5-1: Transmission electron microscope images showing the nanofibrous assembly of A) Fmoc-DIKVAV, B) Fmoc-DIKVAV + 2 mg/mL fucoidan, and C) Fmoc-DIKVAV + 5 mg/mL fucoidan, and D) indicative nano-bundle width measurements from TEM images. *** p < 0.001, **** p < 0.0001
These data confirm that the IKVAV containing material maintained the desirable structural properties similar to that as observed with the Fmoc-FRGDF SAP sequence, thus demonstrating the co-assembly approach can be used for tissue specific therapies. Co-assembling fucoidan and Fmoc-DIKVAV also maintained the bulk viscoelastic characteristics of this class of system. Rheological testing (Figure 5-2 A) showed similarly to Fmoc-DIKVAV, the co-assembled Fmoc-DIKVAV/fucoidan system had a storage modulus (G’) greater than the loss modulus (G’’), indicating viscoelastic properties characteristic of a hydrogel. The addition of the fucoidan was also observed to increase the stiffness of the material, in accordance to observations previously [12]. An increase in modulus for the systems co-assembled with fucoidan was attributed to the increased fibre bundling as indicated by Figure 5-1D.

![Figure 5-2: A) Rheological testing and B) Fourier-Transform Infra-Red Spectroscopy of Fmoc-DIKVAV, Fmoc-DIKVAV + 2 mg/mL fucoidan, and Fmoc-DIKVAV + 5 mg/mL fucoidan.](image)

In future studies, this modulus could be decreased by varying the peptide concentration, sequence, or gelation mechanism, and could potentially lead to greater cellular infiltration as the gel degrades in vivo [64, 65]. Matching the modulus is key to mitigating a foreign body response [46], however, as the results presented in the proceeding sections
demonstrate, the material systems attenuated the astrocyte scar with the moduli presented in Figure 5-2A. In order to confirm the bulk peptide interactions were maintained, Fourier Transform Infra-Red (FTIR) spectroscopy was conducted. All three groups have a major peak at 1630 cm$^{-1}$ and a minor peak at 1690 cm$^{-1}$ (Figure 5-2B) indicating β-sheet formation, characteristic of the self-assembling mechanism of our Fmoc-SAPs, and unaffected by the co-assembly with fucoidan.

5.2.2 Scar extension and astrocyte organisation are altered by Fmoc-DIKVAV hydrogel implants

After determining the material characteristics of Fmoc-DIKVAV were conserved after co-assembly with fucoidan, its effect on astrocyte scar formation post traumatic injury was assessed.

Here, we conducted a comprehensive dimension analysis to assess the effect of Fmoc-DIKVAV/fucoidan hydrogels on astrocyte scar formation after traumatic (stab) injury. It should be noted, that within an inflammatory context, “quiescent” or “resting” references to astrocytes prior to injury is an incorrect simplification, as astrocytes are continually active in a healthy CNS [2]. Therefore, “reactive” will be used to describe glial cells that are responding to insult, and is representative of the spectrum of cellular behaviour and function observed after injury, whilst “active” can be used to describe physiological astrocytes.

We performed a range of assessments to determine the extent of this behaviour: the ratio of hemisphere areas and the lesion and implant volume; the distances of the primary astrocyte scar and local astrocytic disturbance; and confocal microscope images of the
primary scar and astrocytes in the distal parenchyma. These analyses and images enable a holistic overview as to the action of the hydrogels on scar formation within the local environment of the injury.

At seven days post injury and subsequent hydrogel implantation, there was no difference in the ipsilateral to contralateral hemisphere ratios between any of the groups, and all ratios were approximately 1 (Figure 5-3 A). This indicates that the implantation of the hydrogels did not result in unusual swelling or contraction of hemisphere volume. There is however, a significant difference between the lesion/implant area (Figure 5-3 B) of the stab compared to all of the hydrogel groups, and thus demonstrates that the suitability of these material systems to fill the lesion void created by traumatic brain injury (TBI). In the implant groups, the stab wound was filled and therefore supported by the hydrogel, whereas the lesion void was left ‘as is’ in the stab group. We suggest this allowed for tissue collapse after the stab, and thus accounts for the smaller lesion volume observed for the stab group (Figure 5-3 E). This highlights that a single metric such as the lesion area can be misleading when assessing effectiveness of a tissue engineering construct, as a larger lesion area with structural support may be more desirable than a smaller lesion site which had collapsed, an observation which is initially being counter-intuitive. Additionally, the structural support provided by these Fmoc-DIKVAV hydrogels was comprised of nanofibre bundles within a void-filling form, and as such, more closely mimicking the morphology of the native ECM. This property is therefore able to provide physical support to cells, reducing the inflammatory response associated with secondary tissue degeneration, whilst also having the ability to promote improved
migration and differentiation across the lesion site to encourage the desired growth-permissive environment.

The glial scar, a dense network of interwoven reactive astrocytes, is the primary mechanism that is of interest when attenuating the inflammatory response to traumatic injury. However, it is difficult to characterise the individual morphology of such astrocytes due to this dense network. Thus, measuring the distance of the primary (glial) scar extension can be used to assess the implanted hydrogels’ relative effect on scar formation. Analysis of scar dimensions (Figure 5-3 A-D) demonstrates that filling a TBI lesion with a hydrogel is advantageous through reducing the primary astrocyte scar extension. The stab alone resulted in a primary scar extension almost twice that of either the hydrogel groups (Figure 5-3 C). Such a clear difference in primary scar extension may therefore be due to the lack of structural support in the stab group – without structural support to the surrounding, damaged, tissue, further tissue collapse occurs, exacerbating the initial inflammatory response. This secondary damage results in continued astrocyte reactivity, and hence, a larger primary astrocyte scar than when structural support is provided to the lesion site. Interestingly, there is no significant difference in the extension of the primary scar between the hydrogel groups, demonstrating the co-assembly with fucoidan does not have an impact on the scar dimensions. However, penetrative injury is a traumatic event that, in addition to the glial scar formation, disrupts a significant proportion of the astrocyte population away from the wound site. As such, we also investigated the extension of the local astrocytic disturbance from the lesion/implant site. As the investigated time point (seven days) lies within the acute time period after injury, disturbance of the local astrocytic population
was to be expected. Between the stab and hydrogel groups however, there was no
difference in the disturbance extension (Figure 5-3 D), indicating that the dominant
effect of the hydrogel systems was on the primary astrocyte scar formation, not the local
astrocyte population disturbance.

Extensive confocal imaging of the interface of the lesion/implant and the glial scar
reveals variation in astrocyte morphology, distinct between all groups. Figure 5-3 E
shows the significant reactivity of astrocytes after TBI, with long, intertwined,
hypertrophic processes, and individual cells indistinguishable from each other. With the
implantation of Fmoc-DIKVAV (Figure 5-3 F), a glial scar is still formed however some
astrocyte cell bodies can be visualised and appear rounded and with shorter processes
extending out, compared to those seen in the stab group. Contrastingly, whilst there is
approximately 100 μm of interwoven astrocytes extending from the implant border with
both hydrogel implants, there are more easily distinguishable astrocytes (Figure 4 G)
close to the injury site when Fmoc-DIKVAV + 2mg/mL fucoidan was implanted with
an even greater degree of astrocyte organisation observable with Fmoc-DIKVAV + 5
mg/mL fucoidan (Figure 5-3 H). Although these organised astrocytes appear somewhat
reactive, this increased morphological organisation with fewer hypertrophic and
intertwined processes is less representative of astrocytes found in the glial scar.
Figure 5-3: Dimension analysis of the stab, Fmoc-DIKAV, Fmoc-DIKAV + 2 mg/mL fucoidan, and Fmoc-DIKAV + 5 mg/mL fucoidan groups, 7 days after injury and gel implantation, showing A) ratio of ipsilateral to contralateral hemisphere area, with intersecting line at a ratio of 1. B) lesion or gel implant area (p < 0.05), C) distance of primary scar (GFAP+), and D) distance of the local astrocytic (GFAP+) disturbance. E-H) Depth colour-coded projections of astrocyte (GFAP+) 7 days post injury and gel implantation: E) infiltrating the stab wound (depth range 0–12 μm), and at the edge of the implanted F) Fmoc-DIKAV (depth range 0–8 μm), G) Fmoc-DIKAV + 2 mg/mL fucoidan (depth range 0–12 μm), and H) Fmoc-DIKAV + 5 mg/mL fucoidan (depth range 0–11 μm). Dotted line demarcates the lesion or implanted gel from the glial scar, arrowheads highlight individually distinguishable astrocytes. Scale bar = 20 μm. Note depth colour-coded projections were used to more easily visualise astrocyte processes and overall morphology.
This effect is indicative of the hydrogel inducing a shift in astrocyte phenotype, from a typically reactive, scar forming astrocyte, to one more representative of the physiologically active phenotype. This observation could be attributed to the anti-inflammatory properties of fucoidan [12] co-assembled with Fmoc-DIKVAV having a distinct impact on the morphology, and hence, attenuating the inflammatory phenotype of scar-forming astrocytes.

5.2.3 Morphological reconstruction reinforces the complexity of the astrocytic response

Although we have shown that implanting a nanofibrous hydrogel halves the size of the primary astrocyte scar, and that astrocyte organisation within the primary scar is altered when fucoidan is presented, the effectiveness of this system on individual cells should also be assessed. This was achieved by counting cells in 70 μm x 1000 μm areas located on the edge of the lesion or implant site and at a distance (600 μm) (Figure 5-4 A). Cell counts were similar at the edge of the lesion site or implant; however, as Figure 5-3 C shows the primary scars extended further than 70 μm from the edge of the lesion/implant: due to the limitation of the analysed area, these cell counts cannot be compared to the whole primary scar extension. Cell counts at a distance from the lesion/implant, were also similar suggesting that in all treatments the astrocyte population had reached what could be termed an ‘inflammatory homeostasis’ within the acute phase after injury. This is in agreement with the data showing similar extensions of disturbance for the local astrocyte population (Figure 5-3D).

It should be noted, that similar to the scar dimension analysis, astrocyte cell numbers cannot be used in isolation to assess a change in inflammatory action. Therefore, we
used GliaReconstruct to reconstruct the morphology of individual astrocytes, as it is commonly acknowledged that astrocyte morphology and phenotype are intimately linked, if not yet completely understood. The hypertrophic and intertwined morphology astrocytes within the glial scar prevents their reconstruction and astrocytes located 600 μm from the lesion/implant edge (Figure S 5-3) were examined as they were individually distinguishable in all groups.

Implanting Fmoc-DIKVAV and Fmoc-DIKVAV + 5 mg/mL of fucoidan resulted in similar cell area (~1200 and 1400 μm² respectively, Figure 5-4 B) and perimeter (~400 and 450 μm respectively, Figure 5-4 C) of astrocytes far away from the after injury. However, as shown in Figure 5-3 F and H, these two hydrogel implants had differing influence over the organisation of astrocytes comprising the glial scar; Fmoc-DIKVAV resulted in densely interwoven astrocytes, whilst the Fmoc-DIKVAV + 5 mg/mL implant resulted in a more organised astrocyte network. Although the local effects of these implant on astrocyte organisation does not affect astrocyte cell size far away from the implant, this is potentially not true for Fmoc-DIKVAV + 2 mg/mL fucoidan. The total cell area of astrocytes far away from the implanted Fmoc-DIKVAV co-assembled with 2 mg/mL fucoidan was larger than all other groups (Figure 5-4 B). This interesting finding is supported by the total cell perimeter (i.e. giving an indication of the astrocytic process ‘arbour’, Figure 5-4 C), which was significantly greater than the perimeter of cells far away from the implanted Fmoc-DIKVAV or Fmoc-DIKVAV + 5 mg/mL fucoidan, but not significantly greater than the stab group. As previously discussed, Fmoc-DIKVAV + 2 mg/mL enhanced astrocyte organisation within the glial scar, as compared to Fmoc-DIKVAV, but not as drastically as Fmoc-DIKVAV + 5 mg/mL
fucoidan. We have previously observed that a concentration of 2 mg/mL represents the minimum concentration for an observed biological effect, and may be related to the presentation of fucoidan within the scaffold [12]. Here, a similar concentration is required to have an effect on astrocyte organisation, and thus scar formation, as well as astrocyte size.

This interesting finding demonstrates the complexity of astrocytes and their response to therapeutic intervention: at the gel interface, we observed increased astrocyte organisation seemingly dependent on fucoidan concentration, however, further away...
from the gel implant, co-assembly of Fmoc-DIKVAV and 2mg/mL fucoidan results in significantly larger astrocytes than with 5 mg/mL fucoidan. Typically, larger astrocytes would be associated with hypertrophy characteristic of reactive astrocytes, however, astrocytes have for a long time been underestimated and simplified, so we are hesitant to draw such a hasty conclusion. These results highlight the complexity of the astrocytic response to injury, and the need to incorporate a range of analysis techniques including a comprehensive scar dimension analysis, confocal microscopy, cell counts, and cell reconstruction.

5.3 Conclusions

Although astrocytes are a promising therapeutic target for TBI treatments, they present a challenge for tissue engineers and biologists alike in understanding and subsequently altering their complex behaviour after injury, to ultimately facilitate repair and regeneration. Despite such a daunting challenge, here we have shown the multifaceted tissue engineering system of a scaffold co-assembled from the ECM mimic Fmoc-DIKVAV, and the anti-inflammatory fucoidan polysaccharide, reduces the primary glial scar, and significantly alters astrocyte organisation and morphology 7 days after traumatic brain injury. This system has the potential to provide the necessary biological, mechanical and material properties to be used as a platform from which to develop more sophisticated constructs with temporal influence over the astrocytic response after injury. Such a development will have to be grounded in a comprehensive understanding of astrocyte behaviour, and their potential within a reparative context, and will require further collaboration and innovation from tissue engineers and biologists, together.
5.4 Acknowledgements

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5.5 Supplementary Figures

![HPLC of Fmoc-DIKVAV indicating purity of peptide used.](image)

Figure S 5-1: HPLC of Fmoc-DIKVAV indicating purity of peptide used.
Figure S 5-2: Strain sweep of Fmoc-DIKVAV showing the linear-viscoelastic (LVE) region, at 37°C and 1 Hz.

Figure S 5-3: Confocal images 7 days post injury and gel implantation, away from: A) the stab wound, B) implanted Fmoc-DIKVAV, C) implanted Fmoc-DIKVAV + 2 mg/mL fucoidan, and D) implanted Fmoc-DIKVAV + 5 mg/mL fucoidan. Scale bar = 25 μm.
A commentary on the need for 3D-biologically relevant \textit{in vitro} environments to investigate astrocytes and their role in central nervous system inflammation

This chapter discusses the need for 3D cell culture environments to better understand the astrocytic inflammatory response after CNS injury. It highlights the insufficiencies of current 2D cell culture system, or the limitations associated with the \textit{in vivo} translation of some 3D cell culture environments system as nanofibre scaffolds. Here, ideal alternatives are presented to spur the investigation of biologically relevant, translatable 3D cell culture environments for the investigation of astrocytes to improve the development of traumatic brain injury biomaterial treatments.
Publication relevant to this chapter:

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Please see Appendix C for published article.

6.1 Abstract

Astrocytes execute essential functions in the healthy CNS, whilst also being implicated as a limitation to functional regeneration and repair after injury. They respond to injury to minimize damage to healthy tissue whilst also attempting to seal the broken blood-brain-barrier, however, they impede recovery if they are persistent and form a permanent scar in the injured brain. As such, it is of great importance to understand the mechanism underlying the astrocytic response to injury, and this understanding is currently limited by the *in vitro* environments available to scientists. Biomaterials such as nanofibres and hydrogels offer great potential for the development of superior, 3D cell culture environments in which to study astrocyte behaviour and phenotype. The implementation of such *in vitro* environments with a particularly interdisciplinary approach can improve the field’s understanding of astrocytes, their role in central nervous system inflammation, and elucidate potential strategies to achieve functional regeneration.
6.2 Introduction

Persisting inflammation following central nervous system (CNS) injury inhibits functional regeneration and repair [25, 122]. Disruption of the blood-brain-barrier (BBB) contributes to the existing inflammatory cascade after injury through the infiltration of fibroblasts and non-CNS cells and molecules, accompanying the recruitment of microglia and macrophages, and the release of pro-inflammatory cytokines. Importantly, breaching the BBB may affect the astrocytes response [2], inducing astrocytic proliferation to surround the lesion, protecting healthy brain tissue from the lytic processes occurring within the lesion [24]. While this astrocytic barrier protects the surrounding uninjured brain from secondary injury, the astrocytes also release pro-inflammatory molecules such as chondroitin sulfate proteoglycans (CSPGs) that prevent neural growth in their vicinity.

In healthy CNS tissue, astrocytes have important physiological functions, which include regulating synaptic transmission through neurotransmitter uptake and release and maintaining pH, ion and fluid homeostasis [27]. They achieve this latter role by inserting themselves between neural tissue and blood vessels where they form the BBB and regulate nutrient and oxygen flow from vessel to intracellular space. Following injury, reactive astrocytes contribute to the BBB re-formation, neuron and oligodendrocyte survival, motor recovery and containment of inflammatory cells [10] and by inducing a reactive response in astrocytes, inflammation performs both pro-survival or cytotrophic functions, whilst also participating in cytotoxic actions. The duration and extent of these two roles at various time-points following injury thus becomes critical in minimising injury, whilst also promoting repair. As a consequence, astrocytes are important
therapeutic targets for controlling CNS inflammation and developing strategies to achieve functional repair and regeneration after injury.

It is apparent therefore that the frequent description of astrocytes adopting either “quiescent” or “reactive” states is unhelpfully simplistic. In the normal healthy brain, astrocytes are far from quiescent and play an active role in the health and physiology of neurones. Investigating the pathways that control the temporally significant transition of astrocytes from this normal, active state, through an immediate post-injury function, to their post-injury trophic state, should be therapeutically valuable. As the normal physiological role of astrocytes is to interact with neurons with the 3D structure of the brain, including the neurone, extracellular space and blood vessels, it is likely that current two dimensional (2D) cell culture methods used to investigate cell behaviour and function have limited ability to model these complex in vivo signals [123]. Therefore, it is essential that instead, three-dimensional (3D) cell cultures that more accurately recapitulate the in vivo environment are utilized to improve the translation of in vitro findings to clinically relevant treatments. To this end, biomaterial cell culture environments have been developed to investigate astrocyte morphology and behaviour, and their potential for translation to in vivo applications is discussed below.

The in vivo extracellular matrix (ECM) provides structural and biochemical cues to support residing cells that a 3D cell culture environment should emulate. As electrospun nanofibres mimic the fibrous structure of the ECM, possess high porosity and a high surface area-to-volume ratio [8], they show promise for providing physical support to cells in a culture system. We previously demonstrated that primary astrocytes cultured on random or aligned poly(ε-caprolactone) (PCL) nanofibers infiltrated the scaffold.
Their expression of glial fibrillary acidic protein (GFAP) expression was less than sub-cultured primary astrocytes cultured in 2D, whereas brain derived neurotrophic factor (BDNF) and excitatory amino acid transporter 2 (EAAT2) were both increased [7]: this biochemistry is consistent with a pro-survival or “healthy brain” phenotype. These results demonstrate that 3D morphological cues are important factors when designing a 3D in vitro culture system as they can influence the phenotype of cells. This effect of morphology on astrocytes was consistent with our foundation studies that explored the interaction of embryonic cortical neurons on poly(lactic acid) and poly(lactic-co-glycolic acid) surfaces [124], and this is clearly demonstrated by 3D nanofibrous scaffolds using the same materials with no additional functionalization to produce a different morphology [125]. Physical cues also encompass scaffold stiffness, in addition to morphology. Stiff matrices (1,500 – 75,000 Pa) have resulted in an equal proportion of neural stem cells (NSCs) differentiated into neurons and astrocytes, as compared to the higher proportion of differentiated neurons achieved on soft matrices [78]. Additionally, NSCs expressing constitutively active (CA) GTPases - RhoA or Cdc42 - exhibited increased astrocytic differentiation on soft (<1,000 Pa) substrates, mimicking the differentiation observed on stiff substrates, and indicating the regulation of lineage commitment of Rho GTPases. Rho GTPases regulate the cytoskeleton, therefore affecting astrocyte morphology [86], which is currently the key indicator of astrocyte phenotype. Therefore, substrate stiffness could be implemented in an in vitro system to further investigate astrocyte morphology (phenotype) change that occurs after injury.

Additionally, nanofibres can be functionalised with various molecules to further model facets of the ECM in vitro. Expression of heat-shock protein (HSP70, a marker of cellular
stress), and intermediate filament proteins (such as GFAP, vimentin and nestin, that are usually used as indicators for reactive astrocytes) was less when astrocytes were cultured on polyurethane nanofibres coated with laminin, than those coated on laminin coated glass slips [13]. Additionally, there were stark morphological differences, with astrocytes cultured on the laminin coated polyurethane nanofibres having many finely branched processes compared to the flat, polygonal shape observed on the laminin coated coverslips, as seen in Figure 6-1.

![Figure 6-1: Morphology as revealed by 3D reconstruction of confocal images of EGFP-expressing astrocytes cultured on: a) 2D laminin coated coverslips and b) 3D polyurethane nanofibers coated with laminin. Scale = 10 μm [13]. This figure has been reproduced with permission from Elsevier.](image)

As the biochemical cues from the laminin coating were similar in the 2D and 3D environments, it is likely that physical cues from the nanofibres were responsible for the changes to morphology and biochemical expression that more closely represent astrocytes found in vivo. Coating nanofibres with another ECM molecule, fibronectin, for astrocyte culture has also been investigated. The rate of glutamate uptake of primary astrocytes was increased when cultured on poly(L-lactic acid) nanofibres coated with fibronectin, with aligned nanofibres also directing astrocyte migration [98]. The
direction of astrocyte growth along the direction of fibre alignment was similar to what we previously observed on aligned PCL nanofibrous scaffolds [7], although process penetration into the scaffold was not reported, which is likely due increased scaffold thickness and fibre diameter differences as compared to our study (2 μm [98] vs. 0.4 μm [7]). Increased expression of GLAST and glutamine synthetase of astrocytes cultured on aligned and random nanofibres, respectively, was significant when compared to the 2D PLLA film, with no statistical significance between the two nanofibre groups [98]. This indicates that the observed biochemical differences in cultured astrocytes is, again, attributable to the 3D morphology of the nanofiber scaffold, not the coating or the fibre alignment, and subsequently highlights the importance of 3D morphological cues when designing innovative in vitro environments.

However, the functionalisation of nanofibres also introduces the possibility of providing biologically relevant cues along with physical cues described above. PCL nanofibres functionalised with BDNF (covalently bonded to the PCL surface) have supported cortical neural stem cell differentiation into neurons, astrocytes and oligodendrocytes [11]. Similarly, growth factors or relevant molecules immobilised on nanofibre surfaces could provide cues to astrocytes in vitro, and an opportunity to investigate their influences on various astrocyte phenotypes. For example, the introduction of Rho inhibitors, Fasudil or Y27632 (which alter astrocyte morphology), to mature astrocytes cultured on 3D PCL nanofibres increased G-actin labelling and BDNF expression, whilst decreasing F-actin labelling, indicative of a reduction in actin stress fibres, and hence, a more cytotrophic phenotype [43]. The combination of biochemical intervention and a 3D environment produced astrocytes with a cytotrophic phenotype, revealing a
signalling pathway that could be exploited to control astrocyte phenotype in \textit{in vivo} injury or disease.

While nanofibres possess many favourable properties for modelling \textit{in vitro} biochemical and physical signals that control astrocyte phenotypes, they have mechanical features depending on their method of fabrication that limit their \textit{in vivo} deployment. Electrospun nanofibre scaffolds do not flow to fill a void, and as such, when they are implanted in the brain they do not always make intimate contact with the lesion penumbra, as seen in [34], making them more difficult to deploy \textit{in vivo}.

Therefore, it is also worth investigating biomaterials to provide a nanofibrous 3D environment \textit{in vitro} that can also be deployed as a treatment strategy \textit{in vivo}. Shear reversible materials, such as hydrogels, can flow allowing them to be injected to fill voids caused by post traumatic cysts, and yet subsequently reform \textit{in situ}, presenting a material with an elastic modulus similar to the brain. Neurite and astrocyte infiltration into a xyloglucan hydrogel modified with poly-d-lysine (PDL) (50 or 100\%) implanted into the

![Figure 6-2: Partially aligned PCL nanofiber scaffold implanted into the caudate putamen, failing to form intimate contact with the lesion penumbra (arrows) after 60 days [34]. This figure has been reproduced with permission from Elsevier.](image)
rat caudate putamen, was greater than that into the unmodified xyloglucan hydrogel [74]. The hydrogel filled the lesion void, forming intimate contact with the penumbra, and the biochemical modification enabled cellular infiltration, in and across the lesion, presenting this material as a potential in vitro material candidate. More void-filling biomaterials of interest are self-assembled peptide (SAP) hydrogel scaffolds. SAPs incorporating bioactive sequences such as DIKVAV, FRGDF, and YIGSRF were used to deliver cortical neuron progenitor cells into the mouse brain, and did not result in the formation of a glial scar [35]. These hydrogels may be useful means for inducing post injury regeneration and repair because their nanofibrous matrix replicates morphological and chemical properties of the 3D physical environment of the ECM, and contain easily synthesised bioactive sequences that can be tailored for tissue-specific applications [65, 119]. The in vivo performance of these hydrogels has been investigated and validated, so their development should present them as ideal candidates for in vitro culture models. The possibility of using the same environment both in vitro and in vivo is important because while in vitro studies allow for more detailed biochemical and morphological examination of cells, it is also useful to confirm the implementation of the same scaffolds in vivo. Such investigation can lead to a better understanding of astrocytes’ role in CNS inflammation, and point to potential treatments that can be translated in vivo. This interdisciplinary proposition will require biologists, chemists and engineers to work collaboratively to further the field’s understanding of one of the most complex and important cells of the CNS.
Chapter 7 investigates the material system presented in Chapter 5, Fmoc-DIKVAV and its co-assembly with fucoidan, as a 3D cell culture environment. This addresses the need for 3D biologically relevant cell culture environments previously highlighted in Chapter 6. The influence of the Fmoc-DIKVAV/fucoidan system on LPS- and IL-1α stimulated astrocytes is investigated, as is the influence of Fmoc-DIKVAV on the astrocyte morphology and organization in vitro.
Abstract

The complexity of the astrocytic response, and the link between their behaviour and morphology is currently a significant neurological challenge that is essential for the development of treatments strategies post-brain insult. Biomimetic 3D culture environments may be engineered to improve the understanding of these cells and their response to injury, as well as to develop, test, and optimise potential treatments. Here, we present Fmoc-DIKVAV, a nanofibrous self-assembled peptide hydrogel, as not only an anti-proliferative agent when co-assembled with fucoidan, but also as a 3D culture environment capable of maintaining astrocyte growth and cytoskeletal reorganisation. For the first time we report a ‘networking’ behaviour of astrocytes, the extent of which increases from 14 to 24 days \textit{in vitro (div)} in a 3D environment. This novel behaviour was also confirmed \textit{in vivo} at 22 days post injury and hydrogel implantation. The presentation of unique cytoskeletal reorganisation and the replication of \textit{in vitro} results \textit{in vivo} is an exciting advancement for 3D cell culture environment research.
7.1 Introduction

Astrocytes, and the role they play after injury, are a valuable therapeutic target to improve recovery from traumatic brain injury. They execute a range of essential physiological functions within the healthy brain, as well as being a vital component to the inflammatory response of the injured brain [2, 9, 10, 27]. Although the complexity of astrocytes is still poorly understood, their therapeutic value highlights the need for in vitro investigation of these cells and their response to treatments, in order to develop better therapies for traumatic brain injury.

Currently available cell culture environments fail to mimic the extracellular environment of the brain. Electrospun nanofibre scaffolds have been used in many investigations of astrocyte morphology and behaviour in vitro, successfully maintaining cytotrophic or “pro-survival” phenotypes of astrocytes, whilst also decreasing cellular stress and the expression of intermediate filament proteins typically used to characterise reactive astrocytes [7, 13, 43, 98]. However, the geometrical constraints of nanofibre scaffold mats inhibit the material construct from forming intimate contact with the lesioned tissue [26]. Thus, a biomaterial which provides morphological and biological functionality of nanofibre scaffolds, while filling an odd-shaped void, is required for in vivo deployment, and should therefore be used in vitro to ensure the clinical relevance of the outcomes. Hydrogels address these requirements, and have gained recent attention, with naturally-derived and synthetic hydrogels including collagen, agarose, Matrigel, and poly(ethylene glycol) (PEG) used in 3D cell culture systems [47, 126-129]. However, these hydrogels lack the nanofibrous morphology required to mimic the ECM, so composite materials using hydrogels and nanofibres [103, 130] or self-assembled peptide hydrogels [12, 35,
65, 131] have been proposed. Here, we investigate Fmoc-self assembling peptide hydrogels, which mimic the nanofibrous structure of the brain’s extracellular matrix (ECM), whilst providing high density presentation of biological sequences such as RGD, IKVAV, and YISGR on the surface of the nanofibres [132, 133]. Fmoc-SAPs can also deliver growth factors [75], polysaccharides [12], and viral vectors [104], and as a shear thinning hydrogel which can fill a lesion void, they are a suitable for use in in vitro investigations. Of particular interest for neural applications is Fmoc-DIKVAV, presenting the laminin sequence IKVAV, a constituent of the brain’s ECM. To further probe the Fmoc-DIKVAV’s use as a tissue engineering construct for traumatic brain injury, we co-assembled it with the anti-inflammatory and anti-proliferative sulfated polysaccharide, fucoidan. Downregulation of pro-inflammatory cytokines via inhibition of NF-κB, Akt, and MAPK activation in LPS-stimulated microglial cells, as well as the induction of apoptosis in epithelial cancer cells demonstrates fucoidan’s potential in addressing the inflammatory behaviour of astrocytes after traumatic injury [12, 121].

Here, we demonstrate dual functionalities of the Fmoc-DIKVAV system in vitro: as a delivery vehicle to enhance the anti-proliferative effects of fucoidan on LPS or IL-1α stimulated astrocytes, and as a three-dimensional culture environment for astrocytes. This now provides a solid foundation of in vitro investigation of astrocytes using a 3D, biologically relevant biomaterial system, which will improve translation of in vitro results into in vivo settings.
7.2 Results and Discussion

7.2.1 Anti-proliferative effect of multicomponent Fmoc-SAP system

To mimic the reactive behaviour of astrocytes after traumatic injury, the extent of proliferation of primary astrocytes stimulated by LPS and IL-1α was examined when subjected to control, soluble fucoidan, and Fmoc-DIKVAV co-assembled with fucoidan treatment groups. Metabolic assays (Figure 7-1 A and B) showed a consistent suppression of proliferation by soluble fucoidan and Fmoc-DIKVAV + fucoidan. Fucoidan’s co-assembly with Fmoc-DIKVAV suppressed proliferation the most, particularly on LPS-stimulated astrocytes (p < 0.0001). We attribute this synergistic effect to the binding of fucoidan to the fibrils within the Fmoc-DIKVAV gel (as previously reported on Fmoc-FRGDF fibrils [12]), which sustains its delivery. As well, Fmoc-DIKVAV itself may influence astrocyte metabolic activity through changes in astrocyte morphology. Immunocytochemistry showed that LPS- or IL-1α-stimulated astrocytes treated with Fmoc-DIKVAV + fucoidan had a more complex network organisation than the control group (Figure 7-1 C). This reorganisation of astrocytes, which typically grow as flat cells on tissue-culture plastic (TCP) [13], is the first report of this type of cytoskeletal reorganisation, and will be referred to as ‘network formation’ of astrocytes. This network formation was not observed when astrocytes were treated with soluble fucoidan (data not shown), and thus was attributed to the presence of Fmoc-DIKVAV. As astrocyte morphology and behaviour (particularly after injury) are intimately linked, we suggest this reorganisation of the cytoskeleton and intermediate filaments (such as GFAP, shown in Figure 7-1 C) contributed to the decreased metabolic activity of astrocytes as shown in Figure 7-1 A and B.
Figure 7-1: Fmoc-DIKVAV co-assembled with 2 mg/mL fucoidan exhibits exerts anti-proliferative and cell-reorganisation effects on astrocytes in vitro. Metabolic activity of A) 10 ng/mL IL-1α-stimulated and B) 100 ng/mL LPS-stimulated astrocytes treated with nothing (control), soluble fucoidan, or Fmoc-DIKVAV + 2mg/mL fucoidan. C) Immunocytochemistry of: GFAP (red), Aquaporin-4 (AQP4) (green), Hoechst (blue), and merge. Values are mean ± SEM, scale bar = 200 μm, * p < 0.05, ** p < 0.01, **** p < 0.0001
The effect of 3D extracellular morphology on astrocyte morphology is well known [7, 13, 43]. However, these previous studies induce cytoskeletal change by culturing astrocytes on top of a 3D scaffold. Here we observed that interactions with a biomaterial on top of astrocytes (as opposed to underneath, as in typical culture configuration), provided sufficient morphological and biological cues to induce cytoskeletal reorganisation. Thus, we further investigated this culture system, and compared it to Fmoc-DIKVAV in the typical set up (astrocytes cultured on top of the scaffold), without stimulating the astrocytes.

7.2.2 Quasi-3D and 3D culture environments result in astrocyte network formation

We further compared the cytoskeletal changes produced by culturing astrocytes underneath or on top of Fmoc-DIKVAV. When astrocytes were grown underneath Fmoc-DIKVAV, they interacted with the TCP onto which they had adhered, as well as with the Fmoc-DIKVAV on top, but could not grow in the z-direction: thus there were vertical cues but with a true z dimension of growth - hence a ‘quasi-3D’ environment. Meanwhile, astrocytes grown on top of Fmoc-DIKVAV had physical interactions with Fmoc-DIKVAV and cell media, and could infiltrate the hydrogel in the z-direction: thus considered a genuine 3D environment.

The same network formation was observed at 24 div with astrocytes grown under Fmoc-DIKVAV (as was initially observed in Figure 7-1), as well as when astrocytes were grown on top of Fmoc-DIKVAV (Figure 7-2). Networking is influenced by the presence of Fmoc-DIKVAV, but appears to be independent of hydrogel concentration in the cell media groups (Figure S 7-1). The cell density of networks grown on top of Fmoc-
DIKVAV are significantly denser than those grown underneath because of the difference in initial cell seeding (Figure 7-2 B). However, it demonstrated that the three-dimensional nature of the culture environment of this material system can support a much greater cell density than can be obtained in 2D cultures. Astrocytes cultured on top of Fmoc-DIKVAV grew in three-dimensions, increasing cell-cell and cell-material interactions, unlike the astrocytes grown underneath Fmoc-DIKVAV, which were restricted to growing in the XY plane on the TCP. The adhesive and mechanical support the 3D Fmoc-DIKVAV culture environment provided the astrocytes with physical and biochemical features reminiscent of the native ECM compared to the 2D TCP or the ‘quasi-3D’ environment, thus presenting a more attractive cell culture environment.

The cell body areas of astrocytes grown in the ‘quasi-3D’ environment was significantly greater than those grown in the 3D environment (Figure 7-2 C). This could be due to astrocytes spreading out on the TCP, covering a greater area than those growing in a 3D environment, which can grow in the z-direction. The astrocyte network lengths were similar in each environment (Figure 7-2 D), demonstrating this cytoskeletal reorganisation was not dependent on a 3D environment, but rather an attribute of the cell-material interaction that was influential even in a ‘pesudo-3D’ environment. This observation of astrocytes forming networks within a 3D culture environment is novel and demonstrates the influence of this biomaterial on astrocyte morphology.
Figure 7.2: Astrocyte networks form when grown underneath or on top of Fmoc-DIKVAV for 24 div. A) Immunocytochemistry of GFAP (red), F-actin (green), Hoechst (blue), and merge. B) Astrocyte density within the cell networks. C) Area covered by astrocyte cell bodies within the network. D) Network length of astrocytes. Scale bar = 200 μm, values are mean ± SEM.
7.2.3 Three-dimensional cell culture environment facilitates astrocyte network formation over time, with similar networking observed *in vivo*

The influence of the 3D cell culture environment provided by Fmoc-DIKVAV on the growth of astrocytes at 14 and 24 *div* was further assessed, whilst preliminary *in vivo* verification was also presented. After 14 *div*, cells formed flat polyclonal bodies on the 2D control, whilst on the Fmoc-DIKVAV astrocytes organised into clusters which formed short networks of cells - dense areas of cell nuclei (‘nodes’) with cellular processes extending to the next node. These networks were also observed at 24 *div*, with greater interconnectivity between each node (Figure 7-3 A). As in the previous section, the three-dimensional growth of cells on Fmoc-DIKVAV and the 2D control cultures required different seeding densities (20,000 cells/well in the 2D controls and 400,000 cells/well on Fmoc-DIKVAV). The network cell density, calculated as (number of cells) / (area covered by F-actin’ staining) (Figure 7-3 B), was similar regardless of the time point or the culture environment. As networks did not form in 2D, cells that were adjacent and touching each other were used to calculated cell area coverage and thus density. In both culture conditions, this cell density was greater than the theoretical maximum calculated from using initial seeding numbers and the total well plate area available for growth. This indicated that both systems support further growth of astrocytes at 2 and 3 weeks *in vitro*, and that Fmoc-DIKVAV supports a significantly greater density of growth through the provision of a third dimension to facilitate growth. This greater density in the Fmoc-DIKVAV cultures was accompanied by smaller cell area coverage in the XY plane, as indicated by cell body coverage measurements (Figure 7-3 C).
Figure 7.3: Primary astrocytes cultured for 14 or 24 div on 2D TCP or 3D Fmoc-DIKVAV hydrogel. A) Immunocytochemistry of: GFAP (red), F-actin (green), Hoechst (blue), and merge. B) Astrocyte density, dotted lines indicated theoretical maximum initial seeding density. C) Total area covered by astrocytes cell bodies. D) Network length of astrocytes grown on Fmoc-DIKVAV. Scale bar = 200 μm. Values are mean ± SEM.
At both time points, astrocyte cell bodies covered a significantly greater area in the 2D control compared to those grown in Fmoc-DIKVAV, (similarly to Figure 7-2 C, where astrocytes were grown underneath Fmoc-DIKVAV), due to the flattened morphology observed on the 2D surface of TCP, compared to the more ‘in vivo like’ morphologies observed in three-dimensional Fmoc-DIKVAV cultures. Despite this increased cell body area coverage, significantly greater networking of cells as indicated by the network length (Figure 7-3 D) was observed in the Fmoc-DIKVAV cultures. These data demonstrate the ability of Fmoc-DIKVAV to support and maintain astrocyte cell growth for longer culture periods, with a unique and first-of-its-kind 3D cellular network formation.

Whilst these in vitro results are interesting of themselves, it is important to ask how they translate to in vivo applications. Preliminary data following Fmoc-DIKVAV + 5 mg/mL fucoidan implanted in vivo, 22 days after traumatic injury to the caudate putamen of mice is presented. Astrocytes infiltrated the implanted hydrogel (Figure 7-4 A-B) replicating the network observed in vitro at the same time point (Figure 7-4 C).
Figure 7-4: Astrocyte network formation at A) 22d post injury and implantation of Fmoc-DIKVAV + 5 mg/mL fucoidan, B) Zoomed field of view of the network (depth colour coded projection of GFAP+ astrocytes), C) 24 div cultured on top of Fmoc-DIKVAV, immunostaining of GFAP (red), F-actin (green), and Hoechst (blue), and D) Storage moduli of Fmoc-DIKVAV control (no incubation) or Fmoc-DIKVAV incubated for 21 and 60 days. Storage and loss modulus measurements for each group shown in Figure S 7-2. Dotted line indicates the injected hydrogel in vivo.
To our knowledge, the formation of extensive astrocyte networks has not previously been reported in the literature. We attribute this extensive networking to factors such as degradation of the material in vitro/in vivo and the morphological cues provided by the nanofibres within the hydrogel. After 21 days incubation (37°C, 5% CO₂), the moduli of Fmoc-DIKVAV (Figure 7-4 D) was significantly less than the control (no incubation, ~3000 to 400 Pa), and even further reduced (~200 Pa) when incubated for 60 days. It is possible that the changing moduli of this material could have a changing influence on astrocytes grown in the hydrogel. Importantly, due to the versatility of our minimalistic SAP system we can easily tune and optimize parameters such as peptide charge and fibre bundling to influence the degradation profile. Astrocyte size, elongation, and overall cell complexity has been associated with stiff (10 kPa) substrates [134], and while we do not observe increased cell size on the compliant Fmoc-DIKVAV, we do observe a high degree of complex networking and interactions between cells. The various and possibly interactive influences of mechanical, morphological, and potentially biological cues (IKVAV sequence) on astrocyte network formation requires further elucidation.

7.3 Conclusion

The behaviour of astrocytes in vivo and in 2D cultures is known to be very different. Thus development of 3D culture environments that better reflect their in vivo behaviour, is a key challenge for tissue engineers. Here, we have presented a co-assembled system of Fmoc-DIKVAV and fucoidan which has a greater suppression of LPS-stimulated astrocyte proliferation compared to soluble fucoidan, whilst also instigating morphological changes. The co-assembled system of Fmoc-DIKVAV and fucoidan suppresses the astrocyte proliferation induced by LPS and leads to extensive networks
of fibres formed by the astrocytes. The network formation depends on the presence of Fmoc-DIKVAV and is observable in vivo. Thus Fmoc-DIKVAV may prove to be a biomimetic 3D culture environment and a tissue engineering construct for use in treating traumatic brain injury.

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7.5 Supplementary figures

Figure S 7-1: Astrocyte morphology change with exposure to B-D) 10% Fmoc-DIKVAV, F-H) 20% Fmoc-DIKVAV, J-L) 40% Fmoc-DIKVAV, N-P 80% Fmoc-DIKVAV, and A,E,I,M) 2D controls.

Immunocytochemistry for GFAP (red), F-actin (green), and Hoechst (blue). 'Gel on': hydrogel was replenished every 7 days, 'Gel-on-off': hydrogel was replaced with CD MEM after 7 days, 'Gel on-off-on': hydrogel was replaced with CD MEM after 7 days, and re-added at 14 days, with total culture period of 21 div.
Figure S 7-2: Rheological measurements of Fmoc-DIKVAV control and Fmoc-DIKVAV incubated for A) 21 days or B) 60 days, showing a decrease in moduli after incubation.
CHAPTER EIGHT

Conclusion & Future Perspectives

8.1 Integrating astrocytes and biomaterials

This thesis describes the development of two novel biomaterial systems with the potential to control the astrocytic response in a stab model for traumatic brain injury. Many potential treatment strategies for neurodegenerative diseases or injuries focus on reinnervation of the lesion site, however, these treatment strategies must also consider the brain’s inflammatory response, and the complex action of astrocytes.

In this thesis, the influence of electrospun nanofibres functionalised with a novel galactose-presenting polymer, and a co-assembled self-assembled peptide hydrogel and fucoidan system on astrocyte morphology and behaviour was investigated. These biomaterial systems have been investigated in both in vitro and in vivo, extending the understanding of astrocyte interactions with these material systems. The approach used in this research demonstrated the importance of gaining a detailed understanding of astrocytes during their response to injury. We used a range of analysis techniques to better assess their response to our engineered biomaterial systems. The work presented in this thesis interfaces the fields of astrocyte biology and materials engineering, which is essential for developing a tissue engineering treatment strategy for traumatic brain injury.
Using previous research relating to the anti-inflammatory nature of galactose moieties, we synthesised the novel polymer, PLL-LBA, and attached it to the biodegradable and biocompatible PCL nanofibre scaffolds in Chapter 4. *In vitro* investigation demonstrated the importance of 3D morphological cues in reducing inflammatory markers in astrocytes within the first 4 *div*, reinforcing the importance of 3D culture environments as highlighted in Chapter 6. The impact of galactose presentation was demonstrated at a later culture time point (12 *div*), where only the PLL-LBA-presenting scaffold could maintain the reduced inflammatory profile observed at 4 *div*, accompanied by distinct morphological changes *in vitro*. These promising anti-inflammatory results led to *in vivo* studies, where we found that although astrocyte numbers increased when the galactose scaffold was implanted, so too did the neuronal numbers at 7 days. This neuroprotection can be attributed to the anti-inflammatory action of galactose, which we postulate has altered the phenotype of astrocytes in the acute phase after injury (7 days post injury) from a typically growth inhibitory reactive phenotype to that of a neuroprotective and growth-supportive/-permissive phenotype. These results demonstrated the need to evaluate astrocyte response in conjunction with other system components, such as the effect on the neuronal population, rather than in isolation.

Following on from these promising results, to address geometric limitations associated with implanting nanofibre scaffolds into an odd-shaped lesion, another biomaterial system, Fmoc-self-assembled peptide hydrogels were investigated in Chapter 5. Fmoc-DIKVAV allowed for the high density presentation of the biologically relevant laminin epitope, IKVAV, on nanofibrous structures within a shear-thinning hydrogel. Building upon the significant impact galactose had on astrocyte behaviour and morphology, we
used another saccharide, anti-inflammatory fucoidan, which conserved the self-assembling properties whilst enhancing the biofunctionality of Fmoc-DIKVAV when co-assembled. This co-assembled system was evaluated in vivo, implanted after traumatic injury, where we presented the importance of providing structural support to the lesioned area. The structural support provided by the Fmoc-DIKVAV hydrogels reduced the glial scarring by approximately 50%, and when presenting fucoidan, also resulted in a dramatic reorganisation of the astrocytes within the glial scar. Additionally, the delivery of fucoidan via a co-assembled hydrogel system resulted in long-distance influence on astrocyte shape. This work showed the potential of Fmoc-DIKVAV and fucoidan as a tissue engineering construct that could be further developed for treating traumatic brain injury.

Recognising the importance of both in vitro and in vivo studies of biomaterials to provide a holistic understanding of their performance as a treatment strategy, Fmoc-DIKVAV and fucoidan was investigated in vitro in Chapter 6. The co-assembled Fmoc-DIKVAV and fucoidan had superior anti-proliferative effect on LPS- and IL-1α-stimulated astrocytes, which can be attributed to both the sustained delivery of fucoidan on the fibrils of Fmoc-DIKVAV (as opposed to soluble delivery). For the first-time, we also observed the Fmoc-DIKVAV-induced cytoskeletal reorganisation of astrocytes into ‘networks’. The impacts of these cell-material interactions was significant, given that this network formation was observed in a ‘quasi-3D’ and ‘3D’ Fmoc-DIKVAV culture environments. The network formation was also dependent on the presence of the material, and the culture period, with the hydrogel capable of supporting extensive astrocyte network formation to longer culture time points (24 div). This reorganisation
was interestingly replicated in vivo, demonstrating the promise of translating in vitro results obtained in an Fmoc-DIKVAV culture environment. It is postulated that this network formation could be attributed to a decrease in hydrogel moduli, and could be attributed to the material degradation over time, highlighting a key aspect of future work.

8.2 Future perspectives

This thesis has presented the use of Fmoc-DIKVAV as an in vivo treatment strategy and a 3D cell culture environment for the first time, however, avenues of future work will need to be explored before its full potential in treating traumatic brain injury is realised. Firstly, the mechanical degradation of Fmoc-DIKVAV needs to be thoroughly investigated and potentially modified. It is expected that these results would be an underestimate compared to the degradation that would occur in vivo, and such degradation needs to be characterised.

After the characterisation of the mechanical changes that occur in vitro and in vivo, steps can then be taken to control such degradation. This could include the development of a composite hydrogel that would have controllable degradation, as is the case with many hydrogels. However, these hydrogels do not possess the unique and valuable nanofibrous morphology of Fmoc-SAPs. Therefore, a composite material would combine the desirable aspects of Fmoc-DIKVAV with the controlled mechanics of other systems. This composite material is represented in Figure 8-1.
This thesis has presented galactose and fucoidan as effective anti-inflammatory agents, and demonstrated their impact on astrocytes when delivered via a biomaterial system. To fully harness their potential it is necessary to understand the biological pathways on which these agents act, and the relationship between those biological events and the associated changes in morphology that have been reported in this thesis. As there is a recognised, yet ill-defined, relationship between astrocyte morphology and behaviour in the current literature, this investigation would be of significant value in understanding astrocyte behaviour as well as how biomaterials can influence their morphology and behaviour to improve TBI treatments.

Another avenue for future work would be to determine the exact time point at which therapeutic agents should be delivered to attenuate the cytotoxic behaviours of reactive astrocytes and encourage growth-supportive/-permissive phenotypes. Currently researchers agree that it should be sometime after the acute phase of injury (1-2 weeks post injury), however, this time point would be dependent on the size, severity, and
location of the injury. Then, we would need to develop mechanisms to exert temporal control over the delivery of therapeutic agents from the biomaterials, achieving such behavioural changes in astrocytes. This could include a combination of short-term and long-term delivery mechanisms such as enzymatic-triggered release from hydrogels such as PEG, diffusion from delivery vehicles like short nanofibres, and covalent tethering, as shown in Figure 8-2 [42, 70, 77]. The short term mechanisms can be used to target the therapeutic agents for astrocyte behaviour change whilst the long-term mechanisms can be used to encourage cell infiltration and growth into and across the lesion site.

![Diagram](image)

**Figure 8-2:** Varying delivery mechanisms for therapeutic agents to alter astrocyte behaviour (short-term) and encourage cell growth across the lesion for functional repair (long term)

These delivery mechanisms can be incorporated into the hydrogel composite with controllable degradation properties (as mentioned above). This would yield a biomaterial system with appropriate mechanical and morphological cues, as well as biological relevance and temporal delivery of therapeutic agents to encourage a growth-supportive phenotype in astrocytes and long-term cell growth, as shown in Figure 8-3. This complex composite biomaterial system would hold great promise for the treatment of traumatic brain injury, capable of improving repair at the lesion site, and could even incorporate the transplantation of cells to facilitate cell growth across the lesion. Tissue
engineers will have to be cognizant of any compatibility requirements of using materials and drugs in the one system.

Finally, to spur the progress of research into astrocyte response after traumatic injury, and to better inform the development of treatment strategies, further work to develop a 3D culture environment is required. This thesis presents a promising biomimetic environment, Fmoc-DIKVAV, capable of supporting astrocyte growth in vitro. Combining Fmoc-DIKVAV, or, potentially the composite material with controllable degradation as suggested above, with bioreactor technology would mimic the dynamics of the in vivo environment, providing critical mass transport and environmental control. Such a cell culture environment would better model astrocyte behaviour in vivo and thus yeild results that can be better translated from the lab to the clinic.

The future work and directions presented in this thesis requires a collaborative approach between tissue engineers and biologists to realise and subsequently harness the reparative potential of astrocytes using novel biomaterial treatment strategies for traumatic brain injury.
References


Appendix A

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Astrocyte phenotyping is primarily based on morphology. Active astrocytes have several fine processes that interweave around neuronal cell bodies and their processes, whilst reactive astrocytes are characterised by hypertrophic processes, as well as an increase in glial fibrillary acidic protein (GFAP) expression. However molecular markers such as GFAP, galectin-1 (Gal-1), neuroblast differentiation-associated protein (AHNAK), and filamentous actin (F-actin) may also point to astrocyte phenotype. GFAP is upregulated in the inflammatory phenotype of astrocytes and we have shown that minor astrocyte processes express AHNAK and co-localise with GFAP to provide a more holistic view of the astrocytic ‘armour’. Gal-1 is a signalling protein that has been implicated in various inflammatory pathways, and is upregulated in reactive astrocytes (hypertrophic and GFAP-positive) after CNS injury. Stress fibre formation and actin cytoskeletal changes are commonly observed in astrocytes after injury, typically in their inflammatory mode. These four markers can be used to augment morphology in identifying the astrocyte phenotype.

Current 2D cell culture systems do not properly recapitulate the in vitro environment, and hence, may not accurately reflect in vivo cell morphology and behaviour. Consequently, we established a 3D culture system with electropun scaffolds to mimic the fibrous structure of the extracellular matrix (ECM), whilst also presenting biologically relevant cues, to study common inflammatory markers in astrocytes. Electropun nanofibres provide a high surface area-to-volume ratio, high porosity and a fibril size which are similar to the ECM and are thus attractive materials to use in tissue engineering. More information about the electropinning process can be found in the following reviews. We have previously shown that primary astrocytes cultured on poly(ε-caprolactone) (PCL) electropun nanofibre scaffolds infiltrate into the 3D scaffold, reduce GFAP expression and upregulate the expression of BDNF and the I-glutamate transporter SLC1A2. Astrocytes cultured on 3D PCL nanofibres in the presence of Rho Kinase (ROCK) inhibitors, develop a cytotrophic phenotype. Astrocytes have also been successfully maintained in biofunctionalised 3D culture systems, with increased I-glutamate uptake when cultured on poly(ε-lactic acid) nanofibres coated with fibronectin, and reduced cytoskeletal stress when cultured on polyether based polyurethane nanofibres coated with poly-ornithine and laminin. These studies demonstrate the benefits of biofunctionalising a 3D scaffold to provide the mechanical and biochemical cues necessary to recapitulate the 3D in vivo environment, and thus a desirable culture environment to investigate astrocyte biology.

As PCL nanofibre scaffolds induce a cytotrophic phenotype in astrocytes, it is biocompatible and biodegradable, we functionalised these nanofibres using the layer-by-layer (LbL) method. Within the LbL method, we have used biologically relevant polyelectrolytes - heparin (anti-inflammatory), poly-L-lysine (cell adhesion) and a novel polymer, poly-L-lysine conjugated with lactobionic acid (PLL-LBA), which presents galactose moieties that are of interest. Xyloglucan (containing galactose moieties) attenuates the inflammatory response after implantation, with astrocyte numbers decreasing to almost homeostatic levels after 60 days. It is also understood that galactose, which is implicated in inflammation, bind to galactose, and influence the astrocytic cytoskeleton via the Rho/ROCK pathway (i.e. are anti-inflammatory) enhancing BDNF expression. We have investigated randomly aligned PCL nanofibre scaffolds, which have previously supported extensive neurite infiltration, compared to aligned scaffolds which facilitated perpendicular neurite growth around the implanted scaffold.

Here we investigated the impact of functionalised PCL nanofibres on the morphology and hallmark proteins associated with inflammatory astrocytes in vitro, and on neuronal survival in vivo, presenting unique data to demonstrate the potential of such a novel biomaterial system to influence astrocyte behaviour for central nervous system inflammation attenuation.

Experimental

1. Material fabrication and characterisation

1.1 2D PCL scaffold preparation. 2D PCL (Sigma-Aldrich, St Louis, MO, USA, molecular weight = 70 000–90 000) substrate was compression molded at 80 °C and quenched in an ice bath.

1.2 3D nanofibre scaffold preparation. Poly(ε-caprolactone) (PCL) was obtained from Sigma-Aldrich (St Louis, MO, USA, molecular weight = 70 000–90 000). Polymer solutions of 13% (w/v) were prepared for electropinning by dissolving PCL in a solution of 3:1 chloroform (Chem Supply Pty Ltd, Australia) and dichloromethane (Sigma Aldrich, MO, USA), magnetically stirred at room temperature for two hours.

The 13% PCL solution was placed in a plastic syringe with an 18 gauge needle for electropinning at 1.25 kV cm⁻¹. An accelerating voltage of 16 kV was applied to the needle tip, with a grounded rotating mandrel used as the collector. The collector was covered in aluminium foil for easy removal of nanofibre scaffolds, with a working distance of 10 cm used throughout the electropinning process. Mandrel rotation of 5 ms⁻¹ was used to collect randomly oriented nanofibres. Once removed from the collector, the scaffolds were then stored in a desiccator until use for cell culture and implantation.

1.3 Conjugation of poly-L-lysine and lactobionic acid. 50 mg of poly-L-lysine hydrobromide (PLL, Sigma Aldrich, MO, USA, molecular weight = 30 000–70 000), 40 mg of lactobionic acid (LBA, Tokyo Chemical Industry, Tokyo, Japan) and 3 mL of dimethyl sulphoxide (DMSO, Ajax Finechem, Australia) were combined and stirred on heat (75 °C) for two hours. 40 mL of diethyl ether (Merck, MA, USA) was added and left to stir at 100 °C until the PLL-LBA had precipitated out. 40 mL of de-ionised water was then added and left to stir for 5 minutes. The solution was dialysed in cellulose membranes (MWCO: 12–14 000, Spectra/Por, USA) against water and then lyophilised for four days (Christ Alpha 1-2 LD Plus Freeze Dryer, John Morris). This conjugated polymer is denoted as “PLL-LBA”, with the lactobionic group attached to PLL presenting the galactose moiety on the modified PLL.

1.4 Nuclear magnetic resonance. 10 mg of PLL-LBA was dissolved in 0.6 mL deuterium oxide (D₂O, Sigma Aldrich,
MO, USA) and transferred into a NMR tube (NE:UL3-8°, New Era Enterprises, USA), which was placed in the autosampler of the NMR machine (Varian MR-400).

1.5 Layer-by-layer functionalisation

2D scaffolds. 2D PCL samples were submerged in 70% ethanol (Merek, Australia) for 10 minutes followed by three 5 minute phosphate-buffered saline (PBS, pH 7.4) washes. Samples were soaked in 20 mg mL⁻¹ polyethyleneimine (PEI, 50% in H₂O, Sigma Aldrich, USA) in PBS buffer (pH 7.4) for two hours, then rinsed in PBS. Samples were then soaked in 5 mg mL⁻¹ heparin sodium salt from porcine intestinal mucosa (Hep, Grade 1A, ≥180 USP units per mg Sigma Aldrich, USA) in PBS for 15 minutes, followed by a 10 minute PBS buffer wash. Then soaked in 1 mg mL⁻¹ poly-L-lysine hydrobromide (PLL; Sigma Aldrich, USA; molecular weight = 30000-70000), or PLL-LBA, in PBS for 15 minutes, followed by a 10 minute PBS wash. The deposition cycle was repeated for 5 bilayers. Samples were crosslinked with 2% 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Thermo Scientific, Japan) in PBS overnight, followed by three 10 minute PBS washes. Excess liquid was removed from the samples using Kimwipes, dried overnight and subsequently stored in a desiccator.

3D scaffolds. 6.5 × 2.5 cm² pieces of PCL nanofibre scaffolds were submerged in 70% ethanol (Merek, Australia) for 10 minutes, followed by three 5 minute PBS washes. Samples were soaked in 20 mg mL⁻¹ polyethyleneimine (PEI, 50% in H₂O, Sigma Aldrich, USA) in PBS buffer (pH 7.4) for two hours, then rinsed in PBS. The samples were then secured to glass slides that were inserted into the StratoSequence 6 (nanoStrata Inc., USA) sample holder. The samples were then submerged in 5 mg mL⁻¹ heparin (heparin sodium salt from porcine intestinal mucosa, Grade 1A, ≥180 USP units per mg Sigma Aldrich, USA) in PBS for 15 minutes, followed by three 10 minute PBS washes. Samples were then submerged in 1 mg mL⁻¹ poly-L-lysine hydrobromide (PLL; Sigma Aldrich, USA; molecular weight = 30000-70000), or PLL-LBA, in PBS for 15 minutes, followed by three 10 minute PBS washes. This cycle was repeated for 4 bilayers. Samples were crosslinked with 2% 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Thermo Scientific, Japan) in PBS overnight, followed by three 10 minute PBS washes. Excess liquid was removed from the samples using Kimwipes, samples were then hung on clips in an enclosed chamber overnight to dry, then stored in a desiccator.

Samples with heparin and poly-L-lysine layers are denoted as "PCL-Hep + PLL," and samples with heparin and PLL-LBA are denoted as "PCL-Hep + PLL-LBA."

1.6 Scanning electron microscopy. Samples were sputter coated with platinum at 20 mA for one minute. All scanning electron microscopy (SEM) images were taken under 3 kV with a working distance of 3.5 mm on a Zeiss UltraPlus FESEM, using a 20 µm aperture. The average diameters of the fibres were determined using Image J (NIH) to measure a total of 20 fibres across four different samples. Results are expressed as mean ± SEM.

1.7 Contact angle measurements. Contact angle measurements were performed with a KSV CAM200 contact angle goniometer (KSV Instruments) at room temperature, using the sessile drop method with deionised water. A 1.2 mm diameter needle was used to introduce a 6 µL water drop onto the sample surface by gravity. The measurements were taken over three replicate samples and averaged.

1.8 Quartz crystal microbalance and dissipation (QCM-D).

Gold coated quartz crystals were cleaned by UV/ozone treatment for 20 minutes followed by washing in piranha solution consisting of deionised water: ammonia: hydrogen peroxide (5:1:1) before being rinsed thoroughly in deionised water. The crystals were then activated in 3 mM methyl-3-marcaptopropionate (Sigma-Aldrich, St Louis, MO, USA) in absolute ethanol for 8 hours in preparation for coating with a thin coating of PCL. The alkane-gold crystal was then attached to a house built spin-coater with a drop of 0.05 g mL⁻¹ PCL and tetrachloroethane (Sigma-Aldrich, St Louis, MO, USA) solution being placed on the centre of the crystal, and spun for 1 minute at 4500 rpm to obtain a PCL thin film on the crystal. SEM was used to optimise the process and confirm complete coating with the absence of pin holes in the thin film (data note shown). The LB deposition process was then conducted and monitored using QCM-D (Q-Sense E4, Sweden). Initially 0.5 × PBS buffer (pH 7.4) was pumped through the analysis chamber (30 minutes) for stabilisation. 500 µL of each poly-electrolyte and the subsequent PBS washes between electrolyte deposition, were periodically (times replication the LB conditions described above) injected into the chamber over 54 seconds. Recordings were taken at 3rd, 5th and 7th overtone (15, 25 and 35 MHz respectively). Modelling of frequency differences (Q-Sense software), which qualitatively indicates the mass deposited, was conducted using the viscoelastic model.

2. Astrocyte culture on nanofibre scaffolds

2.1 Animals and chemicals. C57/Bl6 mice were obtained from the Florey Institute of Neuroscience and Mental Health, Australia. All experiments received ethical approval from the Florey Neuroscience Institutes Animal Experimentation Ethics Committee ( Ethics Approval Number 07-061). Experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code for the Care and Use of Animals for Experimental Purposes in Australia. All chemicals used to prepare biological solutions, buffers and media were purchased from BDH Laboratories (Australia).

2.2 Scaffold sterilisation. Scaffolds were washed in 80% ethanol for 10 minutes, followed by three PBS washes. Glass inserts (1 mm x 7 mm x 4 mm; to ensure scaffolds did not float) were washed in 80% ethanol and left to dry in a sterile biosafety cabinet for 30 minutes. Scaffolds were then placed in the well plates, with the inserts on top. 5 minutes UV exposure was utilised as the final sterilisation before cell seeding.

2.3 Astrocyte culture. Secondary astrocytic cultures were established from forebrain of postnatal d1.5 mice as previously described previously.22 Astrocytes were subsequently detached using 5 mM EDTA (10 min at 37 °C) and seeded in 96-well plates at 8 × 10⁵ cells per well with the PCL samples (see below; PCL, PCL-Hep + PLL, PCL-Hep + PLL-LBA) in 96 well plates, or
in 24-well plates with coverslips (2D sample, 2 × 10^4 cells per well). Plates were incubated overnight and a full medium change was performed 24 hours later to remove adherent cells. Cells were grown in astrocytic medium (MG: DMEM, Dulbecco’s modified eagle medium, 10% FBS, 100 U ml^-1 penicillin/streptomycin, 0.25% (v/v) Fungizone”) which was subsequently changed every 3 days (in vitro), with each assay was performed on subcultured astrocytes at 14 div and 22 div.

2.4 Imaging and analysis of immunochemical staining. Immunocytochemistry for GFAP, F-actin and β-actin has been described previously, as has the procedure for AHNAK. Photomicrographs of astrocytes in each culture environment were captured to use for analysis. Images were taken at 100× and 200× using an Olympus Camerea C5050 Zoom digital camera through an Olympus IX71 inverted microscope. Two independent experiments produced at least 12 images from triplicate wells for each protein. A threshold was set to remove background staining (the same threshold value was used for all images being analysed) and the area above threshold was measured using ImageJ (NIH). Image data was analysed using a repeated measures two-way ANOVA and the appropriate Bonferroni post hoc tests on Prism (v.6.0; GraphPad, USA), and comparisons of the area above threshold were made. Values are expressed as mean ± SEM.

3. In vitro scaffold implantation

3.1 Animals. C57BL/6 mice were obtained from the Florey Institute of Neuroscience and Mental Health, Australia. All experiments received ethical approval from the Florey Neuroscience Institutes Animal Experimentation Ethics Committee (Ethics Approval Number 12-051). Experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code for the Care and Use of Animals for Experimental Purposes in Australia.

3.2 Scaffold insertion. Scaffold implantation follows a similar procedure as previously reported. Briefly, C57 Bl/6 mice (male and female) were anaesthetised with 1-2% Isoflurane and placed in a stereotactic frame whereby a small hole was drilled into the skull (from bregma: AP: +0.5 mm, L: -2.0 mm). Three types of scaffolds were tested, control PLL, PLL-Hep + PLL, and PLL-Hep + PLL-LBA, as well as a control stab. Scaffolds were sterilised with 80% ethanol and rolled into small capsule sizes and inserted into a 21G guide needle. The guide needle was attached to a Hamilton needle which was lowered through the hole in the skull 3.0 mm deep. The guide needle was then raised and the plunger with the scaffold was descended another 0.5 mm deep to ensure scaffold insertion into the caudate-putamen.

Each type of scaffold was inserted into six mice; three of which were killed at 7 days post-surgery, and the other three at 21 days post-surgery. Two mice also received a needle injury without the scaffold, representing a stab wound, and one killed at 7 days and one killed at 21 days. In total 20 mice were used.

3.3 Tissue preparation. Mice were perfused 7 days and 21 days post-surgery with warmed (37 °C) 0.1 M PBS, followed by 35 ml of chilled 4% PFA (Sigma Aldrich, Australia) in 0.1 M PBS and 0.2% picric acid (4 °C; pH 7.4). The brains were removed and post-fixed for 1 hour, then left over 2 nights at 4 °C in a 30% sucrose PBS solution. The brain tissues were then frozen and cut serially with a cryostat. The striatum was cut at 20 μm thick in a 1:10 series on to slides double coated with 0.1% chrome alum (Ajax Chemicals, Australia) and 1% gelatine (Sigma Aldrich, Australia). Slides were individually heated by a glass hot rod to ensure tissue adhesion; and no loss of scaffold material throughout staining process later.

3.4 Immunohistochemistry. Anti-GFAP immunohistochemistry was performed by incubating one series of sections through the striatum, at 1:500 rabbit × GFAP antibody (Dako, Australia) over 2 nights at room temperature (In PBS, 0.3% Triton X-100 and 1.0% normal donkey serum). After several washes and 6% goat serum block for 15 minutes, slides were incubated for a further 3 hours at room temperature in secondary antibody donkey, anti-rabbit Alexa Fluor 594 at 1:100, (Millipore, Australia). Sections were cover-slipped using Dako fluorescent mounting medium (Dako, Australia).

NeuN immunohistochemistry was performed by incubating one series of sections through the striatum, at 1:600 mouse × NeuN antibody (Millipore, Australia) over 2 nights at room temperature (In PBS, 0.3% Triton X-100 and 1.0% normal goat serum). After several washes and 6% goat serum block for 15 minutes, slides were incubated for a further 3 hours at room temperature in secondary antibody goat anti-mouse Alexa Fluor 488 at 1:100, (Millipore, Australia). Sections were cover-slipped using Dako fluorescent mounting medium (Dako, Australia).

3.5 Cell counts. GFAP labelled astrocytes were counted ‘close’ to scaffold (at the scaffold-parenchyma interface) and ‘far’ away from scaffold (600 μm away from scaffold). The Stereoinvestigator program (MicroBrightField, VT, USA) was used to derive counts at pre-determined fractionator intervals of x = 80 μm, y = 80 μm, and a counting frame of x = 70 μm, y = 70 μm. Counts were made using the 60× oil lens. Total markers counted were then divided by the total area (μm²) to achieve a final density of cells/astrocytes per area (μm³). The same method was used to count the NeuN cell numbers.

4. Data analysis

All values presented are mean ± SEM from replicate determinations from multiple independent experiments. Data were subjected to unpaired student t-tests using GraphPad Prism v.4.0 (GraphPad, San Diego, CA, USA).

Results and discussion

1. Conjugation of polyc(l-lysine) and lactobionic acid presents galactose moiety

The conjugation mechanism of PLL and LBA allowed the presentation of the galactose moiety of the LBA lactone form of LBA (Fig. 1). The attachment was confirmed using NMR, and the percentage of LBA attachment to PLL was 30–40%. This synthesis procedure is an easy, reproducible method of presenting multiple biologically relevant moieties in one molecule.
which can then be used in a multi-faceted system to provide physical and biochemical signals in vitro and in vivo. A schematic of the LBL process and a summary of the sample groups used in this study are shown in Fig. 2.

2. Nanofibrous morphology maintained after layer-by-layer functionalisation

PCL nanofibre scaffolds retain a nanofibrous morphology, and scaffold porosity following functionalisation with heparin, PLL, and PLL-LBA, whilst enhancing the biochemical profile of PCL nanofibre scaffolds. SEM images of electrospun nanofibres are shown in Fig. 3, before (A and B) and after (C-F) LBL functionalisation. Nanofibrous morphology is similar in each group, regardless of whether functionalisation was with Hep + PLL (C and D) or Hep + PLL-LBA (E and F). Changes from the observed smooth fibre surface (A and B) to the ‘rippled’ fibre surface after functionalisation (C-F) indicate the presence of the covalently bonded ‘layers’ as a result of the LBL process. This was observed in SEM images of both the Hep + PLL and Hep + PLL-LBA functionalised nanofibres, implying that the inclusion of the LBA moiety on PLL does not affect the functionalising of PCL nanofibres by PLL-LBA, using the LBL method. The conclusion that functionalisation was similar in Hep + PLL and Hep + PLL-LBA groups was also supported by the finding that average fibre diameter did not change following LBL functionalisation (Fig. 3G). As functionalised scaffolds maintained the morphology, scaffold porosity and average fibre diameter of control scaffolds, we contend that the presentation of chemical cues due to functionalisation will be the mechanisms for any observed differences in cells cultured on the functionalised scaffolds.

Quartz crystal microbalance and dissipation (QCM-D) and water contact angle were then used to test this conclusion. A small increase was found in the change of dissipation after the first heparin and PLL deposits (Fig. 4), which is consistent with previous findings that used QCM-D to characterise LBL functionalisation. The ΔΔt was larger after the second heparin adsorption, and increased further after every layer deposition, regardless of whether PLL or PLL-LBA were used as the polyelectrolyte. These data confirm consistent mass deposition after each layer during the functionalisation process, up to four bilayers.

Water contact angle measurements of layer-deposition on PEI-activated 2D PCL samples show clearly alternating contact angles for the heparin and PLL or PLL-LBA polyelectrolyte multi-layers after the first four terminating layers (Fig. 5). The alternating contact angles for the heparin (~60°) and PLL or PLL-LBA (~55°) terminating layers demonstrate the hydrophobic and hydrophilic properties of the respective layers, and also confirm the presence of distinctly different layers throughout the LBL process. There is a trend for a lower contact angle for
the PLL-LBA layers compared to PLL, which is consistent with the presence of hydroxyl groups from the LBA. These hydroxyl groups result in greater hydrophilicity for the PLL-LBA layers, but do not result in a significant difference in hydrophilicity compared to the PLL layers.

Using the LBL method, PCL nanofibres have been effectively functionalised with heparin and PLL or PLL-LBA, confirmed by SEM, QCM-D and water contact angle analysis. The LBL approach demonstrated here adds to a range of strategies from our team for the presentation of molecular entities to regulate cellular architecture and biology.²⁶,³⁵ and is recognized to have diverse applications in biomedicine.²⁶,³⁵ Importantly, this simple, reproducible technique has allowed for the presentation of multiple biologically relevant cues to potentially influence the behaviour of astrocytes in vitro and in vivo, with implications in controlling the CNS inflammatory response.

3. In vitro assessment of the effect of scaffold functionalisation on astrocyte protein expression

Murine astrocytes were grown on 2D PCL, PCL nanofibres (3D PCL), PCL nanofibres functionalised with Hep + PLL (PCL–Hep + PLL) and PCL nanofibres functionalised with Hep + PLL-LBA (PCL–Hep + PLL-LBA) for 4 or 12 div (days in vitro, equivalent to 14 and 22 div, as secondary astrocytes were generated and plated at 10 div). Protein expression by astrocytes sub-cultured on the PCL samples was quantified using immunolabelling and morphometric analysis.

Astrocyte phenotype has previously been considered binary, being either healthy (good) or reactive (bad). On the basis of gene profiling, the notation of A1 and A2 phenotypes to describe reactive astrocytes promoting neuroinflammation and healing respectively (respectively)²⁶,³⁵ have been proposed, a concept paralleling the M1 pro-inflammatory and M2 immunosuppressive notations for microglia (and macrophages).⁴⁰

An alternate perspective is that reactive astrocytes exist on a spectrum of trophic to toxic phenotypes, dependent on the time-point after injury, and its severity. The activation of astrocytes, with cell numbers peaking at 7–10 days post injury, inhibits immediate growth through physical and biochemical barriers, resulting in dystrophic axons.³⁵ This should not, however, be interpreted as being exclusively cytotoxic behaviour. Reactive astrocytes possess cytrophic functions, as their selective ablation results in failed blood-brain barrier (BBB) repair, prolonged infiltration of macrophages, local neuronal death as well as significant functional deterioration.³³ This spectrum of responses demonstrates the importance of reactive astrocytes and the need to understand their complex role within the CNS after injury. We hypothesise that optimal recovery will occur when the existing and currently persistent reactive astrocytosis is, at an appropriate time-point, abated to allow repair and regeneration, with endogenous astrocytes performing their normal cytrophic and supportive functions.

3.1 3D morphology of nanofibres provides a superior culture environment. All 3D culture conditions provided the same morphological cues (as seen in Fig. 3), effectively mimicking the nanofibrous morphology of the native ECM, and thus providing a cell culture environment more representative of the in vitro environment compared to the current 2D environments used to study cell behaviour. At 4 div, expressions of GFAP, AHNAC, Gal-1, and F-actin by astrocytes on all three nanofibrous scaffolds (PCL, PCL–Hep + PLL, PCL–Hep + PLL-LBA) was similar regardless of functionalisation, but lower than by astrocytes cultured on 2D PCL (Fig. 6A–D). This attenuation of expression of proteins typically associated with an inflammatory astrocytic phenotype implies that 3D morphological cues provided by the nanofibrous scaffolds, induce a cytrophic phenotype within a short culture period. We would expect that this would be associated with upregulated BDNF and EATAT2 expression.³⁷

3.2 Galactose moiety maintains an attenuated astrocyte inflammatory phenotype at 12 div. The reduction in expression
of protein associated with an inflammatory profile did not persist when astrocytes were cultured on scaffolds that provided 3D morphological cues (Fig. 6A-D). By 12 div of astrocytes being cultured on 2D PCL, 3D PCL and PCL–Hep + PLL, the expression of all of these four proteins was increased. Expression of these proteins by astrocytes cultured on the 2D PCL and 3D PCL was similar, indicating that at 12 div, the 3D morphology of PCL alone did not control astrocyte protein expression. Similarly at 12 div, presenting heparin or PLL through 3D PCL or PCL–Hep + PLL, did not maintain its effect on the morphology or inflammatory profile of cultured astrocytes, as the expression levels of all four proteins was greater than their respective levels at 4 div of culture. This evidence implies that the initial switch to a cytotrophic profile at 4 div was not maintained and, by 12 div, there was a return to an inflammatory profile. We propose that such a shift to an inflammatory profile can be attributed to the insufficiencies of a static, one-cell type culture environment lacking the cell-cell and cell–ECM dynamics found in vivo, to maintain sensitive primary astrocytes. In future, the use of bioreactors and mixed cultures would create a more dynamic culture environment that would elucidate valuable information to understand astrocyte behaviour in vivo, and thus improve subsequent translation of strategies in vivo.46

In contrast at 12 div GFAP, AHNK, Gal-1 or F-actin expression of astrocytes cultured on PCL–Hep + PLL–LBA remained at 4 div levels and was significantly lower than under all other culture conditions. These observations imply the attenuation of the inflammatory profile was maintained when astrocytes were cultured in the presence of the galactose moiety in PLL–LBA. This difference is most likely a direct result of the coolant presentation of galactose moieties on the surface of the PCL nanofibers, as 3D morphological cues such as presentation of heparin or PLL by PCL–Hep + PLL–LBA did not maintain reduced levels of GFAP, AHNK, F-actin, and Gal-1.

As Gal-1 expression was also reduced in the presence of galactose moieties, it raises the question of its role in identifying any influence of the galactose moiety on the expression of the other proteins (GFAP, AHNK, F-actin) and indeed Gal-1 itself. Following ischemia, Gal-1 upregulation is linked to attenuating both astroglisis and the production of inflammatory molecules (e.g. interleukin-1β), and increased BDNF production by astrocytes.60,61 This finding implies that the presence of the galactose moiety downregulating Gal-1 expression may also lead to lower expression of other “trophic” influences from astrocytes such as IBDNF. The Gal-1 expression of astrocytes from 4 to 12 div did not increase when cultured on PCL–Hep + PLL–LBA, indicating the culture environment presenting LBA on the nanofibers did not induce the anti-inflammatory action of astrocytes associated with Gal-1 (which would thus result in an increased expression). Additionally, the effect of a longer culture period increasing Gal-1 expression (as seen with 2D, 3D, and PCL–Hep + PLL culture conditions) was not observed when LBA was presented on the surface of the nanofibres, further demonstrating the ability of the galactose moiety to influence aspects of the inflammatory response of astrocytes, specifically, via the action of Gal-1.

Increased GFAP expression by astrocytes and hypertrophy are the most commonly used markers of reactive astrogliosis after injury or disease.62,63 Although GFAP expression of astrocytes was reduced on the three 3D nanofibre scaffolds at 4 div, it significantly increased between 4 and 12 div for all culture conditions, with the exception of PCL–Hep + PLL–LBA, where expression remained constant from 4 to 12 div.
Immunolabelling of GFAP with AHNAK or Gal-1 (Fig. 6E) at 12 div confirmed a reduction in GFAP, AHNAK and Gal-1 expression. Astrocytes grew in tight colonies on PCL-Hep + PLL-LBA, in contrast to the widely spread astrocytes observed on 2D PCL, 3D PCL control, and PCL-Hep + PLL (individual staining of AHNAK, Gal-1, F-actin and GFAP shown in Fig. S1, ESI†). These colonies in culture could indicate the localised interaction of astrocytes with galactose moieties on the surface of the nanofibres, inhibiting process elongation and migration. Similarly, F-actin staining also increased for all culture conditions at 12 div with the exception of PCL-Hep + PLL-LBA, with astrocytes also forming tighter colonies compared to all other culture conditions [data not shown].

Patterns of GFAP, AHNAK, and F-actin immunostaining, in concert with reduced Gal-1 expression, were indicative of decreased stellation, with less spindly processes suggesting decreased migration and process outgrowth, although importantly glutamate transporter activity was not compromised relative to other 3D cultures at 12 div [data not shown]. These observations were similar to our earlier findings in astrocytes exposed to the Rho Kinase (ROCK) inhibitor, Fasudil, and demonstrate the persistent effect of the covalent attachment of the galactose moiety, LBA on PCL-Hep + PLL-LBA. Therefore, we propose that the galactose moiety may act as a ROCK inhibitor, acting through Gal-1, as similar changes in morphology and expression have been observed in many other studies.\(^{23,44}\)

4. In vitro assessment of the effect of scaffold implantation after stab injury

The influence of the various 3D nanofibrous scaffolds on the in vitro response of astrocytes to traumatic (stab) brain injury was assessed next. As functionalising the PCL nanofibres with heparin and PLL did not affect astrocyte protein expression in vitro, and astrocyte numbers were fewer in the presence of PCL nanofibre scaffolds than with a stab injury alone,\(^{21}\) we did not use a stab wound as a control or PCL-Hep + PLL scaffold. Rather, the effect of un-functionalised PCL nanofibre scaffold was compared with galactose presentation on PCL nanofibres.

Although the astrocyte response was the focus of the in vitro studies, in vivo studies provide the opportunity to also examine the effect on the surrounding neuronal population. Hence, both neurons (NeuN+ ) and astrocytes (GFAP+) were counted.

4.1 Galactose functionalisation promotes long distance neuronal survival 7 days after injury.

Seven days after injury and subsequent scaffold implantation, GFAP+ astrocytes and neurons (NeuN+) at the edge of galactose presenting scaffolds (PCL-Hep + PLL-LBA) were significantly greater than adjacent to PCL scaffolds (Fig. 7A and B). At a distance (600 \(\mu\)m) from the
two scaffolds, astrocyte numbers were similar but the number of neurons both close to and at a distance from the PCL–Hep + PLL–LBA scaffold was significantly greater than was the case for PCL scaffolds. An increased presence of neurons both close to, and away from the scaffold, and the associated increased number of astrocytes suggests attenuation of the typically growth prohibitive actions of astrocytes observed in the acute time period after injury, and notably at the 7 day time point investigated here. Such attenuation is attributed to the galactose moieties present on PCL–Hep + PLL–LBA, and highlights that greater astrocyte numbers should not be directly correlated with cytotoxic behaviour, and reinforces the need for a holistic view of astrocyte behaviour after injury. We propose that future studies should compare the protein expression profile of astrocytes under the two conditions.

After injury, astrocytes inhibit growth through a physical barrier formed around the lesion, interwining their hypertrophic processes, as well as the secretion of inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs). Additionally, as neurons die after traumatic injury, an increased neuronal survival rate when galactose is presented on the surface of PCL nanofibres is extremely promising. Astrocytes at the scaffold surface would interact with the galactose moieties, which we have shown maintain a non-inflammatory profile of astrocytes in vitro. Given the complex cell-cell signalling that occurs in vivo, it is expected that the attenuation of the inflammatory profile of astrocytes, as demonstrated in vitro, would subsequently influence neuronal survival after traumatic injury in vivo. The exact mechanism of such protection or rescue needs to be investigated further to gain a more complete understanding of the action of galactose-interacting astrocytes on neuronal survival. However, data presented here is a promising proof-of-concept strategy to promote neuronal survival within the acute phase post-injury.

4.2 ‘Homeostatic’ astrocyte and neuron numbers reached at 21 days. The initial difference in astrocyte numbers observed close to the scaffold at 7 days was not present at 21 days, with no significant difference between astrocyte numbers close to the PCL or PCL–Hep + PLL–LBA scaffold. However, there was a significant increase in astrocyte numbers close to the PCL control from 7 to 21 days, with similar counts to those of PCL–Hep + PLL–LBA. We have previously shown that astrocyte numbers peak at ~10 days post injury, so it is reasonable to conclude that astrocytes had not reached peak numbers at seven days, explaining the increased numbers from 7 to 21 days. It is also logical to expect greater astrocyte numbers at the interface of the implanted scaffold, compared to distant from the lesioned area, as significant trauma to the surrounding tissue has occurred, as reflected by the lower neuronal populations. Although more comprehensive analysis of the astrocytic response to these implanted scaffolds is required, these initial findings could suggest that reactive astrocytes (which we did not investigate in vitro) interact more favourably with the galactose moieties than with the control nanofibres, hence, result in higher astrocyte numbers at 7 days, and this is sustained through to 21 days. The greater action of the galactose moieties observed in vivo (within 7 days) is in agreement with the in vitro data which showed a significant effect at 12 div.

Increased cell numbers from 7 to 21 days were also observed within the neuronal populations in both groups: there was a significant increase in neuronal numbers close to and far away from the PCL scaffold, as well as close to the PCL–Hep + PLL–LBA scaffold. There was no significant difference between neuron numbers close to or far away from either of the scaffolds – all of which are similar to the neuron counts far away from the PCL–Hep + PLL–LBA scaffold at 7 days. This evidence demonstrates that the presence of the galactose moieties presented on the surface of PCL nanofibre scaffolds had a significant effect on astrocyte and neuron counts at seven days, but had no significant effect at a longer time point viz. 21 days in vivo. As both astrocyte and neuron numbers are similar in each group at 21 days, our findings suggest a potential ‘homeostatic’ level of cell numbers, within an inflammatory context, has been reached by 21 days.

Although the in vitro data presented here demonstrate that covalently attached galactose moieties on PCL nanofibres have a significant effect on astrocytic and neuronal populations after traumatic injury, the differing changes in astrocyte numbers close to each of the scaffolds from the 7 to 21 day time points (an increase for PCL, no change for PCL–Hep + PLL–LBA), and the associated effect on neuronal survival cannot be elucidated from only cell numbers. Further work to characterise the biological profile of these astrocytes and their morphology (together with their phenotype), and investigate the exact mechanism through which the neuronal population is protected would provide a greater understanding of this promising TBI
treatment strategy, and better inform future development of this system to achieve long-acting biological influence on astrocytes after traumatic injury.

Conclusion
We demonstrated a covalent functionalisation of PCL nanofibres to present galactose moieties which attenuates the inflammatory profile of astrocytes in vitro, and promotes neuron survival after traumatic brain injury in vivo. Galactose functionalisation of PCL nanofibres maintained an attenuated inflammatory profile of astrocytes at a longer culture period, with the same scaffold resulting in increased astrocyte numbers close to the scaffold after implantation into a TBI, accompanied by increased neuron numbers at 7 days post-injury. Although further biological investigation is required to fully understand how cell death behind such neuronal protection, this study presents a promising proof-of-concept for the application of the layer-by-layer functionalisation method to present covalently attached galactose for to influence astrocyte behaviour, with implications for the development of future traumatic brain injury treatment strategies.

Statement of contributions
FLM, JW, KH, and DN contributed to the development of the novel polymer, PLL-LBA. FLM fabricated all nanofibre scaffolds, Lbl. functionalisation, and characterisation. CIL, SO, CC conducted the in vitro testing and characterisation. FRW and KDO contributed to the analysis of in vitro results. DT conducted the in vivo testing and characterisation. FLM, MKH, PMB, DRN wrote the paper with MKH, PMB, and DRN designing the research investigation.

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References
15. S. Fedoroff, L. Ahmed, M. Opas and V. Kalnins, Organization of microfilaments in astrocytes that form in the presence of dibutyryl cyclic AMP in cultures, and which are similar to reactive astrocytes in vivo, Neuroscience, 1987, 22(1), 355-366.
18. T. B. Pessehmann, C. Zandén, Y. De Pablo, F. Kirehhofer, M. Pekna and J. Liu, et al., Bioactive 3D cell culture system


23 Z.-M. Huang, Y. Z. Zhang, M. Kotaki and S. Ramakrishna, A review on polymer nanofibers by electrospinning and their applications in nanocomposites, *Compos. Sci. Technol.*, 2003, 63(Suppl.1), 2223–2233.


Reducing Astrocytic Scarring after Traumatic Brain Injury with a Multifaceted Anti-Inflammatory Hydrogel System

Francesca L. Maclean, Yi Wang, Rohan Walker, Malcolm K. Horn, Richard J. Williams, and David R. Nisbet

Laboratory of Advanced Biomaterials, Research School of Engineering, The Australian National University, Canberra, Australia
School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, Australia
Centre for Brain and Mental Health Research, Hunter Medical Research Institute, New Lambton Heights, Australia
Flerey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Australia
Department of Medicine, University of Melbourne, St. Vincent’s Hospital, Fitzroy, Australia
School of Engineering, RMIT University, Melbourne, Australia
BioFuel3D, St. Vincent’s Hospital, Fitzroy, Australia

Supporting Information

ABSTRACT: Traumatic brain injury results in devastating long-term functional damage due to the growth inhibition of the inflammatory response, and in particular, the complex response of astrocytes. Sustained, nonsteroidal anti-inflammatory approaches that can attenuate this response are of interest to improve therapeutic outcomes, particularly when combined with a tissue engineering construct that recapitulates the physiological microenvironment to facilitate functional repair. Here, we present a multifaceted, therapeutic extracellular matrix mimic consisting of a coassembled scaffold with a laminin-inspired self-assembling peptide hydrogel, Fmoc-DIKVAV, and the anti-inflammatory macromolecule, facoidan. At 7 days post-injury, our novel multicomponent hydrogel system presenting biologically relevant nanofibers and the anti-inflammatory facoidan attenuated the primary giall scar to half that of a stab (control) injury. Further, the presentation of facoidan increased the organisation of astrocytes within the giall scar, while also significantly changing the morphology of astrocytes distal from the administered hydrogel and further into the parenchyma. This demonstrated that the anti-inflammatory facoidan, present on the surface of the Fmoc-DIKVAV nanofibers, causes a change in astrocyte phenotype post-injury attenuating “reactive” astrocites. For the first time, we present a multicomponent tissue engineering construct to promote a growth-permissive environment in vivo and, thus, increase the potential for repair and regeneration after traumatic brain injury.

KEYWORDS: hydrogel, facoidan, astrocyte, phenotype, central nervous system

1. INTRODUCTION

Unlike many ‘frontline’ organs, such as the liver or skin, the central nervous system’s (CNS) response to traumatic injury is unable to repair damaged tissues; rather, it limits the damage through a sophisticated inflammatory response. Although the CNS undergoes repair phases similar to those of other organs (inflammation, cell proliferation, and tissue remodeling), its ability to resolve traumatic injury is severely impeded by the growth inhibitory environment created by the inflammatory response, which is orchestrated by many cells, including astrocytes. The cellular and molecular mechanisms of the CNS, and more specifically, the brain, post-injury have been comprehensively reviewed elsewhere. Of particular interest is the

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180
impact of the astrocyte response on the ability of the CNS to regenerate. After injury, astrocytes become reactive, characterized by an increase in intermediate filament protein expression (including glial fibrillary acidic protein (GFAP), and vimentin), hypertrophy, and proliferation near the injury site. Reactive astrocytes are essential for inflammatory cell containment and preventing secondary degeneration through the demarcation of the lesion site through the formation of what is commonly known as the glial scar, a mesh-like structure of interwoven astrocyte processes. Astrocytes forming the glial scar, as well as pericytes, fibroblasts, and inflammatory cells, produce growth-inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) which are key to inhibiting growth immediately after injury. The physical and chemical barriers created postinjury are essential for arresting secondary degeneration, but this same behavior of astrocytes also led to the perception that they impede regeneration after injury. Astrocytes have a complex and crucial role in the repair and regeneration processes to follow injury as likely therapeutic targets to facilitate regeneration after injury, as their behavior can be manipulated to yield a growth-permissive or -supportive environment. Consequently, mechanisms that control astrocyte behavior warrant further investigation.

While astrocytes initially inhibit postinjury regeneration, repair can be compromised when this inhibition persists. Regenerating axons stall at the border of mature lesions and form dystrophic endbulbs; contrary to initial opinion, the regenerative ability of these endbulbs is retained. This implies that altering the astrocyte phenotype after injury may provide a growth-permissive and stimulatory environment and present additional stimulatory cues which encourage axonal entry into the enervated regions. Thus, a treatment system that attenuates the inhibitory response of astrocytes, while concurrently providing a microenvironment to facilitate axons to traverse a lesion, should be of therapeutic value. Such a multifaceted system could then be optimized to temporally control astrocyte phenotype, which would allow the important inflammatory action that occurs in the acute phase, while harnessing growth-permissive and -supportive astrocyte action within the chronic phase after injury. However, the current challenge is to first develop a treatment system which can provide biological, chemical, and physical cues to the injury site to change the morphology and thus phenotype of astrocytes.

Biomaterial scaffolds are an ideal candidate to deliver biological cues to direct astrocytes toward a growth-permissive or -supportive phenotype to allow axonal infiltration into the injury, while also providing a biologically relevant microenvironment that supports cell adhesion, growth, and differentiation. Various scaffolds selected for (one or more) properties that mimic the extra-cellular matrix (ECM), and, increasingly, provide specific biochemical cues in vivo which can be controlled spatially and temporally to facilitate cell growth, differentiation, and proliferation have been examined as treatment strategies for neural regeneration. Here, we investigate fluoromethyloxycarbonyl-capped self-assembled peptide hydrogels (Fmoc-SAPs) because their nanofibrous structure can also provide biochemical cues, while their sheer thinning and void-filling properties allow for effective microinjection into a range of tissues. Fmoc-DIKAV, a laminin-inspired peptide sequence that self-assembles via π−π stacking of the aromatic groups and β-sheet interactions, is of particular interest for neural applications. This self-assembled process allows for high-density presentation of the biologically relevant IKVAV sequence, which promotes neurite outgrowth when presented on a biomaterial scaffold, and is of biological relevance for neural tissue engineering contexts. The modulus of this material system is of a similar magnitude to that of various (scar-free) regions of the brain such as the white and gray matter. These characteristics presented in a single material make Fmoc-DIKAV an ideal candidate to alter the astrocytic response and, ultimately, facilitate repair and regeneration after injury, as has been investigated here. We have previously shown that the supramolecular assembly of Fmoc-SAPs with bioactive macromolecules provides a novel functionalization strategy to improve the capacity of the scaffolds to control cells. Of interest is the anti-inflammatory sulfonated polysaccharide, fucoidan, which has been shown to inhibit the production excessive nitrous oxide (NO) and prostaglandin E, and attenuated the expression of pro-inflammatory cytokines in lipopolysaccharide-induced BV2 microglial cells. Moreover, fucoidan distributes itself within an Fmoc-FRGDF supramolecular scaffold to downregulate inflammatory cytokine expression in cancer cells; thus, combining fucoidan with Fmoc-DIKAV is a promising anti-inflammatory material construct for traumatic brain injury (TBI). Here, we report on the development and delivery of a growth-supportive physical and biochemical environment enabled by the Fmoc-DIKAV scaffold, functionalized with the anti-inflammatory properties of fucoidan, as a multifaceted biomaterial system with the potential to attenuate the inflammatory astrocytic response after traumatic brain injury and improve therapeutic outcomes.

2. METHODS

2.1. Solid-Phase Peptide Synthesis. Fmoc-DIKAV was synthesized using solid phase peptide synthesis, at a molar scale of 0.4 mmol in a custom built reaction vessel. Fmoc protected amino acids, O-benzotriazole-N,N,N′,N′′-tetramethyluronium-hexa-fluorophosphate (HBTU) and hydroxybenzotriazole (HOBr), and Wang-based resins were purchased from GL Biochem (China), and all other chemicals were bought from Sigma-Aldrich. Dimethylformamide (DMF) was dried for a minimum of 2 h with 4 A molecular sieves before use.

Amino acids were added to the resin amino acid through stepwise deprotection of the N-terminal Fmoc protecting group using 20 v/v% piperidine in DMF and a subsequent coupling of the next Fmoc-amino acid in the sequence in a solution of HBTU, HOBr, and N,N′-diisopropylethylamine (DIEA) in DMF. Each deprotection/coupling step was verified using a Kaiser test to detect free amines. Deprotection and coupling is repeated until the DIKAV sequence was synthesized, with the final Fmoc group not removed. The final resin and Fmoc-protected peptide were washed with ethanol and dried under a constant vacuum for 3 days. A solution of trifluoroacetic acid (TFA), 2.5% triethylsilane (TES), and 2.5% deionized water was prepared to cleave the resin–peptide bond. The resin protected peptide was left to stand in the cleavage solution for 2 h, and following cleavage, the peptide solution was filtered through glass wool. Excess TFA was evaporated using a rotary evaporator at 60 °C under a constant vacuum for 7 days, then ground into a fine powder.

2.2. Fmoc-SAP Gel Formation. A total of 10 mg of Fmoc-DIKAV (and 2.5 or 5 mg/mL fucoidan if applicable) was dissolved in 100 μL of deionized water and then 75 μL of 0.5 M sodium hydroxide (NaOH, Becto-Australia) and 2.5 or 5 mg/mL hydrochloric acid (HCl, Merck, Australia) was added dropwise to slowly reduce the pH, with
continuous vortexing, until physiological pH was reached, measured using a microprobe pH meter. PBS was then added to make the gel up to 20 mg/mL, and the gel was exposed to ultraviolet (UV) light for 20 min. Purity of the Fmoc-DIKVAV peptide was determined as ~96% as confirmed via HPCL (Figure S1).

2.3. Fourier Transform Infrared Spectroscopy. Fourier transform infrared spectroscopy was performed using Alpha Platinum Attenuated Total Reflectance FTIR (Bruker Optics). D_{2}O hydrogels for FTIR were formed by replacing water with Deuterium Oxide (D_{2}O, Sigma, Australia) and HCl with deuterium chloride (DCI, Sigma, Australia). A total of 30 μL of Fmoc-SAP gel was placed on the single reflection diamond and spread over the crystal using the pressure applicator. A baseline absorbance of water was subtracted from the total absorbance of the Fmoc-SAPs.

2.4. Rheology. A Kneuss Pro+ Rheometer (Malvern) with a 20 mm smooth flat plate with a solvent trap was used to determine the viscoelastic properties of the Fmoc-SAPs gels. The Fmoc-SAP gel was placed on the plate with a gap size of 0.2 mm. Figure S2 shows a strain sweep of Fmoc-DIKVAV showing the linear viscoelastic (LVE) region between 0.1 and 2%, at 37 °C and 1 Hz with a shear strain of 0.4% was therefore used with a frequency of 0.1–100 Hz.

2.5. Transmission Electron Microscopy. Transmission electron microscopy (TEM) was performed on a HITACHI H4700 TEM at 100 kV. Formvar coated copper grids were glow discharged for 30 s at 15 mA. Negative stains with 0.75% uranyl formate (UF) were used to image the Fmoc-SAP gels, with the procedure fully described in ref.26.

2.6. Implantation of Fmoc-SAP Hydrogel after Traumatic Brain Injury. 2.6.1. Stab Injury and SAP Implantation. C57 BL/6 mice (male and female) were anesthetized with 1–2% isoflurane and placed in a stereotactic frame whereby a small hole was drilled into the skull. A 21G needle was attached to a Hamilton needle which was lowered into the RSH striatum (coordinates AP+0.5 mm, L-2.0 mm, deep –3.0 mm from bregma) to create the stab injury. This injury was used for the stab control group, or, into which the hydrogels were implanted for the three hydrogel groups (see below). Hydrogels were exposed to UV for 20 min prior to implantation and were drawn up into a Hamilton syringe, to which a fine glass capillary was attached. A total of 3 μL of SAP (Fmoc-DIKVAV, Fmoc-DIKVAV + 2 mg/mL fucoidan, or Fmoc-DIKVAV + 5 mg/mL fucoidan) was injected into the stab injury, in increments of 0.5 μL/0.5 mm (moving upward from AP+0.5 mm, L-2.0 mm, deep –3.0 mm from bregma).

Each group was n = 4, and there was an additional group which received only a stab injury (stab control).

2.6.2. Tissue Preparation. Seven days and 21 days post-surgery, mice were perfused with warmed (37 °C) 0.1 M PBS, followed by 35 mL of chilled 4% PFA (Sigma-Aldrich, Australia) in 0.1 M PBS and 0.2% picric acid (4 °C; pH 7.4). The brains were removed and post-fixed for 1 h, left for two nights at 4 °C in a 30% sucrose PBS solution. They were then frozen and cut serially with a cryostat at 20-μm-thick in a 1:10 series onto slides double coated with 0.1% chrome alum (Ajax Chemicals, Australia) and 1% gelatin (Sigma-Aldrich, Australia). Slides were individually heated by a glass hot rod to ensure tissue adhesion and no loss of scaffold material throughout the staining process later.

2.7. Immunohistochemistry. Immunohistochemistry was performed by incubating tissue slides with either anti-GFAP (rabbit antiguillar fibrillary acidic protein, 1:500, Dako, Australia) and anti-OX42 (mouse anti-CD11b (clone OX-42), 1:100, Bio-Rad, Australia) or anti-GFAP (rabbit anti-GFAP, 1:500, Dako, Australia) and anti-SMI32 (mouse antineurofilament H nonphosphorylated, 1:300, Covance, Australia) overnight at room temperature (in PBS, 0.3% Triton X-100 and 1% normal donkey serum). After two 5 min PBS washes, and a 6% goat serum block for 20 min, slides were incubated for a further 4 h at room temperature in secondary antibodies (donkey antirabbit Alexa Fluor 594 and donkey antimonouse Alexa-Fluor 488, Jackson Immuno Research Laboratories, USA, both at 1:100). The secondary antibodies were removed after 4 h and replaced with a hoechst solutions (Hoechst 33342, 1:1000, Invitrogen, Australia) for 5 min, followed by three 5 min PBS washes. Slides were then washed in deionized water,
had the excess water removed, and were covered slips using fluorescent mounting medium (Dako, Australia).

2.7.1. Cell Counts. GFAP labeled astrocytes were counted “close” to the SAP implant (at the SAP-parenchyma interface) and “far” away from scaffold (600 μm away from scaffold). The Stereoinvestigator program (MicroBrightField, VT, USA) was used to derive counts at predetermined fractionator intervals of x = 80 μm, y = 80 μm, and a counting frame of x = 70 μm, y = 70 μm, with the counting area 70 μm (x) by 1000 μm (y). Counts were made using the 63× oil lens. Total markers counted were then divided by the total area (μm²) to achieve a final density of cells per area (μm²).

2.8. Analysis of Astrocyte Scar and Reconstruction. The hemispheric area, lesion/implant area, primary astrocyte scar, and local astrocytic disturbance were analyzed using ImageJ (NIH). Images were taken at 25× on Leica SP8 and cells labeled for GFAP. The primary astrocytic scar and local astrocytic disturbance distances were measured perpendicular to the needle tract.

Cilia Reconstruct was used to analyze individual astrocytes labeled with GFAP, as previously described in ref 27, with some alterations. Astrocytes within the primary scar could not be reconstructed due to their dense, fibrous network. Therefore, astrocytes were analyzed at a minimum distance of 600 μm from the edge of the lesion or implant to enable accurate reconstructions. Maximum intensity projections of z-stacks (7–10 μm) at 25× were converted to black and white, and astrocytes were digitally reconstructed using algorithms executed in Matlab (v2013b, The MathWorks, Inc.). Size filtering was utilized to discern cellular bodies, which revealed information such as cell and process area and cell perimeter. Specifically, immunolabeled astrocytes (GFAP⁺) were segmented from the background using the multilevel Otsu’s thresholding method, which calculates thresholds to minimize the interclass pixel intensity variance between the various classes. The classes used in this analysis were soma, processes, and background. Morphological parameters including cell area and perimeter were automatically calculated using the Matlab image-processing toolbox function “regionprops.” The total number of pixels is given as the area, and the perimeter is length of the boundary of the region. For each animal (n = 4 in each experimental group), three separate images were chosen for reconstruction.

2.9. Statistical Analysis. All values presented are mean ± SEM. Data were subjected to unpaired student t tests using GraphPad Prism v.4.0 (GraphPad, San Diego, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Co-Assembly with Fucoidan Maintains the Fmoc-DIKVAV Nanostructure. We have previously demonstrated the coassembly of the fibronectin-based sequence, Fmoc-FRgDF, with fucoidan and demonstrated that the nanofibrillar architecture is maintained. 25,26 In order to confirm that the material properties making Fmoc-DIKVAV scaffolds an attractive tissue engineering material choice for neural applications were also unaffected, we carried out a series of characterization steps. Transmission electron microscopy (TEM) (Figure 1A–C) showed that the nanofibrinous architecture of the Fmoc-DIKVAV sequence upon self-assembly (A) is maintained with the addition of 2 or 5 mg/mL fucoidan (B, C) to yield a coassembled Fmoc-DIKVAV/fucoidan SAP system. Indicative measurements of the nanobundle width from the TEM images (D) highlighted an increase in bundling with the addition of fucoidan. This was attributed to the charged sulfate groups on the polysaccharide interacting with the charged groups on DIKVAV presented on the surface of the nanofibers (particularly, the positively charged lysine) and subsequently promoting the closer bundling of more of the self-assembled nanofibers. This confirms that the IKVAV containing material maintained the desirable structural properties similar to those observed with the Fmoc-FRGDF SAP sequence, thus demonstrating that the coassembly approach can be used for tissue specific therapies.

Co-assembling fucoidan and Fmoc-DIKVAV also maintained the bulk viscoelastic characteristics of this class of system. Rheological testing (Figure 2A) showed all systems had a storage modulus (G’) greater than the loss modulus (G’’), indicating viscoelastic properties characteristic of a hydrogel. The addition of the fucoidan was also observed to slightly alter the stiffness of the material, in accordance with previous observations. 27 The modulus of Fmoc-DIKVAV was greatest (∼10 kPa), which was maintained in the 2 mg/mL system, yet with a reduction to ∼3 kPa in the 5 mg/mL system. This was attributed to the change in the network formed by the fibrils, modulated by the surface interaction of the fucoidan, resulting in increased bundling as previously observed in Fmoc-FRGDF systems 27 (Figure 1D). In future studies, this modulus could be tuned via this method, in addition to varying the peptide concentration, sequence, or gelation mechanism, and could potentially lead to greater cellular infiltration as the gel degrades in vivo. 21,59

In order to confirm that the bulk peptide interactions were maintained, Fourier transform infrared (FTIR) spectroscopy was conducted. Figure S3 shows the FTIR spectra of the amide I region of a peptide hydrogel made with water, and one with D2O cosolvent. This highlights that while the peak at 1550 cm⁻¹ is significantly affected by the water signal, the peaks in the 1640–1700 cm⁻¹ region match between the systems, with a slight shift attributed to D2O stretching 30 and validates that information from the amide I region is unaffected by the water signal. In the H2O hydrogels, all three groups have a major peak at 1630 cm⁻¹ and a minor peak at 1690 cm⁻¹ (Figure 2B), indicating the presence of β-sheet and stacked carbamates, characteristic of the self-assembling mechanism of our Fmoc SAPs, and unaffected by the coassembly with fucoidan.

3.2. Scar Extension and Astrocyte Organization Are Altered by Fmoc-DIKVAV Hydrogel Implants. After...
Figure 3. (A–D) Dimension analysis of the stab, Fmoc-DIKVAV, Fmoc-DIKVAV + 2 mg/mL fucoidan, and Fmoc-DIKVAV + 5 mg/mL fucoidan groups, 7 days after injury and gel implantation, showing (A) ratio of ipsilateral to contralateral hemisphere area, with intersecting line at a ratio of 1, (B) lesion or gel implant area (*p < 0.05), (C) distance of primary scar (GFAP+), and (D) distance of the local astrocytic (GFAP+) disturbance. (E–H) Depth color-coded projections of astrocytes (GFAP+) 7 days post injury and gel implantation; (E) infiltrating the stab wound (depth range 0–12 μm) and at the edge of the implanted (F) Fmoc-DIKVAV (depth range 0–8 μm), (G) Fmoc-DIKVAV + 2 mg/mL fucoidan (depth range 0–12 μm), and (H) Fmoc-DIKVAV + 5 mg/mL fucoidan (depth range 0–11 μm). Dotted line demarcates the lesion or implanted gel from the glial scar; arrowheads highlight individually distinguishable astrocytes. Scale bar = 20 μm. Note that depth color-coded projections were used to more easily visualize astrocyte processes and overall morphology.

Determining that the material characteristics of Fmoc-DIKVAV were conserved after coassembly with fucoidan, its effect on astrocyte scar formation post-traumatic injury was assessed.

Here, we conducted a comprehensive dimension analysis to assess the effect of Fmoc-DIKVAV/fucoidan hydrogels on astrocyte scar formation after traumatic (stab) injury. It should be noted, that within an inflammatory context, “quiescent” or “resting” references to astrocytes prior to injury are an incorrect simplification, as astrocytes are continually active in a healthy CNS. Therefore, “reactive” will be used to describe glial cells that are responding to insult and is representative of the spectrum of cellular behavior and function observed after injury, while “active” can be used to describe physiological astrocytes.

We performed a range of assessments to determine the extent of this behavior: the ratio of hemisphere areas and the lesion and implant volume, the distances of the primary astrocyte scar and local astrocytic disturbance, and confocal microscope images of the primary scar and astrocytes in the distal parenchyma. These analyses and images enable a holistic overview as to the action of the hydrogels on scar formation within the local environment of the injury.

At 7 days post-injury and subsequent hydrogel implantation, there was no difference in the ipsilateral to contralateral hemisphere ratios between any of the groups, and all ratios were approximately 1 (Figure 3A). This indicates that the implantation of the hydrogels did not result in unusual swelling or contraction of hemisphere volume. There is, however, a significant difference between the lesion/implant area (Figure 3B) of the stab compared to all of the hydrogel groups, and thus this demonstrates the suitability of these material systems to fill the lesion void created by traumatic brain injury (TBI). In the implant groups, the stab wound was filled and therefore supported by the hydrogel, whereas the lesion void was left “as is” in the stab group. We suggest this allowed for tissue collapse after the stab and thus accounts for the smaller lesion volume observed for the stab group (Figure 3E). This highlights that a single metric such as the lesion area can be misleading when assessing effectiveness of a tissue engineering construct, as a larger lesion area with structural support would be more desirable than a smaller lesion site which had collapsed, an observation which is initially counterintuitive. Additionally, the structural support provided by these Fmoc-DIKVAV hydrogels was comprised of nanofiber bundles within a void-filling form, and as such, more closely mimicking the morphology of the native ECM. This property is therefore able to provide physical support to cells, reducing the inflammatory response associated with secondary tissue degeneration, while also having the ability to promote improved migration and differentiation across the lesion site to encourage the desired growth-permissive environment.

The glial scar, a dense network of interwoven reactive astrocytes, is the primary mechanism that is of interest when attenuating the inflammatory response to traumatic injury. However, it is difficult to characterize the individual morphology of such astrocytes due to this dense network. Thus, measuring the distance of the primary (glial) scar extension can be used to assess the implanted hydrogels’ relative effect on scar formation. Analysis of scar dimensions (Figure 3A–D) demonstrates that filling a TBI lesion with a hydrogel is advantageous through reducing the primary astrocyte scar extension. The stab alone resulted in a primary scar extension almost twice that of either the hydrogel groups (Figure 3C). Such a clear difference in primary scar extension may therefore be due to the lack of structural support in the stab group—without structural support to the surrounding...
damaged, tissue, further tissue collapse occurs, exacerbating the initial inflammatory response. This secondary damage results in continued astrocyte reactivity, and hence, a larger primary astrocyte scar than when structural support is provided to the lesion site. Interestingly, there is no significant difference in the extension of the primary scar between the hydrogel groups, demonstrating that coassembly with fucoidan does not have an impact on the scar dimensions. Matching the modulus is key to mitigating a foreign body response; however, as the results presented in the preceding sections demonstrate, the material systems attenuated the astrocyte scar with the moduli presented in Figure 2A. However, penetrative injury is a traumatic event that, in addition to the glial scar formation, disrupts a significant proportion of the astrocyte population away from the wound site. As such, we also investigated the extension of the local astrocytic disturbance from the lesion/implant site. As the investigated time point (7 days) lies within the acute time period after injury, disturbance of the local astrocytic population was to be expected. Between the stab and hydrogel groups, however, there was no difference in the disturbance extension (Figure 3D), indicating that the dominant effect of the hydrogel systems was on the primary astrocyte scar formation, not the local astrocyte population disturbance.

Extensive confocal imaging of the interface of the lesion/implant and the glial scar reveals variation in astrocyte morphology, distinct between all groups. Figure 3E shows the significant reactivity of astrocytes after TBI, with long, interwoven, hypertrophic processes, and individual cells indistinguishable from each other. With the implantation of Fnoc-DIKVAV (Figure 3F), a glial scar is still formed; however, some astrocyte cell bodies can be visualized and appear rounded and with shorter processes extending out, compared to those seen in the stab group. Contrastingly, while there is approximately 100 μm of interwoven astrocytes extending from the implant border with both hydrogel implants, there are more easily distinguishable astrocytes (Figure 4G) close to the injury site when Fnoc-DIKVAV + 2 mg/mL fucoidan was implanted with an even greater degree of astrocyte organization observable with Fnoc-DIKVAV + 5 mg/mL fucoidan (Figure 3H). Although these organized astrocytes appear somewhat reactive, this increased morphological organization with fewer hypertrophic and intertwined processes is less representative of astrocytes found in the glial scar. This effect is indicative of the hydrogel inducing a shift in astrocyte phenotype, from a typically reactive, scar forming astrocyte to one more representative of the physiologically active phenotype. This observation could be attributed to the anti-inflammatory properties of fucoidan35 coassembled with Fnoc-DIKVAV having a distinct impact on the morphology, and hence attenuating the inflammatory phenotype of scar-forming astrocytes.

3.3. Morphological Reconstruction Reinforces the Complexity of the Astrocytic Response. Although we have shown that implanting a nanofibrous hydrogel halves the size of the primary astrocyte scar, and that astrocyte organization within the primary scar is altered when fucoidan is presented, the effectiveness of this system on individual cells should also be assessed. This was achieved by counting cells in 70 μm × 1000 μm areas located on the edge of the lesion or implant site and at a distance (600 μm; Figure 4A). Cell counts were similar at the edge of the lesion site or implant; however, as Figure 3C shows, the primary scars extended further than 70 μm from the edge of the lesion/implant due to the limitation of the analyzed area, these cell counts cannot be compared to the whole primary scar extension. Cell counts at a distance from the lesion/implant were also similar, suggesting that in all treatments the astrocyte population had reached what could be termed an "inflammatory homeostasis" within the acute phase after injury. This is in agreement with the data showing similar extensions of disturbance for the local astrocyte population (Figure 3D).

It should be noted that, similar to the scar dimension analysis, astrocyte cell numbers cannot be used in isolation to assess a change in inflammatory action. Therefore, we used GliaReconstruct to reconstruct the morphology of individual astrocytes, as it is commonly acknowledged that astrocyte morphology and phenotype are intimately linked, if not yet completely understood. The hypertrophic and intertwined morphology astrocytes within the glial scar prevents their reconstruction, and astrocytes located 600 μm from the lesion/implant edge (Figure 4B) were examined as they were individually distinguishable in all groups.

Implanting Fnoc-DIKVAV and Fnoc-DIKVAV + 5 mg/mL fucoidan resulted in similar cell area (~1200 and 1400 μm² respectively, Figure 4B) and perimeter (~400 and 450 μm respectively, Figure 4C) of astrocytes far away from the after injury. However, as shown in Figure 3F and H, these two hydrogel implants had differing influence over the organization of astrocytes comprising the glial scar; Fnoc-DIKVAV resulted in densely interwoven astrocytes, while the Fnoc-DIKVAV + 5 mg/mL implant resulted in a more organized astrocyte network. Although the local effects of these implant on astrocyte organization does not affect astrocyte cell size far away from the implant, this is potentially not true for Fnoc-DIKVAV + 2 mg/mL fucoidan. The total cell area of astrocytes far away from the implanted Fnoc-DIKVAV coassembled with 2 mg/mL of fucoidan was larger than that of all other groups (Figure 4B). This interesting finding is supported by the total cell perimeter (i.e., giving an indication of the astrocytic process "arbor," Figure 4C), which was significantly greater than the perimeter of cells far away from the implanted Fnoc-DIKVAV or Fnoc-DIKVAV + 5 mg/mL fucoidan but not significantly

Figure 4. (A) Cell (GFAP+) counts near (edge) and far away (600 μm) from the lesion or gel implant, (B) total cell area of the reconstructed astrocytes (GFAP+), and (C) total perimeter of reconstructed astrocytes (GFAP+), far away from the lesion/gel implant. No significance between near groups, or far groups in A. *p < 0.05 and **p < 0.001, using an unpaired t test.

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185
greater than the stabc group. As previously discussed, Fmoc-DIKAV + 2 mg/mL enhanced astrocyte organization within the glial scar, as compared to Fmoc-DIKAV, but not as drastically as Fmoc-DIKAV + 5 mg/mL fucoidan. We have previously observed that a concentration of 2 mg/mL represents the minimum concentration for an observed biological effect and may be related to the presentation of fucoidan within the scaffold.35 Here, a similar concentration is required to have an effect on astrocyte organization, and thus scar formation, as well as astrocyte size.

This interesting finding demonstrates the complexity of astrocytes and their response to therapeutic intervention: at the gel interface, we observed increased astrocyte organization seemingly dependent on fucoidan concentration; however, further away from the gel implant, coassembly of Fmoc-DIKAV and 2 mg/mL fucoidan results in significantly larger astrocytes than with 5 mg/mL fucoidan. Typically, larger astrocytes would be associated with hypertrophy characteristic of reactive astrocytes, however, astrocytes have for a long time been underestimated and simplified, so we are hesitant to draw such a hasty conclusion. These results highlight the complexity of the astrocytic response to injury, and the need to incorporate a range of analysis techniques including a comprehensive scar dimension analysis, confocal microscopy, cell counts, and cell reconstruction.

4. CONCLUSION

Although astrocytes are a promising therapeutic target for TBI treatments, they present a challenge for tissue engineers and biologists alike in understanding and subsequently altering their complex behavior after injury, to ultimately facilitate repair and regeneration. Despite such a daunting challenge, here we have shown that the multifaceted tissue engineering system of a scaffold coassembled from the ECM mimic Fmoc-DIKAV, and the anti-inflammatory fucoidan polysaccharide, reduces the primary glial scar and significantly alters astrocyte organization and morphology 7 days after traumatic brain injury. The impacts of fucoidan concentrations on astrocyte organization and size vary, highlighting the complexity of the cellular response to the tissue engineering construct. This system has the potential to provide the necessary biological, mechanical, and material properties to be used as a platform from which to develop more sophisticated constructs with temporal influence over the astrocytic response after injury. Such a development will have to be grounded in a comprehensive understanding of astrocyte behavior, and their potential within a reparative context, and will require further collaboration and innovation from tissue engineers and biologists, together.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomater.7b00524.

(Figure S1) Purity of the Fmoc-DIKAV peptide determined as >96% as confirmed by HPLC; (Figure S2) strain sweep of Fmoc-DIKAV showing the linear-viscoelastic (LVE) region between 0.1 and 2%, at 37 °C and 1 Hz; (Figure S3) FTIR spectra of the amide I region of a peptide hydrogel made with water, and one with D2O cosolvent; (Figure S4) hypertrophic and intertwined morphology astrocytes within the glial scar preventing their reconstruction and astrocytes located 600 μm from the lesion/implant edge (PDF)

AUTHOR INFORMATION

Corresponding Authors
*E-mail: richard.williams@rmit.edu.au.
*E-mail: david.nisbet@anu.edu.au.

ORCID
David R. Nisbet: 0000-0002-1343-0769

Author Contributions
R.J.W. and D.R.N. contributed equally.

Notes
The authors declare no competing financial interest.

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REFERENCES

Appendix C

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A Commentary on the Need for 3D-Biologically Relevant In Vitro Environments to Investigate Astrocytes and Their Role in Central Nervous System Inflammation

F. L. Maclean1 · R. J. Williams2 · M. K. Horne3,4 · D. R. Nibet1

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Abstract  Astrocytes execute essential functions in the healthy CNS, whilst also being implicated as a limitation to functional regeneration and repair after injury. They respond to injury to minimize damage to healthy tissue whilst also attempting to seal the broken blood-brain-barrier, however, they impede recovery if they are persistent and form a permanent scar in the injured brain. As such, it is of great importance to understand the mechanism underlying the astrocytic response to injury, and this understanding is currently limited by the in vitro environments available to scientists. Biomaterials such as nanofibres and hydrogels offer great potential for the development of superior, 3D cell culture environments in which to study astrocyte behavior and phenotype. The implementation of such in vitro environments with a particularly interdisciplinary approach can improve the field’s understanding of astrocytes, their role in central nervous system inflammation, and elucidate potential strategies to achieve functional regeneration.

Keywords  Astrocytes · Biomaterials · Cell culture · 3D environment

Introduction
Persisting inflammation following central nervous system (CNS) injury inhibits functional regeneration and repair [1, 2]. Disruption of the blood-brain-barrier (BBB) contributes to the existing inflammatory cascade after injury through the infiltration of microglia and non-CNS cells and molecules, accompanying the recruitment of microglia and macrophages, and the release of pro-inflammatory cytokines. Importantly, breaching the BBB may affect the astrocytes response [3], inducing astrocytic proliferation to surround the lesion, protecting healthy brain tissue from the lytic processes occurring within the lesion [4]. While this astrocytic barrier protects the surrounding uninjured brain from secondary injury, the astrocytes also release pro-inflammatory molecules such as chondroitin sulfate proteoglycans (CSPGs) that prevent neural growth in their vicinity.

In healthy CNS tissue, astrocytes have important physiological functions, which include regulating synaptic transmission through neurotransmitter uptake and release and maintaining pH, ion and fluid homeostasis [5]. They achieve this latter role by inserting themselves between neural tissue and blood vessels where they form the BBB and regulate nutrient and oxygen flow from vessel to intracellular space. Following injury, reactive astrocytes contribute to the BBB re-formation, neuron and oligoden-drocyte survival, motor recovery and containment of inflammatory cells [6] and by inducing a reactive response in astrocytes, inflammation performs both pro-survival or cytotoxic functions, whilst also participating in cytotoxic
actions. The duration and extent of these two roles at various time-points following injury thus becomes critical in minimising injury, whilst also promoting repair. As a consequence, astrocytes are important therapeutic targets for controlling CNS inflammation and developing strategies to achieve functional repair and regeneration after injury.

It is apparent therefore that the frequent description of astrocytes adopting either “quiescent” or “reactive” states is unhelpfully simplistic. In the normal healthy brain, astrocytes are far from quiescent and play an active role in the health and physiology of neurones. Investigating the pathways that control the temporally significant transition of astrocytes from this normal, active state, through an immediate post-injury function, to their post-injury trophic state, should be therapeutically valuable. As the normal physiological role of astrocytes is to interact with neurones with the 3D structure of the brain, including the neurone, extracellular space and blood vessels, it is likely that current two dimensional (2D) cell culture methods used to investigate cell behavior and function have limited ability to model these complex in vivo signals [7]. Therefore, it is essential that instead, three-dimensional (3D) cell cultures that more accurately recapitulate the in vivo environment are utilized to improve the translation of in vitro findings to clinically relevant treatments. To this end, biomaterial cell culture environments have been developed to investigate astrocyte morphology and behaviour, and their potential for translation to in vivo applications is discussed below.

The in vivo extracellular matrix (ECM) provides structural and biochemical cues to support residing cells that a 3D cell culture environment should emulate. As electrospun nanofibres mimic the fibrinous structure of the ECM, possess high porosity and a high surface area-to-volume ratio [8], they show promise for providing physical support to cells in a culture system. We previously demonstrated that primary astrocytes cultured on random or aligned poly(ε-caprolactone) (PCL) nanofibers infiltrated the scaffold. Their expression of glial fibrillary acidic protein (GFAP) expression was less than sub-cultured primary astrocytes cultured in 2D, whereas brain derived neurotrophic factor (BDNF) and excitatory amino acid transporter 2 (EAAT2) were both increased [9]; this biochemistry is consistent with a pro-survival or “healthy brain” phenotype. These results demonstrate that 3D morphological cues are important factors when designing a 3D in vitro culture system as they can influence the phenotype of cells. This effect of morphology on astrocytes was consistent with our foundation studies that explored the interaction of embryonic cortical neurons on poly(lactic acid) and poly(lactic-co-glycolic acid) surfaces [10], and this is clearly demonstrated by 3D nanofibrous scaffolds using the same materials with no additional functionalization to produce a different morphology [11]. Physical cues also encompass scaffold stiffness, in addition to morphology. Stiff matrices (1500–75,000 Pa) have resulted in an equal proportion of neural stem cells (NSCs) differentiated into neurons and astrocytes, as compared to the higher proportion of differentiated neurons achieved on soft matrices [12]. Additionally, NSCs expressing constitutively active (CA) GTPases—RhoA or Cdc42—exhibited increased astrocytic differentiation on soft (<1000 Pa) substrates, mimicking the differentiation observed on stiff substrates, and indicating the regulation of lineage commitment of Rho GTPases. Rho GTPases regulate the cytoskeleton, therefore affecting astrocyte morphology [13], which is currently the key indicator of astrocyte phenotype. Therefore, substrate stiffness could be implemented in an in vitro system to further investigate astrocyte morphology (phenotype) change that occurs after injury.

Additionally, nanofibers can be functionalised with various molecules to further model facets of the ECM in vitro. Expression of heat-shock protein (HSP70, a marker of cellular stress), and intermediate filament proteins (such as GFAP, vimentin and nestin, that are usually used as indicators for reactive astrocytes) was less when astrocytes were cultured on polyurethane nanofibres coated with laminin, than those coated on laminin coated glass slips [14]. Additionally, there were stark morphological differences, with astrocytes cultured on the laminin coated polyurethane nanofibres having many finely branched processes compared to the flat, polygonal shape observed on the laminin coated coverslips, as seen in Fig. 1.

As the biochemical cues from the laminin coating were similar in the 2D and 3D environments, it is likely that physical cues from the nanofibres were responsible for the

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![Fig. 1 Morphology as revealed by 3D reconstruction of confocal images of EGFP-expressing astrocytes cultured on: a 2D laminin coated coverslips and b 3D polyurethane nanofibers coated with laminin. Scale 10 μm [14]. This figure has been reproduced with permission from Elsevier](image-url)

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the same scaffolds in vivo. Such investigation can lead to a better understanding of astrocytes’ role in CNS inflammation, and point to potential treatments that can be translated in vivo. This interdisciplinary proposition will require biologist, chemists and engineers to work collaboratively to further the field’s understanding of one of the most complex and important cells of the CNS.

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References

3D Electrospun scaffolds promote a cytotoxic phenotype of cultured primary astrocytes

Chew L. Lau,*1 Michelle Kovacevic,*1 Tine S. Tingleff,†† John S. Forsythe,‡ Holly S. Cate,§ Daniel Merlo,* Cecilia Cederfur,* Francesca L. Maclean,¶ Clare L. Parish,¶ Malcolm K. Home,* David R. Nisbet,*† and Philip M. Beattie*†

*Florrey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Australia
†Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
‡Department of Materials Engineering, Monash University, Clayton, Australia
¶Centre for Neuroscience Research, Department of Anatomy and Neurosciences, The University of Melbourne, Parkville, Australia
†Research School of Engineering, The Australian National University, Canberra, Australia

Abstract
Astrocytes are a target for regenerative neurobiology because in brain injury their phenotype arbitrates brain integrity, neuronal death and subsequent repair and regeneration. We explored the ability of 3D scaffolds to direct astrocytes into phenotypes with the potential to support neuronal survival. Poly-ε-caprolactone scaffolds were electrosprun with random and aligned fibre orientations on which murine astrocytes were sub-cultured and analysed at 4 and 12 DIV. Astrocytes survived, proliferated and migrated into scaffolds adopting 3D morphologies, mimicking in vivo striatal phenotypes. Cells on random poly-ε-caprolactone scaffolds grew as circular colonies extending processes deep within sub-micron tissue, whereas astrocytes on aligned scaffolds formed rectangular colonies with processes following not only the direction of fibre alignment but also penetrating the scaffold. Cell viability was maintained over 12 DIV, and cytotoxicity for F-13) actin showed lower stress fibres on bioscaffolds relative to 2D astrocytes. Reduced cytotoxic stress was confirmed by the decreased expression of glial fibrillary acidic protein. qPCR demonstrated up-regulation of genes (excitatory amino acid transporter-2, brain-derived neurotrophic factor and anti-oxidant) reflecting healthy biology of mature astrocytes in our extended culture protocol. This study illustrates the therapeutic potential of bioengineering strategies using 3D electrosprun scaffolds which direct astrocytes into phenotypes supporting brain repair.

Keywords: astrocyte, astrologous, bioengineering, cell culture, cytoplastic phenotype, gene expression, J Neurochem. (2014) 10.1111/jnc.12702

Repair of the injured CNS requires reconstitution of neural networks through axon extensions and synapse formation. Synapses in the CNS are in close apposition with astrocytes, which are recognised as plastic cells playing key roles in maintaining brain function via energetic, anti-oxidant activity, trophic factor synthesis, neurovascular coupling and L-glutamate (Glia) homeostasis (Rieder et al. 1997; Marmaglio and Rodensteiner 2006; Paguna et al. 2012). Astrocytic morphological change with plasticity would not be unexpected in a cell with a large cell body, an extensive and fine cell body, tight junctions with other astrocytes and end-feet on the blood-brain barrier and vasculature (McMillan et al. 1994; Van der Kooy 1994)
Ridet et al. 1997; Panicker and Norenberg 2005). Not surprisingly, astrocytes exhibit various morphological and biochemical changes in response to changes in their milieu induced by physiological and pathological events. Such changes occur across a continuum of events, being influenced by the prevailing extracellular milieu in disease and trauma and by the extent of multiple factors, which appear able to cause both short- and long-term responses (Ridet et al. 1997; Maragalis and Rothstein 2006). Whilst the terms astroglial and reactive gliosis can be found in the literature, astrocytes are now considered to exist in a variety of phenotypes that have pro- (‘cytotoxic’) and destructive (‘cytotoxic’) components (McWilliam et al. 1994; Panicker and Norenberg 2005; Sofroniew and Vinters 2010). These phenotypes have been found in both the normal brain and brains affected by neurological diseases and there is some evidence that astrocytes can dynamically change phenotypic components, an attractive target to promote endogenous repair if they can be directed into a cytotoxic phenotype supportive of neuronal survival and axon regrowth. Thus changing the physical and molecular phenotype of astrocytes may facilitate neural repair mechanisms (Maragalis and Rothstein 2006; Sofroniew and Vinters 2010).

Work from our laboratory, initially focusing on excitatory amino acid transporters (EAATs), documented in conventional cultured astrocytes that morphological changes in vitro induce alterations in biology and gene expression (Zagami et al. 2009; Lau et al. 2010, 2011, 2012; Sheean et al. 2013). The actin cytoskeleton and Rho GTPases (Rho, Rac, Cdc42) are fundamental determinants of cellular motility and migration (Le Clanche and Cailler 2008; Mattila and Lappalainen 2008). We found treatment of astrocytes with inhibitors of Rho kinase produced stellated morphology, less actin stress fibres and a shift in the F-/~actin ratio to a predominance of G-/~actin (Lau et al. 2011). Here analyses of the astrocytic transcriptome confirmed major alterations to genes of the extracellular matrix (ECM), with elevated expression of EAAT2, brain-derived neurotrophic factor (BDNF) and key anti-oxidant genes (Lau et al. 2012) – findings suggestive of shift to a cytotoxic phenotype. Tissue engineering is another strategy where the use of scaffolds provides cues for cellular organisation, survival and function (Stevens et al. 2005; Teo et al. 2006) and in concert with materials science to manipulate surface chemistry, fibre alignment, diameter and inter-fibre spacing can morphologically replicate components of the ECM (Stevens et al. 2005; Teo et al. 2006). We found that the implantation of 3D electrospun poly-e-caprolactone (PCL) scaffolds produced a delayed astrocytic response in the striatum in a rat model of traumatic brain injury (Nisbet et al. 2009). After 60 days, astrocyte numbers had returned to normal levels, but more importantly the PCL scaffold failed to elicit a prolonged foreign body reaction. Interestingly, neurites infiltrated into the randomly aligned scaffold during the peak in astrocytes activation, suggesting the infiltration was being promoted by astrocytes. This result led to our hypothesis that electrospun scaffolds may promote cytotoxic astrocytic phenotype. Here we have taken this posulate to a comparison in vivo of astrocytes cultured on electrospun 3D scaffolds and in conventional 2D mode. Investigations of astrocytic morphology and distribution on 3D electrospun scaffolds, as well as their ability to support cell survival, proliferation and functional outcomes of growth, demonstrated cellular penetration of astrocytic processes into the scaffolds and promotion of a cytotoxic phenotype; two key outcomes likely supportive of brain repair. Our evidence demonstrates that bioengineering astrocytes has great potential for regenerative neurobiology. A preliminary account of these findings was presented at the 43rd Meeting of the American Society for Neurochemistry (Beart et al. 2012).

Materials and methods

All experimentation was approved by the Ethics Committee of the Flinders Institute for Neuroscience and Mental Health and was undertaken according to the guidelines of the National Health and Medical Research Council (NHMRC, Australia).

Preparation of poly-e-caprolactone scaffolds

Poly-e-caprolactone (PCL) was obtained from Sigma-Aldrich (molecular weight 70 000–90 000; St Louis, MO, USA). 3D PCL films were fabricated using a compression moulder heated to 190°C. The PCL was held between two polished stainless steel plates covered with Teflon release film and allowed to reach temperature for 2 min. A 20 kPa force was then applied to the plates for 5 min to fabricate the films before they were removed and quenched in an ice-water slurry. 3D electrospun scaffolds were fabricated as described previously (Nisbet et al. 2008) with minor modifications. Polymer solutions of 10% (w/v) were prepared for electrospinning by dissolving PCL in 2 ml of chloroform (Merck Pty Ltd., Kilbarchan, Australia) and methanol (Merck Pty Ltd.) at a ratio of 3 : 1 (v/v). The solutions were placed into a glass syringe (10 ml) with a 21-gauge needle for electrospinning at a flow rate of 1.5 ml/h. A 15 kV accelerating voltage was used at the positive electrode, with a ground rotating mandrel (diameter = 5 cm) employed as the collector. The working distance was 15 cm and the collector was coated with aluminium foil for easy removal. Speeds of 200 rpm (orbit diameter 14 cm) were used to fabricate randomly oriented fibres and 4000 rpm (orbit diameter 14 cm) to fabricate aligned fibres. Once removed from the collector, PCL scaffolds were then cut into circles using a punch with diameters matching the dimensions of the walls (6- and 24-well plates). The scaffolds were sterilised in 70% ethanol for 15 min and washed with sterilised phosphate buffered saline pH 7.4 prior to use.

Scaffold characterisation

The morphology of the scaffolds was characterized using scanning electron microscopy (SEM). The samples were sputter coated with platinum at 20 mA for 1 min. All SEM images were taken under 3 kV with a working distance of 3.5 mm on a Zeiss Ultra Plus FESEM (Oberkocher, Germany). The average diameters of the fibres were determined using ImageJ software (http://rsb.info.nih.gov/ij/, National Institutes of Health, version 1.46) to measure a
total of 20 fibres across four different samples and were 400 ± 110 and 450 ± 150 nm, for random and aligned scaffolds, respectively (Fig. 1).

**Cell culture**

All media used for primary cell culture and maintenance were from GibcoLife Technologies (Mulgrave, Australia) unless otherwise stated. Primary cultures of astrocytes were established from the forebrain of post-natal day 1-5 C57 Black 6 mice (animal facilities of Florey Institute for Neuroscience and Mental Health) as previously described (O’Shea et al. 2006). After astrocytes had formed a confluent layer (10 DIV), dishes were shaken overnight (to remove cells other than astrocytes) in a Rasek Orbital Mixer Incubator (180 rpm, orbit diameter 25 mm, 37°C). Sub-culturing generated secondary cultures where astrocytes were seeded onto 24-well plates (polystyrene plate, glass coverslips or 2D PCL at 2 × 10^5 cells/well) or 96-well plates (polystyrene plate, random or aligned 3D PCL at 8 × 10^3 cells/well) depending on the experiment, and incubated in a humidified incubator at 37°C with 5% CO₂. Scaffolds were held at the bottom of the well with a coverslip insert. Preliminary experiments explored the optimal cell density, examining seeding densities of 4, 8 and 16 × 10^3 cells/well (Figure S1). A full medium change was performed to remove non-adherent cells and medium was subsequently changed every 5-7 days until cells were ready for use (4 DIV or 12 DIV following sub-culturing). Immunocytochemistry with glial fibrillary acidic protein (GFAP) revealed the presence of a monolayer of astrocytes (Apricó et al. 2004; O’Shea et al. 2006). Note: 4 DIV and 12 DIV following sub-culturing is equivalent to 14 DIV and 22 DIV but for simplicity will be referred to in this paper as 4 DIV and 12 DIV.

**Immunocytochemistry**

The immunocytochemical procedures have been described previously (Lau et al. 2011). Astrocytes grown on glass coverslips, 2D PCL films, aligned or random scaffolds were fixed in 4% paraformaldehyde in phosphate buffered saline for 10 min. Cells were incubated with primary antibodies GFAP (1:1000, Chemicon International Inc., Melbourne, Australia) at 4°C overnight, followed by secondary antibody (Alexa Fluor® 488, Molecular Probes® Life Technologies, Mulgrave, Australia) incubation for 3 h at 23°C. Coverslips and scaffolds were mounted on glass microscope slides with DAKO fluorescent mounting medium (DAKO, Victoria, Australia), left to dry in the dark overnight and stored at 4°C. Fluorescence was visualized using a Nikon IX71 inverted microscope (Nikon Australia Pty Ltd, Melbourne, Japan). Digital images were acquired using a Nikon Coolpix C-5500 Zoom digital camera attached to the Olympus IX71 inverted microscope.

**Cytokeletal staining**

As described previously (Lau et al. 2011), concurrent labelling of F- and G-actin was obtained by staining with TRITC-conjugated phalloidin (1:1000, Sigma-Aldrich, Melbourne, Australia) and Alexa Fluor® 488-conjugated deoxyribonuclease I (DNase, 1:250: Molecular Probes® Life Technologies), respectively.

**Confocal imaging**

Astrocytes were prepared on scaffolds and immunostained with GFP (see above). Nuclei were stained using Hoechst 33342 (5 µg/mL, Molecular Probes® Life Technologies) and the fluorescence was visualized under Leica SP2 Confocal Microscope (Leica Microsystems, Wetzlar, Germany). The scaffolds were imaged using reflection of a HeNe 633 nm laser. Z-stacks were obtained with optical sectioning at 0.2 µm.

**Cell viability and function assays**

Cellular viability was assessed using a MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) reduction assay according to the manufacturer’s instructions (Lau et al. 2011). In addition a lactate dehydrogenase (LDH) assay was carried out using a Cytotoxicity Detection Kit (Roche, Sydney, Australia) according to the manufacturer’s instructions (Lau et al. 2011).

1-Glucuronate uptake: Activity of astrocytic EAATs was determined using [3H]-d-aspartate ([3H]-d-Asp) uptake (Apricó et al. 2004). In brief, cells were pre-incubated at 37°C for 5 min and then incubated with [3H]-d-Asp (30 nM, 5 min), with or without the unlabelled d-Asp (1 mM) in uptake buffer (125 mM NaCl, 2 mM ECl, 0.6 mM MgSO₄, 1 mM CaCl₂, 6 mM D-glucose, 10 mM HEPES, pH 7.4). Uptake was terminated by washing at 4°C, and accumulated radioactivity determined by scintillation spectrometry (O’Shea et al. 2006).

**Western blot analysis**

As described previously (Lau et al. 2010), cells were grown in polylysine micro-well plates, with or without aligned and random PCL scaffolds for 12 DIV. Samples were pooled from eight wells (n = 8 replicates), and total cell protein concentration was determined with the Bio-Rad DC Assay Kit (Sydney, Australia) according to the manufacturer’s instructions. Standard western blot protocols were carried out with 10 µg protein per lane (three lanes per condition, where each lane represents an independent experiment) and membranes were incubated with primary GFAP antibodies at 1:1000 (Promega, Melbourne, Australia) overnight at 4°C. Following washing, membranes were incubated with horseradish-peroxidase conjugated secondary antibodies (goat anti-rabbit IgG, 1:1000) for 3 h at 23°C. Proteins were then

visualised using enhanced chemiluminescence. As a control for protein loading, blots were subsequently probed for β-actin (primary antibody 1:10 000) using the same procedures. Densitometric analysis of western blots was performed using ImageJ software (http://rsb.info.nih.gov/ij, National Institutes of Health, version 1.46e) to measure the area and density of protein bands after subtracting the background of the autoradiographic film (Cimarelli et al. 2005).

Quantitative real-time PCR
RNA was extracted from astrocytes grown on polystyrene micro-well plates, 2D PCL, aligned and random electrospun scaffolds. Total RNA was extracted from four independent cultures with eight replicates per culture using the RNeasy Mini Kit (Qiagen, Alameda, CA, USA) according to the manufacturer’s instructions. In order to quantify the RNA extract, 1.8 µL of RNA sample was used for spectrophotometric analysis using NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). qRT-PCR was performed on an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described (Binder et al. 2005). Primers were designed using Primer Express 3.0 (Applied Biosystems, Melbourne, Australia). Sequences of primers and details of all procedures were published previously (Liu et al. 2012). To determine statistical significance for qRT-PCR data, 95% confidence intervals were constructed for single-variable analysis. All data are presented as mean ± SEM, and are expressed relative to the control condition (polystyrene or 2D PCL).

Data analysis
Specific details are given above. All values are mean ± SEM from replicate determinations from multiple independent experiments. Data were subjected to one-way ANOVA followed by Dunn’s Multiple Comparison post-hoc Test using GraphPad Prism v.4.0 (GraphPad, San Diego, CA, USA).

Results
Dramatic change in astrocytic morphology on 3D PCL
Initial work explored optimal culture conditions by investigating GFAP immunolabelling and observations based upon length of GFAP-positive astrocytic processes and indicated a density of 8 × 10³ cells/well yielded viable cultures (Figure S1). Under these conditions, astrocytes grown under control conditions on coverslips were evenly distributed at 4 DIV and appeared as flattened cells (Fig. 2a), with increased cell number at 12 DIV (Fig. 2b). When cultured on random scaffolds, astrocytes appeared as tight colonies (ratio of larger to smaller axis < 2) at 4 DIV (Fig. 2f), and these colonies expanded to occupy the scaffold with condensed processes (typically 50–75 µm) by 12 DIV. By comparison, astrocytes grown on aligned scaffolds grew in colonies that were more rectangular (ratio of larger to smaller axis > 2) (Fig. 2e) at 4 DIV than those growing on random scaffolds (Fig. 2c), with processes aligned with the fibres and by 12 DIV extending for lengths sometimes exceeding 300 µm (Fig. 2d). Morphological estimates of the length of GFAP-positive processes at 12 DIV were 65 ± 5 µm and 90 ± 3 µm (≥ 15 images, three independent experiments) for random and aligned scaffolds, respectively.

Astrocytes on 3D scaffolds maintain viability but show decreased total protein and GFAP levels at 12 DIV
Prolonged culturing of astrocytes under control conditions and on aligned PCL scaffolds resulted in a significant reduction in mitochondrial function (Fig. 4a). Reduction in mitochondrial function, as shown by MTT activities, was similar at 12 DIV regardless of the culture conditions. In addition, nuclear condensation and fragmentation were not observed at 12 DIV, consistent with the absence of pyknotic nuclei (Figure S2). LDH, an extracellular measure of compromised cell membrane, was significantly higher in GFAP-positive astrocytes grown on polystyrene (control) and aligned scaffolds at 4 DIV than the cultures cultured until 12 DIV (p < 0.05). Surprisingly, the LDH of cells grown on aligned scaffolds was higher than those grown on polystyrene (control, p < 0.05) (Fig. 4b). Total cellular protein increased between 4 DIV and 12 DIV, regardless of the surface on which cells were cultured and was taken as an indicator of astrocyte proliferation within the 8 days (Fig. 4c). However, cellular protein concentration of astrocytes grown on the 3D scaffolds was significantly lower (~ 30–40% decreases at 12 DIV) than those grown on polystyrene (random p < 0.001 and aligned p < 0.05) at 12 DIV. "[H]U-Asp uptake activity, which is an index of Glu transport and thus reflects astrocytic function (Beaty and O’Shea 2007), was similar in all culture conditions. On the basis of the studies described above, 12 DIV was chosen as the timepoint for further investigation.

Western immunoblotting studies were undertaken for GFAP (Fig. 5), a main constituent of intermediate filaments in astrocytes, and an index classically increased in reactive astrocytes (Middeldorp and Hof 2011). The expression of GFAP in astrocytes grown on both random and aligned scaffolds decreased appreciably (~ 80% reductions) relative to those maintained in 2D culture at 12 DIV (polystyrene, p < 0.01).
Astrocytic interaction with the 3D scaffolds

Confocal imaging indicated that GFAP-positive fibres penetrated into the fibre network of aligned and random PCL scaffolds (Fig. 6a and b respectively). Image analysis allowed the concurrent visualisation of GFAP-positive fibres and digital sectioning demonstrated that processes extended up to 10 μm into the scaffolds, including projecting to the base of the scaffolds (Fig. 6). Hoechst-labelled nuclei were also visualised within the 3D PCL, indicating that astrocytes were capable of migrating deep into the scaffolds. Astrocytes appeared not to show any obvious preference with respect to their capacities to penetrate into random or aligned scaffolds.

Gene expression

Given the extensive changes in astrocytic morphology and GFAP expression, we sought molecular insights into genes that might contribute to the transformation of phenotype. Our previous study (Lau et al. 2012) focused attention on select genes involved in (i) cell motility and pathfinding [skeletal muscle actin α2 (ACTA2), vinculin (VCL), chemokine (C-X-C motif) ligand 12 (CXCL12)], (ii) TGFβ-related migration [transgelin (TAGLN)] and (iii) astrocytic function, including Glu transport (SLC1A2 = EAAT2), BDNF and anti-oxidant [glutathione S-transferase α1 (GSTA1) and heme oxygenase (decyling) 1 (HMOX1)]. The mRNA expression of all of these genes was significantly
greater in astrocytes cultured on the 3D scaffolds, regardless of their fibre alignment (Fig. 7), relative to conventional 2D cultures. PCL itself possessed properties that induced increased mRNA expression, since expression of ACTA2, VCL, CXCL12 and BDNF were increased in astrocytes grown on either 2D or 3D PCL (both random and aligned) scaffolds (Fig. 7). mRNA expression of TAGLN, a gene linked to transforming growth factor β-mediated cellular migration, in astrocytes grown on 3D-aligned scaffolds was significantly greater than astrocytes grown on control scaffolds and 2D PCL film, suggesting this increase is because of the 3D morphology and topography of the PCL scaffold (Fig. 7). Similar increases in mRNA expression of the major Glu transporters SLC1A2 and GSTA1, a key enzyme for glutathione synthesis, in conjunction with the BDNF data, suggest that the electrospun fibres had promoted a salutary astrocytic phenotype.

**Discussion**

Astrocytes offer great potential for regenerative neurobiology because of the fundamental roles they play in the function of the CNS, participating in many physiological events including synaptic transmission, maintenance of neurotransmitter homeostasis, cerebral blood flow and as a site of anti-oxidant defence during stress. Astrocytic responses occur as a continuum that is graded according to the extent of the trauma or disease—when minor there is resolution and even the extreme scenario of glial scar formation is considered manageable by pharmacological intervention (Ridet et al. 1997; Mueller et al. 2009; Sofroniew 2009). Our interest in taking advantage of the biology of astrocytes to promote repair and regeneration was driven by our documentation of phenotypes in vitro (Lau et al. 2012) and *in vivo* (Nisbet et al. 2006) consistent with a pro-survival state. In these studies different interventions resulted in cytoskeletal properties that potentially could result...
in beneficial outcomes. Here we have used engineered scaffolds to influence astrocytic phenotype with the ultimate goal of understanding how the inflammatory cascade might be tuned to generate a favourable outcome. Key findings emergent from our study were that astrocytes survived and migrated on 3D PCL scaffolds with a stellated morphology, adopting a phenotype where the reduced pattern of GFAP expression and overall gene profile were likely to be cytoprotective in a reactive milieu.

**Cell morphology and behaviour**

When astrocytes were cultured on the 2D scaffolds, they initially formed colonies with condensed processes and then adopted elongated and ramified cell morphologies. Given the relative increase in G-actin staining (i.e. fewer stress fibres) and an ~ 80% decrease in GFAP expression, our data indicate these cells are less stressed compared to cells grown in the conventional 2D culture system. The morphology of the cells changed dramatically as they extended processes over the substratum to occupy vacant space. This cytoskeletal event is considered an important determinant of cell growth and survival (O’Neill et al. 1986; Schnell et al. 2007; Horne et al. 2010). At 12 DIV we demonstrated that the survival and viability of astrocytes grown on the 3D PCL scaffolds was maintained with findings suggestive of adoption of a quiescent state after the initial proliferation and expansion (c.f. Fig. 4). While cellular proliferation was supported by 3D scaffolds, the large reduction in GFAP levels relative to only a ~ 30–40% decrease in total protein is indicative of a genuine shift in astrocytic phenotype on PCL scaffolds.

Given the tendency for astrocytes to form colonies as early as 4 DIV on the scaffolds, contact inhibition (or density-dependent inhibition of cell division) may be occurring in these conditions (Marti and Steinberg 1972). However, nutrient supply is thought to be more important than the effects of cell–cell contact, so the pattern of organisation of high-density cultures may be a contributing factor to their slower rate of cell proliferation. Thus cellular density and the reduced nutrient supply, as well as the toxic build up of waste products, likely influenced viability at the density of 16 × 10^3 cells/well (Owen and Shoichet 2010), where there
Fig. 6 Three-dimensional rendering of confocal images (Z-stack). Astrocytes were grown for 12 DIV on (a) aligned poly-ε-caprolactone (PCL) scaffold and (d) random PCL scaffold. (b, c, e and f) showed digital sectioning, revealing the cross-section of the scaffold. Cells on the scaffold were labelled with glial fibrillary acidic protein (GFAP) (red) and Hoechst 33258 (blue). Divisions on x-y-axis = 10 μm and on z-axis = 2 μm.

were extensive overlapping layers of astrocytes. Moreover, for both types of 3D scaffolds GFAP-positive processes were significantly longer at 8 × 10^5 relative to 4 × 10^4 cells well at 12 DIV (Figure S2), suggesting this density was optimal in terms of cell-cell contact required for astrocytic growth. Density-dependent inhibition of cell division may help explain the reduction in glial scarning observed in rat striatum when treated with PCL scaffolds in vivo (Nisbet et al. 2009), considering astrocyte proliferation soon after injury has been associated with a destructive phenotype (Paish et al. 2002).

Cell-scaffold interactions: cytoskeletal evidence for increased cellular migration

Electrospun scaffolds are engineered to mimic the native ECM, and both the fibre orientation and topography have profound effects astrocytic shape, growth and function (Singhvi et al. 1994; Gerardo-Nava et al. 2009). The present results demonstrate that aligned scaffolds provide contact guidance cues that allow for longer and more directed process outgrowths (Z-stack, Fig. 6) even though migration and penetration of cell bodies into both random and aligned scaffolds was similar. This ability of electrospun scaffolds to influence the behaviour and directionality of astrocytic growth is thus of particular interest to CNS repair strategies attempting to target specific areas of injury and direct the outgrowth of regenerating axons (East et al. 2010). Astrocytes have been grown successfully on micro-grooved and patterned substrates (Sorensen et al. 2007; Meng et al. 2012) but these topographies do not offer the 3D advantages of our scaffolds. Attempts to culture astrocytes on electrospun fibres have generally been hampered by issues related to poor proliferation and cytoskeletal stress (Cao et al. 2012; Kim et al. 2012), and our success shows the advantages of employing secondary cultures of astrocytes in combination with electrospun scaffolds. When this study was completed the successful maintenance of astrocytes in 3D was reported on electrospun scaffolds (Pouchmann et al. 2013; Zaidzoe et al. 2014). Our procedure has the important advantage over both these studies that it allows 3D culture over an extended time interval of more mature astrocytes under conditions where there is preservation of the cytoskeletal phenotype with an
extensively arborised cellular architecture featuring numerous major and minor processes.

Normally astrocytes are grown in vitro as a 2D monolayer, which is very different to their 3D environment in vivo. Unlike the recent work by Zuidema et al. (2014), who undertook extensive characterisation of astrocytic architecture in 2D, we used confocal Z-stacks in conjunction with Hoechst staining and cytochemistry for GFAP to demonstrate that cells migrate and penetrate through the scaffold from its top through its middle to the base. The mean fibre diameter of our electrospray fibres was appreciably less (0.4 vs. 2 µm) than that of Zuidema et al. (2014), who also employed fibroblasts, conditions that are likely to encourage adhesion and extensive growth and migration (Wang et al. 2009) along these scaffolds. Importantly, the appreciable penetration of astrocytes processes into our biomaterials achieved one of the key aims of our study to produce 3D astrocytes. Interestingly, both scaffolds lead to promotion of Glu transport, but guided by our earlier evidence for cytoskeletal changes (Lee et al. 2012) we also demonstrated reduced expression of GFAP and actin stress fibres in conjunction with up-regulation of BDNF and anti-oxidant genes. These data suggest that the current difficulty in correlating healthy, stellated in vivo astrocytic phenotypes with flat, cobblestone-like morphologies displayed in vitro may be overcome by using electrospray 3D scaffolds as a substrate to induce more physiologically representative astrocytes for in vitro studies of cellular behaviour and morphology.

Cellular interaction with different substrates can activate different cellular and ECM mechanisms linked to the control of astrocytic cytoskeletal arrangement (Provenzano and Keely 2011). The active cytoskeleton has been well established to...
have important roles in cell morphology and migration, with astrocytic process formation occurring because of depolymerisation of actin filaments via a shift from F-actin to G-actin (Kinetelberg 2004; Lau et al. 2011). This study found that control cultures of primary astrocytes have a high F- to G-actin ratio and numerous focal adhesions. Increases in both F- and G-actin observed in astrocytes cultured on random and aligned fibres are most likely a reflection of process elongation on these scaffolds. These data suggest that control cells are at higher levels of cytoskeletal stress, possibly because of the non-compliant 2D substrate (glass/polyurethane) on which they were seeded, whereas PCL scaffolds more clearly reflect the elastic modulus encountered in vivo. This interpretation is also supported by our immunoblotting data of GFAP expression, which was decreased in astrocytes grown on both random and aligned scaffolds rather than up-regulated as would occur in inflammatory response occurring in CNS insults (Middekoop and Hol 2011).

The pathways that regulate actin polymerisation are not fully characterised, however the GTPases (Rho, Rac and Cdc42) are known to be involved (Goldman and Abramson 1990; Hall 1999; Etienne-Manneville and Hall 2002). Extensive work in our laboratory has shown that following treatment with the Rho kinase inhibitor, cultures of murine astrocytes shift from a flat, cobblestone phenotype to a stellate shape, with a concomitant decrease in F-actin and a proportional increase in G-actin (Lau et al. 2011) and adoption of a cytotoxic phenotype (Lau et al. 2012). Astrocytes grown on the PCL scaffolds showed similar increases in 'healthy' genes, especially the SLC1A2 (i.e. EAAT2) and anti-oxidant linked GSTA1, indicating that 2D scaffolds promote a transition to a cytotoxic phenotype. Our observation of increased SLC1A2 is consistent with Zhiderma et al. (2014), who also found EAAT1 expression was elevated on their bioscaffold. Expression of the astro-trophic BDNF was always elevated on PCL, including on both random and aligned scaffolds. Indeed, there is evidence that SLC1A2 and BDNF may be co-regulated in health and pathology (Xu et al. 2008; Gounley et al. 2012; Lau et al. 2012). The up-regulation of SLC1A2 expression found on the scaffolds was also found with ROCK inhibitors (Lau et al. 2012) and likely represents the homeostatic behaviour displayed by astrocytes to maintain this key transport (Abe and Minowa 2003; Zagami et al. 2009; Lau et al. 2010) integral to the CNS defence against excitotoxicity. Key indices of the healthy phenotype would appear to be not only elevated expression of SLC1A2, but also of the major anti-oxidant gene, GSTA1, together with decreased GFAP and an accompanying increase in G-actin.

Conclusion

These results show that astrocytes cultured on electrospun 3D PCL scaffolds have extended processes that penetrate the scaffold. Importantly, decreased expression of the astrocytic intermediate filament marker GFAP and increased G-actin suggested astrocytes adopted biologies that were less stressed, whilst displaying elevated expression of genes indicative of a cytotoxic phenotype. This study points to the therapeutic potential of bioengineering strategies using 3D electrospun scaffolds of differing fibre orientations that direct astrocytes into phenotypes supporting brain repair.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Preliminary studies were performed to examine the effect of cell densities on astrocyte growth and distribution on 3D PCL scaffolds with cells maintained in 2D on glass coverslips serving as an internal control.

**Figure S2.** Nuclear staining (Hoechst 33342) of primary astrocytes sub-cultured on coverslips (a, b), random PCL (c, d) and aligned PCL (e, f) for 4 (a, c, e) or 12 DIV (b, d, f).

**References**


Electrospun scaffolds and astrocytes phenotypes | 11


size and microglial activation in response to programmed-induced


dimensional collagen gels and is maintained following plastic compression to form a spinal cord repair matrix. Tissue Eng. Part A 16, 3173–3184.


dimensional scaffolds incorporating immobilized

Kim S. H., Shin C., Min S. K., Jung S. M. and Shin H. S. (2012) In vitro evaluation of the effects of electrospun PCL nanofibrous mats


transplantable inhibitors of L-glutamate uptake produce astrocytic

inclusion and increase EAAT2 cell surface expression. Neuroreport 21, 75–78.


Softironie M. V. (2009) Molecular dissection of reactive astroglisis and

Softironie M. V. and Vichten H. V. (2010) Astrocytes: biology and

Storlason A., Alidsen O., Katsch K., Borell M., Rekhe M. O. and
monolayers aligned by microtopography. Biomaterials 28,
2498–2505.

Stevens M. M., Mayer M., Anderson G. D., Wikel D. B., Whitesides G. M. and
Biomaterials 26, 7635–7641.


Fibrocartilage and focal adhesion kinase small interfering RNA
modulate rat retinal Müller cells adhesion and migration. Cell. Mol.

Xu S., Han J. C., Morlais A., Morlais C. M., Williams K. and Fan Y. S.
(2005) Characterization of 1p14.2 deletion in WAGR syndrome by

Cotranslative and cotranslational matrix exert differential effects on spinal

Enhanced GLT-1 mediated glutamate uptake and migration of
primary astrocytes directed by (fibrinogen-coated electrospun poly-
(lactic acid) fiber. Biomaterials 35, 1530–1540.
Appendix E

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Transcriptomic analysis and 3D bioengineering of astrocytes indicate ROCK inhibition produces cytotoxic astrogliosis

Ross D. O’Shea1,2, Chowi L. Lau1, Natasha Zulazie1, Francesca L. Maclean1, David R. Nisbet1, Malcolm K. Home3,4 and Philip M. Bear5

1 Department of Physiology, Anatomy and Embryology, La Trobe University, Melbourne, VIC, Australia
2 School of Engineering, University of Melbourne, Parkville, VIC, Australia
3 Department of Neuroscience, The Australian National University, Canberra, ACT, Australia
4 Department of Neuroscience, St Vincent’s Hospital, Parkville, VIC, Australia
5 Department of Neurology, University Hospital of Würzburg, Germany

Contact details:
Ross O’Shea, Department of Physiology, Anatomy and Embryology, La Trobe University, Parkville, VIC 3083, Australia.
Email: ross.oshea@latrobe.edu.au
These authors have contributed equally to this work.

INTRODUCTION

Astrocytes make important contributions to the maintenance of the function of the mammalian central nervous system (CNS)—not only are they the most populous cells, but they play major roles in the maintenance of CNS health through their involvement in energetics, L-glutamate (Gla) homeostasis, anti-inflammatory and anti-oxidant activity and release of trophic factors and glutamate transmitters (Ribot et al., 1997; Marangos and Rothstein, 2006; Parpura et al., 2012).

Abbreviations: CNS, central nervous system; dsXAMP, NF-κB oligodeoxynucleotides; J2-AE, 2-oxoglutarate dehydrogenase complex; GLUT, glutathione transporters; GABA, glutamic acid decarboxylase; GCLC, glutamine synthetase; GCH, glutamine synthetase; GSK3, glycogen synthase kinase-3; GluA, glutamate receptors; GPCR, G protein-coupled receptors; IR, insulin receptor; ITGB1, integrin β1; KEGG, Kyoto Encyclopedia of Genes and Genomes; LRRK2, leucine-rich repeat kinase 2; MAPK, mitogen activated protein kinase; MEK, mitogen-activated protein kinase; MLL1, mixed-lineage leukemia 1; MTH1, thioredoxin reductase 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NF1, neurofibromatosis 1; NFKBIA, interleukin 1; ROCK, Rho-associated, COH, substrate(s) of cytokine signaling 2; ROCK2, reelin; S1PR1, sphingosine 1-phosphate receptor 1; STX1A, synaptotagmin-1A; TRPC3, transient receptor potential cation channel subfamily C member 3; TUBB3, tubulin, beta-3; TUNCS, transgenic-derived growth factor.

Moreover, astrocytes are well documented to be plastic cells that change their morphology and biologic structure in response to alterations in the extracellular milieu, which may elicit short- and/or long-term responses. These morphological changes occur in normal and pathological brain tissue, and there is an ever-expanding literature that astrocytes exist in diverse phenotypes across a continuum regulating pro-survival ("cytotoxic") and destructive ("cytolytic") components (McMillan et al., 1994; Pardridge and Vinters, 2001; Sotoudeh and Vinters, 2010). Astrocytes are historically considered to contribute to brain pathologies in a "secondary" mode during which they have been considered as gliotic (Panci et al., 1997; Marangos and Rothstein, 2006), but there is now a solid body of growing evidence supporting their primary role in non-cell autonomous injury, where they secrete toxic entities and/or contribute to pathogenesis, driving disease progression in various neuropathologies (Lindberg and Cleveland, 2007; Hevia et al., 2009; Hevia and Sotoudeh, 2014). Astrocytes...
may contribute to non-cell autonomous injury in motor neuron disease (MND: amyotrophic lateral sclerosis) (Phoerunieks et al., 2014) and other neurodegenerative conditions (Di Mita et al., 2012). Whilst the gial scar has long been considered a genuine target for drug development (Mueller et al., 2009), and especially the application of inhibitors of Rho kinase (ROCK) (Mueller et al., 2005), this view is simplistic given advances in our recent knowledge since astrocytes are much more than an inflammatory cell displaying adaptive plasticity in the functioning CNS. Thus many aspects of astrocyte biology offer options as attractive targets to improve their brain health and hence to effect a resultant improvement in synaptic function (Vargas and Johnson, 2010).

Our earlier research demonstrated the association between astrocytic morphology and a number of important aspects of astrocytic function, particularly the abundance and activity of Glu transporters ( excitatory amino acid transporters, EAAs). We observed that altering the morphology of astrocytes, using cyclic AMP analogs or ROCK inhibitors, also increased Glu uptake (Lau et al., 2005), elevated transporter Vma with an approximate doubling of EAAT2 expression at the cell surface and a smaller increase in EAAT1 expression as quantified by immunoblotting (Lau et al., 2011). Similar changes in EAAT activity or abundance were also observed in other treatments altering astrocytic morphology (Zagami et al., 2005, 2009; OShe et al., 2006). We concluded that ROCK inhibitor-induced elevations in Glu transporter function may contribute to their beneficial actions in brain pathologies, since enhanced EAAT activity is likely to be beneficial in CNS injury where excitotoxicity is a common mechanism effecting neurodegeneration (Beart and O’Shea, 2007; Shlekin and Robinson, 2007). Later work led to the hypothesis that changes to the astrocytic cytoskeleton induced by Rho kinase inhibitors were accompanied by the adoption of a “healthy” phenotype. We defined this pro-survival, healthy phenotype as possessing elevated expression of EAAT2, BDNF and key anti-oxidant genes. A shift in the F/G-actin ratio in favor of G-actin, indicating a reduction in actin stress fibers and alterations to cytoskeletal signaling mechanisms (Kuhn et al., 2000), was also considered integral to this “healthy” phenotype. Much more is known about ROCK inhibitors and their ameliorative effects on destructive (“cytotoxic”) gial scarring (Mueller et al., 2005), but our findings reveal diverse “healthy” effects on the astrocyte transcriptome likely to be beneficial in brain injury.

Our combined interest in the relationship between astrocyte morphology and biology led us to apply tissue engineering (Tse et al., 2006) to astrocytes. We found that 3D poly-ε-caprolactone (PCL) scaffolds altered astrocytic responses in vivo in a model of traumatic brain injury (Nisbet et al., 2009). Here our hypothesis was promotion by the bioscaffold of a cytoprotective astrocyte phenotype, so when considered with a likely role for the extracellular matrix (ECM) (Lau et al., 2012), we speculated about links to Rho GTPases, perhaps involving the actin cytoskeleton. In primary culture, astrocytes on 3D PCL scaffolds displayed reduced cytoskeletal stress as confirmed by decreased expression of GFAP and increased G-actin (Lau et al., 2014), and, when maintained over an extended period, possessed an extensively arborized, stellate morphology. These astrocytes showed a gene expression profile strikingly similar to that of 2D astrocytes treated with Fasudil, with up-regulation of genes for EAAT2, BDNF and anti-oxidant enzymes (Lau et al., 2014). Since 2D astrocytes treated with Rho kinase inhibitors also adopt a stellate shape, astrocyte transcriptome (Lau et al., 2012) is likely to contain insights into previously unsuspected mechanisms given new literature on this class of molecules.

In this study, we sought to place our findings in their contemporary context (Parpura et al., 2012; Burke and Sofroniew, 2014), by further interrogating our transcriptome after Fasudil treatment through mining this astrocyte database to reveal previously unexplored biological themes. Secondly, given our success with 3D bioengineered astrocytes, we undertook additional analyses on the possible combined benefits of Rho kinase inhibitors in our 3D culture model. Together these data provide further evidence that ROCK inhibitors produce physiologically beneficial responses in astrocyte biology which are likely to be beneficial in the management of inflammation in diverse neuropathologies.

**MATERIALS AND METHODS**

**ANIMALS**

C57BL/6 mice were obtained from the Florey Neuroscience Institutes (Melbourne, VIC, Australia). All experiments receive ethical approval from the Florey Neuroscience Institutes Animal Experimentation Ethics Committee (ethics approval number 07-051). Experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code for the Care and Use of Animals for Experimental Purposes in Australia.

**BIOTECHNOLOGY, CYTOCHEMISTRY AND NEUROCHEMICAL ASSAYS**

Secondary astrocyte cultures were established from forebrain of postnatal day 1.5 mice as described previously (Lau et al., 2011).

Briefly, forebrains were dissected in ice-cold solution (HIBS, Hank’s balanced salt solution; 137 mM NaCl, 5.37 mM KCl, 4.1 mM NaHCO3, 0.44 mM KH2PO4, 0.13 mM Na2HPO4, 10 mM HEPES, 1 mM sodium pyruvate, 13 mM D(-)glucose, 0.01 g/l phenol red), containing 3 mg/ml bovine serum albumin (BSA) and 1.2 mM MgSO4, pH 7.4). Cells were dissociated, centrifuged, and the pellet resuspended in astrocytic medium (AM: DMEM, Dulbecco’s modified eagle medium, 10% FBS, 100U/ml penicillin/streptomycin, 0.25% (w/v) Fungizone™), preheated to 36.5°C at a volume of 3 ml per brain and plated at 10 ml per 75 cm² flask. Cells were maintained in a humidified incubator supplied with 5% CO2 at 36.5°C and complete medium changes were carried out twice weekly.

After 10 days in vitro (DIV), when a confluent layer had formed, the cells were shaken overnight (180 rpm) and rinsed in fresh medium to remove non-astrocytic cells. Astrocytes were subsequently detached using 5 mM EDTA (10 min at 27°C) and seeded on 96-well plates, random or aligned FCL scaffolds in 96-well plates (all 8 x 10⁵ cells/well or on 13 mm glass coverslips (conventional 2D controls) in 24-well plates (2 x 10⁴ cells/well). 3D fibrous scaffolds were engineered from unfuctionalized FCL using electrospinning (Nisbet et al., 2009); thicknesses were approximately 250 and 150 μm for random and aligned scaffolds, respectively. Astrocytes were treated 8 div later
with vehicle, N,N',N'-dioctylcarbazole-3,3'-cyclic monophosphosphate (DIO-AMP, 1 mM), or Fasudil (100 μM) for a further 72 h when biochemical and morphological analyses were undertaken.

Cytochemistry for GFAP, F-actin and G-actin has been described previously (Lau et al., 2011). For immunocytochemistry, cells were washed with phosphate buffered saline (PBS; 0.137 mM NaCl, 0.5 mM KH2PO4, 0.5 mM Na2HPO4, pH 7.4) and fixed in 4% paraformaldehyde (PFA) for 10 min. followed by three washes with PBS, then incubated with 10% normal goat serum (NGS) and donkey serum (NDS) in PBS containing 0.3% Triton X-100. Cells were then incubated with primary antibodies against GFAP (1:1000; Chemicon) or AHNK (1:500; Molecular Probes), a marker of late-stage microglial and astrocyte activity (Racchetti et al., 2012), at 4°C overnight on a rocking platform. Cells were then washed and incubated with secondary antibodies (anti-rabbit Alexa Fluor® 488 for GFAP, 1:500; anti-mouse Alexa Fluor® 568 for AHNK, 1:500; Molecular Probes) and Hoechst 33342 (1:500 dilution) stained in 2% (v/v) NGS or NDS in PBS containing 0.3% (v/v) Triton X-100 for 3 h at room temperature. Cells were then again washed with PBS three times at room temperature. Coverslips and scaffolds were then mounted on glass microscope slides using Dako fluorescent mounting medium and left to dry in the dark overnight. Both coverslips and scaffolds were stored at 4°C until examined by microscopy.

For concurrent labeling of F- and G-actin, cells were washed rapidly with PBS twice by vacuum aspiration and incubated in stabilizing solution (10 mM Tris base, 0.137 mM NaCl, 0.01% Triton X-100, 2 mM MgCl2, 0.2 mM DTT (Bio-Vector, Canada), 10% glycerol) for 1 min at 4°C. Cells were then washed with 4% (v/v) PFA in PBS for 15 min at 4°C. Cells were washed in PBS twice at room temperature and excess PFA was quenched by adding 50 mM NH4Cl in PBS for 15 min at room temperature. Cells were permeabilized in 0.5% (v/v) Triton X-100 in PBS for 5 min and incubated in blocking solution (2% (v/v) BSA, 0.1% (v/v) Triton X-100 in PBS) for 15 min at room temperature. Following another two washes with PBS, cells were incubated in dye solution (DNAse-Alexa Fluor 488/1:250; Molecular Probes; rhodamine-phalloidin 1:25 in blocking solution; BDH) for 30 min in the dark. Cells were then washed in PBS three times and mounted on glass microscope slides using Dako fluorescence mounting medium and left to dry in the dark. Cells on scaffolds were mounted by placing the scaffolds with the cells on top, mounted with Dako fluorescence mounting medium and glass coverslips (13 mm round; Menzel-Gläser). Slides were stored at 4°C until required for imaging.

Methods for measurement of cellular viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), an index of mitochondrial function] and lactate dehydrogenase (LDH) assays have been published (Lau et al., 2011). After treatment, MTT was added to the wells give a final concentration of 0.5 mg/mL, incubated with the cells at 36.5°C with 5% CO2 for 30 min. Media were aspirated and 300 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan product. The absorbance was subsequently measured at 570 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer. Lactate dehydrogenase (LDH) assay was carried out using a commercially available kit (Roche). Medium (50 μL) was collected from each well and placed in a 96-well plate. The samples were incubated with the reaction mixture (CytoTox96 Detection Kit from Roche), according to the manufacturer’s protocol and left in the dark for 30 min. The absorbance of the sample mixture was determined at 490 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer. Data from these experiments were analyzed using Two-Way repeated-measures ANOVA with Bonferroni’s post-hoc test using Graphpad Prism software (Version 6).

To examine changes in relative abundance of F- and G-actin, the G-actin image was “subtracted” from the corresponding F-actin image after both images were converted to gray-scale, and integrated optical density was measured using ImageJ (NIH version 1.37). Image analysis used data from 4 images/well from 2 wells/culture over 3 independent cultures. The average value for all fields subjected to the same treatment in an individual experiment was analyzed to a single data point. All images for each term of actin were obtained using the same exposure settings. Statistical comparisons were made using Two-Way repeated-measures ANOVA with Bonferroni’s post-hoc test using Graphpad Prism software (Version 6).

MICROARRAY ANALYSES

Full details have been given previously (Lau et al., 2012), where we validated microarray data by quantitative RT-PCR. Differentially expressed genes between control and Fasudil-treated samples at each time point were then filtered to include only those passing a stringent false cut-off of 0.05. Data have been previously deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE28098 (Lau et al., 2012).

RESULTS

NOVEL INSIGHTS FROM TRANSCRIPTOMIC PROFILING INTO BENEFICIAL ACTIONS OF FASUDIL IN ASTROCYTES

Our initial rationale for undertaking microarray analyses to define the genomic changes induced by Fasudil in astrocytes was the total “disconnect” between the extremely rapid changes in astrocytic shape, which were quite obvious at 30 min, and the alterations in Glu transport which were of a much slower time course (≥ 24 h) (Lau et al., 2011, 2014). We reasoned that appreciable transcription and new protein synthesis must be taking place to underpin these changes, and that understanding the molecular changes in astrocytes should allow new mechanistic insights into how ROCK inhibitors provide benefit during brain insults. Our initial bioinformatics revealed that differentially expressed genes at 2 and 6 h were predominantly down-regulated, and after gene ontology analysis, did not appear to follow a particular biological theme so our focus was on large significant fold changes at later time points (12 and 24 h). Our attention thus settled upon major biological processes regulating astrocytic morphology and cytoskeletal reorganization viz. actin cytoskeleton, axon guidance, transforming growth factor-β signaling and tight junctions. We also found large changes in many genes associated with the ECM (Lau et al., 2012). Here, in view of new understanding that astrocytic responses occur across a continuum that is dependent upon the extent of trauma/disease, and which may resolve when minor and be manageable even in glial scarring by
pharmaceutical intervention (Mueller et al., 2009; Sofroniew, 2009), we undertook a new mining of our transcriptomic database accessible through GEO Series accession number GSE25829 (Lau et al., 2012).

**Transport and molecular motors**

Since our published work had ended with a focus on the pro-survival ("cytoprotective") astrocytic phenotype produced by ROCK inhibition (Lau et al., 2012), we took a step back and focused our attention on mechanistic issues related more broadly to signalling and trafficking events underpinning astrocytic motility and cytoskeletal reorganization. ROCK inhibitors effect disassembly of actin stress fibers and focal adhesions (Mueller et al., 2005), and we documented rapid disassembly (as early as 15 min) of phalloidin-labeled actin stress fibers in cultured murine astrocytes treated with fasudil. Here we demonstrated the astrocyte underwent a transition to a preponderance of G-actin relative to F-actin, which were increased and decreased 4-fold, respectively (Lau et al., 2010). Rho GTPases, notably Rho and Rac, are key regulators of actin and microtubule cytoskeletons, and actin flow can regulate the positioning of the microtubule cytoskeleton. Active transport, be it anterograde or retrograde, plays a key role in the delivery of gene products and cellular organelles and has been studied in detail in neurons—as its disruption leads to "transportopathies" in various neurodegenerative conditions (Liu et al., 2012). Very little is known of these events in astrocytes where we found expression profiles of kinesin family members (KIF2A, KIF13A, KIF18A, and KIF21A), involved in anterograde transport, were down-regulated (2–3-fold) at 2 and 6 h after Fasudil (KIF24, Table 1). Dynamin and dynactin members linked to the retrograde motor system also displayed reduced expression at early time points but had returned to control by 24 h. We extended these analyses to include GTPase Rabs, which act as molecular switches to mediate vesicular transport along the cytoskeleton by engaging specific motor proteins (Ng and Tang, 2008)—members of Ras oncogene family 3 (RAS) may play a role in necroptosis in astrocytes and there was a notable down-regulation of the expression of its isoform RAB3D at 12 and 24 h (Table 1). Interestingly, two targets of ROCK phosphorylation, syntaxin binding protein 1A (STX1A) involved in vesicle docking/fusion (3-fold decrease, 6 h), and dystrophin-related protein 2 (DPR2), collapsing response mediator protein 2 (2-fold decreases, 12 and 24 h, Table 1) linked to semaphorin-mediated guidance mechanisms (Azimouche et al., 2009), were also down-regulated. Given the general trend of data here after ROCK inhibition was decreased gene expression, we wondered whether the consequence stabilization with astrocytes adopting an aligned linear and a pro-survival phenotype (Lau et al., 2011, 2012) might also reflect transition to a non-migratory state as has been suggested in normal brain (Ciric et al., 2014).

**Autophagic and lysosomal systems**

Autophagy, a key system regulating cellular homeostasis including protein and organelle degradation, is known to be affected by

| Table 1 | Selected genes with expression changes passing the filter of fold change > 2.0 and FDR < 0.05 in at least one time point for Fasudil-treated compared with untreated astrocytes. |
|---|---|---|---|---|---|---|---|
| Gene ID | Description | 2h | 6h | 12h | 24h |
| | | P-value | Fold-change | P-value | Fold-change | P-value | Fold-change | P-value | Fold-change |
| KIF2A | Kinesin family member A4 | 2.00E-02 | -1.42 | 1.21E-07 | -2.20 | 1.90E-01 | 1.00 | 9.26E-01 | -1.02 |
| RAB3D | Member RAS oncogene family | 6.14E-02 | -1.23 | 7.69E-03 | -1.40 | 1.76E-07 | -2.37 | 1.55E-05 | -1.78 |
| DRP2 | Dystrophin related protein 2 | 2.08E-02 | -1.83 | 5.92E-01 | -1.10 | 8.41E-08 | -2.35 | 4.82E-03 | -1.77 |
| FANC1 | Forkhead box O1 | 1.99E-02 | -4.19 | 2.32E-05 | -2.10 | 1.90E-04 | 1.81 | 4.26E-02 | 1.36 |
| SCSTM1 | Sca1/stem cell marker 1 | 1.03E-02 | -1.98 | 8.39E-09 | -2.17 | 1.04E-02 | 2.27 | 2.39E-01 | 1.46 |
| MPN1 | Midkine 1 | 4.32E-01 | -1.17 | 7.17E-08 | -3.20 | 3.02E-01 | -1.22 | 2.16E-01 | 1.06 |
| JAK2 | Janus kinase 2 | 3.85E-02 | -1.94 | 1.29E-08 | -2.10 | 2.78E-01 | 1.16 | 2.71E-02 | 1.16 |
| NPC1 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | 1.24E-04 | -1.05 | 1.35E-03 | -1.40 | 2.36E-03 | 1.37 | 2.67E-01 | -1.11 |

Gene expression data are from NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE25829 (Lau et al., 2012). The FDR threshold of 0.05 corresponds to a P-value of 2.2 x 10^-6, 1.0 x 10^-4, and 2.0 x 10^-3 and 1.7 x 10^-1 for the 2, 6, 12, and 24 h time points, respectively. Please see the P-values for the fold changes at each time point.
ROCK inhibitors, and is also considered a novel target for management of neurodegenerative diseases (Harri and Rubenstein, 2012). Indeed, actions here would hardly be surprising given the cytoskeletal changes and the fact autophagosomes themselves are membranous cargo moving along microtubules and transported by the kinesin and dynein/dynactin complex—expression of genes related to molecular motors was generally found to be decreased here. ROCKI/2 regulate actin dynamics and cell migration through phosphorylation of various substrates, but little is known as to how the cytoskeleton influences the activity of the ubiquitin-proteasome system and autophagy. ROCK inhibitors have systemic, dependent actions being reported to accelerate autophagic flux (Mleczak et al., 2013), enhance both UPS and autophagic activity (Bauer et al., 2009), and to inhibit autophagosome formation (Aguilera et al., 2012). The literature on autophagy in astrocytes is relatively small (Dello Russo et al., 2013), but suggestive that as highly plastic glia they adapt to stress and support neurons (Lee et al., 2016; Title et al., 2018). Mammalian knockdown members of the class O (FoxOXO) are master signaling integrators influencing many cellular responses, including oxidative stress and inflammation. FOXO pathways are linked to both autophagy and the UPS, and lack of FOXO1 prevents autophagy (Yang et al., 2013). While there is a substantial literature on the role of FOXO1 in inflammation, very little is known about its involvement in astrocytosis. However, recently oxidative-induced injury of astrocytes was reported to involve metallothionein-3 via a FOXO-dependent mechanism (Lee et al., 2014). In our database, substantial changes were found in the expression of FOXO1 at 2 and 6 h (4- and 2-fold, respectively) post-Fasudil, although significant changes were not observed at longer times (Table 1). We extended our examination to other autophagic genes and found the expression of sequestosome 1 (SQSTM1, p62), a multifunctional scaffolding/adaptor protein interacting with both autophagosomal and proteasomal systems (Krohlich et al., 2010), was also decreased at both 2 and 6 h (Table 1). Mitochondrial transport is also driven by molecular motors with associated proteins linking mitochondria to microtubule-based transport, and there is a rapidly expanding literature on mitochondrial dynamics and recruitment of mitophagy, an unique form of autophagy handing damaged mitochondria (Baker et al., 2014). Expression of mitofusin 1 (MFN1), an outer mitochondrial membrane protein involved in mitochondrial dynamics, was downregulated 2-fold at 6 h (Table 1), and had returned to control levels at longer time intervals. Ras homolog gene family members T1/2 (RHO11/12, Miro1/2), Rho GTPases mediating mitochondrial transport by sensing Ca2+, also underwent two-fold decreases in expression at 6 h. Many genes involved in mitochondrial dynamics, including fission and fusion, contribute to the pathological events in brain pathologies (Wang et al., 2009; Bauer et al., 2014). Overall inhibition of Rho kinase results in an astrocyte where protein degradation pathways appear down-regulated initially and then assume a relatively inactive mode consistent with adoption of a healthy, anti-inflammatory phenotype.

Pro-inflammatory mechanisms

Inflammatory events related to glial scarring have received attention in the earlier literature (Muller et al., 2005; Ding et al., 2016; Yu et al., 2010), so we examined these events in our astrocytic system after ROCK inhibition. Unregulated activation of the Janus Kinase-Signal Transducer and Activators of Transcription (JAK-STAT) pathway is a key driver of various inflammatory conditions and has been identified as a target for therapeutic intervention (Kaminska and Swiatek-Machado, 2008). Although less understood in brain, oxidative stress and some cytokines activate via JAK2-dependent mechanisms STAT3 (Flanagan et al., 2000). Numerous changes, mainly at early timepoints, were noted in these pro-inflammatory mechanisms and were suggestive of decreased activity. Although down-regulation of expression of JAK2 was found at 2 and 6 h (Table 1), interestingly in our mature cultured astrocytes the expression of STAT3 (data not shown) was unchanged by Fasudil. Although recent elegant work points to a quite precise role of STAT3 in scar-forming astroglia surrounding inflammatory cells in spinal cord injury (Vinner et al., 2018), the recruitment of astrocyte cellular signaling in inflammation appears context dependent (Scelfo et al., 2014). We found that the expression of both nuclear factor of kappa light chain gene exchange in B cells (NKG2D) and suppressor of cytokine signaling (SOCS2) was reduced at 2 h and had returned to essentially control levels at longer time intervals (Table 1). While NKG2 deletion or knockdown reduces inflammation in a number of CNS injury models (Scelfo et al., 2014), the regulation of SOCS system is extremely complex generally functioning to reduce chronic inflammation (Lmassa et al., 2013). Indeed conditional ablation of SOCS3, but not STAT3, produces contraction of lesion area and notable improvement in functional recovery after spinal cord contusion (Okada et al., 2006).

Hypoxic-inducible factor-1 system and angiogenesis

We previously characterized the hypoxic-inducible factor-1 (HIF-1) system in an astrocytic model of tolerance against oxidative injury where there was downstream production of vascular endothelial growth factor (VEGF) (Chau et al., 2010). Thus in our work we were interested to explore the HIF-1 system and the effects on downstream genes involved in angiogenesis and energetics, since these mechanisms are potentially neuroprotective (Trendelenburg and Dinslak, 2005). Astrocytes are the major source of brain VEGF in the brain and various stimuli can modulate its induction and secretion (Engelhardt et al., 2014). Whilst inhibition of ROCK can activate VEGF-driven neovascularization and angiogenesis (Koili et al., 2009), it is very clear the VEGF-mediated responses are concentration- and system-dependent being either beneficial or detrimental in the brain (Ellison et al., 2013). The expression levels of VEGF4 and HIF1A were significantly reduced at 2 and 6 h, respectively, and had returned to control levels at longer time intervals (Table 1); significant changes were not found for erythropoietin (data not shown). Whilst a small body of evidence pertinent to astrocytes indicates that HIF1A regulates downstream expression of VEGF (Chavez et al., 2006; Chau et al., 2010), we were surprised to find a large 3-fold decrease of VEGF at 2 h, evidence which might support the recently described regulation of VEGF expression independent of HIF1 signaling in astrocytes (Arany et al., 2008; Schnaid-Badnik et al., 2008). Recently, astrocyte-derived VEGF was reported to drive blood-brain barrier disruption (where astrocytes also retract their endfeet from vessels) in brain inflammatory disease and
inhibition of VEGF signaling suggested as a protective approach (Argaw et al., 2012). Astrocytes are less susceptible than neurons to injury by impairment of oxidative metabolism, at least in part because of their capacity to switch to glycolysis, which particularly under conditions of hypoxia is linked to the HIF-1 system (Schmid-Brandt et al., 2006). Here, hexokinase 2 (HK2), a key glycolytic enzyme, considered an integral component of the downstream response displayed large reductions in expression at both 2 and 6 h consistent with the VEGF data (Table 1).

**ROCK INHIBITORS PROVIDE ADDITIONAL BENEFITS FOR ASTROCYTES ON 3D ELECTROSPUN SCAFFOLDS**

*Effects of drug treatments on astrocyte cultured on glass coverslips*

Cultures of mouse astrocytes were established on glass coverslips (2D) or on random or aligned PCL scaffolds for 18 div and immunolabeled with antibodies against GFAP and AHNK. In agreement with previous studies, conventional 2D astrocytes exhibited a more radially morphologic with more extensive processes when treated for 5 days with dbcAMP (1 mM), Fasudil (100 μM) or Y27632 (30 μM) (Figure 1) (cf. Lau et al., 2012). Under control conditions, 2D astrocytes appeared as flattened, polygonal cells and most were GFAP positive. Labeling for AHNK immunocytochemistry was more widespread and partially co-localized with GFAP under control conditions (see below, Figure 1). When treated with dbcAMP, astrocytes appeared to undergo complete stellation, with reduced cell body area and elongated processes. The processes were thicker and shorter when compared to astrocytes treated with both ROCK inhibitors. Interestingly, the staining for AHNK in astrocytes treated with dbcAMP was relatively darker compared to control (Figure 1). The effects of Y27632 treatment on astrocytes mimicked that of Fasudil. Cells demonstrated increased retraction of cell bodies as well as elongated extensive processes labeling pattern for AHNK, a master of enalpanganolyses (Racchetti et al., 2012), were similar to GFAP distribution, but more widespread under control conditions and following experimental treatments (Figure 1). Notably all cases GFAP-positive processes were well defined whereas AHNK labeling of astrocytes was more intense and its distribution through all parts of the astrocytic arbor, including fine processes, made full resolution difficult.

**Effects of bioscaffolds on astrocytic morphology**

Astrocytes displayed a different phenotype when cultured on either random or aligned bioscaffolds with cells possessing elongated cell bodies, ramified cell processes and condensed GFAP filaments (Figure 1). On random scaffolds, astrocytes formed tighter clusters, approximately 100–250 μm in diameter, but use of aligned bioscaffolds produced astrocytes with more extensive elongated processes (approximately 50–250 μm) following fiber orientation that were distributed in loose clumps. Under control conditions, the labeling pattern for GFAP was found to partially co-localize with AHNK, although the latter was more widespread, in cultures on both biomaterials, similar to those cultured on glass coverslips (Figure 1).
Effects of drug treatments on astrocytes cultured on different substrates

Astrocytes treated with dbcAMP (1 mM, 72 h) appeared to form clusters on both random (clusters approximately 100–200 μm in diameter) and aligned scaffolds (approximately 75–125 μm in diameter). In contrast to 2D cultured astrocytes, the processes were extensive, and outgrowth of the processes was more widespread. Processes infiltrated both type of scaffolds, as previously described (Lau et al., 2014) in random scaffolds (process length approximately 75–150 μm), but there was more growth along aligned fibers (approximately 100–300 μm) (Figure 1). As was the case in astrocytes cultures on glass coverslips, immunolabeling of AHNAK was widespread and more ubiquitously expressed through the entire astrocytic area than GFAP with which it was generally co-localized in major processes (Figure 1).

When treated with Fasudil (100 μM, 72 h) astrocytes were evenly distributed and infiltration into both biomatrices was extensive. The processes appeared to be elongated approximately 50–200 μm on random scaffolds and 100–300 μm on aligned scaffolds, with retracted cell bodies. The process outgrowth was widespread on random bioscaffolds, but astrocytes on aligned bioscaffolds appeared to grow in a similar orientation as those on random scaffolds, and were evenly distributed forming less tight clusters with approximately 200–350 μm in diameter on both random and aligned scaffolds (Figure 1). Microscopic examination revealed that the labeling pattern for AHNAK was more widespread through the whole area than that of GFAP, which was restricted to major processes, on both biomatrices (Figure 1).

The effects of the other Rho Kinase inhibitors, Y27632, mimicked that of Fasudil. Astrocytes treated with Y27632 (30 μM, 72 h) also demonstrated extensive processes on both types of scaffolds, however the processes appeared to be longer than in astrocytes treated with Fasudil, with an approximate 150–300 μm in astrocytes cultured on random and approximately 75–350 μm length in astrocytes cultured on aligned biomatrices (Figure 1). AHNAK immunolabeling revealed much more of the astrocytic arbor and thus was partially co-localized to GFAP but more widespread.

Effects of drug treatments on actin expression

Since the actin cytoskeleton plays a determinant role in regulating cellular responses to the extracellular matrix, the effects of fibrillar surfaces on actin dynamics were examined by staining astrocytes for its two forms: F-actin (rhodamine-conjugated phalloidin) and G-actin (Alexa Fluor 488-conjugated Dylight) (Figure 2). Under control conditions 2D astrocytes displayed well-organized F-actin fibers with densely packed stress fibers and a diffuse expression of globular G-actin. When treated with dbcAMP for 72 h, the intensity of labeling for G-actin increased, while F-actin displayed a prominent change from well-organized actin rings packed with stress fibers to more elongated processes with reduced stress fibers. A similar pattern of changes was found in astrocytes treated with the Rho kinase inhibitors Fasudil and Y27632, where remodeling of the actin cytoskeleton was demonstrated by a shift from F-actin to G-actin predominance. F-actin in astrocytes treated with Y27632 exhibited the same morphology as control, displaying a more organized ring shape without stress fibers. In contrast,
F-actin formed clusters and exhibit a more globular shape in astrocytes treated with Fasudil.

When cultured on random bioengineer scaffolds, F- and G-actin appeared to clump in the absence of presence of drug treatments. Astrocytes treated with dbcAMP and Fasudil displayed wider clumping of both F- and G-actin in relation to controls, while cells treated with Y27632 appeared in tighter clusters (Figure 2). On aligned scaffolds, astrocytes formed clumps in the absence or presence of drug treatments. Additionally, under control conditions or following treatment with Y27632 both F- and G-actin clumps appeared to be more “rectangular” in shape, with some increase in larger processes, presumably aligned with the fibers (Figure 2). Astrocytes treated with either dbcAMP or Fasudil formed F- and G-actin in tight small clusters, however F- and G-actin in astrocytes treated with Fasudil displayed elongated processes compared to cells treated with dbcAMP. There was maintenance of overall G-actin labeling under all conditions, and notably with Y27632, whereas all treatments decreased F-actin relative to control (Figure 2).

Image analysis revealed a significant difference in integrated optical density, reflecting a shift from F-actin to G-actin predominance when astrocytes were cultured in 2D or aligned fibers (F > 0.05 for all treatments versus Control) after 72 h treatment (Figure 3). A similar pattern of F- to G-actin ratio shift was observed in astrocyte cultures on random scaffolds but this change was not statistically significant.

**Effects of drug treatments on cell viability**

Biochemical analyses of astrocytes cultured on different substrates were undertaken in the absence and presence of drug treatments. Cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay] and cell damage (lactate dehydrogenase assay) revealed no significant detrimental effects of treatments (Figure 4). Minor but significant increases in cell viability or decreases in cell damage were observed in some combinations of substrate and drug treatment, but no obvious patterns were apparent. In all cases where significant changes were observed, drug treatments (Fasudil or Y27632) either increased mitochondrial activity or decreased cell damage; detrimental changes were not observed following drug treatments.

**DISCUSSION**

Given astrocytes are a plastic glial population existing in various morphologies and displaying diverse biologies, major and wide-ranging effects would be expected upon manipulation of the ROCK/Rho system, a key determinant via actin of cellular survival, migration and proliferation (Riento and Ridley, 2003). Whilst our initial bioinformatic analysis of transcriptomic changes induced by Fasudil were directed at major biological processes regulating cytoskeletal reorganization, we also found significant changes in expression of a diverse group of genes associated with astrocyte function, and suggested that overall ROCK inhibitors would produce “healthy and physiologically beneficial responses in astrocytic biology” (Luu et al., 2012). We thus posited the existence of a pro-survival, cytoprotective phenotype wherein the essential criteria were a preponderance of G-actin and elevated expression of EAAT2, BDNF and key antioxidant genes. Re-analysis of our bioinformatic data revealed diverse additional effects on the astrocyte transcriptome likely to be beneficial in brain injury. Gene expression profiles of motor and autonomic cellular cascades and inflammatory/myoogenic responses were all induced favoring adoption of what might be considered “healthy” anti-migratory phenotype. These types of changes generally have not been a focus in astrocytes, although often documented in neurons, but there is the beginnings of an new literature describing roles for ROCK inhibitors or actions of ROCK inhibitors in the biological processes reported here (vide supra). This concept of an anti-migratory phenotype (Cárdenas et al., 2014) is an interesting one for in vivo ROCK inhibitors produced very rapid stabilization of astrocytes (approximately 15 min with most changes complete by 3–6 h), a morphology which in uninjured brain is considered to reflect non-migratory properties, and here may indicate Fasudil produces a normal cytoprotective astrocyte. This state may resemble cytoprotic components of minimal, self-solving hypothermy often stated to occur in minor trauma/injury wherein there is re-establishment of a healthy physiological phenotype (Balasubraman and Tung, 1986; Sofroniew, 2009; Burna and Sofroniew, 2014). Earlier work has documented the ability of ROCK inhibitors to produce extension of GFAP-positive, presumed astrocytic processes in vitro and in vivo models of nerve crush (Sagawa et al., 2007; Ichihara et al., 2008). Extensive process formation has also been noted with ROCK inhibitors in wound healing models using astrocytes (Heite et al., 2003). Such morphological rearrangements as discussed here are underpinned by extensive rearrangement of the actin cytoskeleton, particularly lamellipodia and filopodia [Le Clair et al. and Cadene, 2008; Mattila and Lappalainen, 2008].

Tissue engineering in combination with materials science has been also been employed to manipulate astrocyte biology. We have previously reported an in vivo model of traumatic brain injury (Nisbet et al., 2009) that, in the presence of PCL,
scaffolds, astrocytes provided signals encouraging neurite infiltration of injured tissue, with findings favoring the existence of early cytotoxic and late cytotoxic components of astrogliosis. Others have reported variable data with astrocytes in tissue engineering and the literature suggests that the type of engineering approach employed greatly influences the nature of the outcome, and specifically the temporal contributions of cytotoxic vs. cytotoxic astrogliosis (Iannotti et al., 2003; Wong et al., 2007; Nibuet et al., 2010). Nevertheless our success in vivo encouraged us to pursue mechanistic studies with tissue engineering of astrocytes on PCL bioscaffolds where we found 3D extensive process formation, stellation and adhesion of a cytotoxic phenotype resembling that found in 2D astrocytes treated with ROCK inhibitors (vide supra). There have been remarkably few successes where bioengineering strategies in vitro have led to the establishment of viable astrocytes on bioscaffolds (Fusco et al., 1999; Zidovska et al., 2013), but our use of secondary astrocytes allowed the long term maintenance of mature cells on PCL bioscaffolds (Lau et al., 2014). Given there was a body of evidence for beneficial effects of ROCK inhibitors in models of head and spinal trauma (Rao et al., 2012; Wiatrak et al., 2014) we extended our study to explore whether the inclusion of Fasudil and Y27632 would provide further benefits in our 3D astrocyte model. Here we found that both ROCK inhibitors produced additional GFAP-positive processes relative to PCL scaffolds alone, and there seemed a bonus of a further shift to an even greater preponderance of G-actin relative to F-actin. AHRNK, a marker of endogenous activity and migration (Racchetti et al., 2012), proved very suitable for immunostaining of live astrocytic processes, being partially co-localized with GFAP, but revealing much more of the astrocyte than GFAP, an effect which was particularly obvious on aligned bioscaffolds. Overall these data are consistent with ROCK inhibitors providing further beneficial effects over and above PCL scaffolds alone. Preliminary evidence from Western immunoblotting suggested ROCK inhibitors reduced GFAP expression relative to dbcAMP (Supplementary Figure 1), whilst patterns of AHRNK expression were generally consistent with immunocytochemistry. In ongoing work examining Gla transporter activity, we confirmed our previous report of elevation of uptake in 2D astrocytes by Fasudil and Y27632 (Lau et al., 2011), and found EAAT activity appeared to be elevated 2-4 fold in astrocytes maintained on bioscaffolds (data not shown). Whilst we need to undertake further experiments to document fully the phenotype of the astrocytes found here, it does seem that the phenotype may be shifted even further toward the direction of cytotoxic astrogliosis. Our findings here, demonstrating that Fasudil and Y27632 under certain conditions either increased cell viability or decreased cell damage compared with control conditions (Figure 4), provide further evidence for the potential benefits of ROCK inhibitors via direct effects on astrocytes, which may contribute to the beneficial outcomes of these treatments in brain injury. Taken together there would seem to be a case for combining ROCK inhibitors with tissue engineering models of traumatic brain and spinal cord injury.

ROCK inhibitors, and especially Fasudil, have been examined in various injury models where astrocytes may contribute to the pathology, perhaps via cytotoxic inflammation, and/or by compromising recovery from trauma/neurodegeneration. Gla, including astrocytes, are known to contribute to the neuropathology of MND through non-cell autonomous mechanisms (Vargas and Johnson, 2015; Picoulot et al., 2014). MND is a rapidly advancing degenerative condition where suitable new therapeutic strategies are badly needed (Turner and Talbot, 2008), so the pro-survival response produced by Fasudil in the SOD1 mouse model of MND is an impressive advance (Takata et al., 2013; Tonges et al., 2014) since the rapid progression of disease in this model generally does not respond to interventions (Turner and Talbot, 2008). Amelioration of astrogliarial pathology was noted early after Fasudil treatment whereas beneficial effects were noted at much later stages of microglial mediated inflammation (Tonges et al., 2014). ROCK kinase inhibitors may also be useful in other neurodegenerative conditions where astrocytes contribute to inflammation, perhaps by non-cell autonomous mechanisms.
For example in Alzheimer’s disease, where astrocytic mechanisms are linked to disease risk factors and whose contribution to synaptic signaling is compromised by amyloid-β peptide (Talalayova et al., 2013), Fasudil may have therapeutic potential as it suppressed the inflammation in rodent hippocampus induced by amyloid-β peptide (Song et al., 2014). In experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis, Fasudil reduced inflammation and demyelination. Early or late administration of Fasudil exerted beneficial effects producing a shift in macrophage function from the cytotoxic M1 to the anti-inflammatory or reparative M2 phenotype in spinal cord and spleen (Lin et al., 2013). Subsequently the same laboratory suggested that at least part of this action of Fasudil was via a diminution of cytoskeletal astrogliosis and less infiltration of inflammatory cells across the blood brain barrier (Gue et al., 2014). Interestingly, astrocyte phenotype is now recognized to determine the outcome of CNS repair and inflammation, and components of astrocyte biology likely represent valid targets to enhance lesion repair in multiple sclerosis (Barnett and Linning, 2013).

Given the seminal role astrocytes play in synaptic transmission and maintenance of brain function generally, their diverse biology offers many options for potentially “druggable” targets—beneficial shifts to cytoprotective phenotypes would improve their overall health and ameliorate cytoxic inflammation in brain pathologies, and thus conceivably allow maintenance of appropriate synaptic function (Vargas and Johnson, 2010). Another often not discussed aspect of astrocyte biology is their multiple “morphological” interfaces, not only via the communication of astrocytic tight junctions, but also with different cellular populations via: neurons, oligodendrocytes, blood vessels, blood brain barrier and microglia (Volterra and Mederosi, 2005), including via the quad-particle synapse (Schafer et al., 2013). Thus astrocytes are seminal players in an organismal perspective to orchestrate the biology of these different CNS populations by integrating synaptic and non-synaptic signaling. The recent work with ROCK inhibitors showing cytoprotective changes in astrocyte function in disease models of MND (Torgesen et al., 2014) and multiple adenos (Gue et al., 2014) are particularly encouraging. Indeed, we speculate that Fasudil-induced changes in astrocytic phenotype exert beneficial effects which should be taken in a similar context to the much popularized “healthy” shift in macrophage function (now extended to microglia) from the cytotoxic M1 to the reparative M2 phenotype—and which is produced by Fasudil in spinal cord in experimental autoimmune encephalomyelitis (Lin et al., 2012). Met analysis of data from experimental studies of spinal cord injury evaluating Xho/AROCK blockade found significant overall improvement in locomotor function. Overall a possible role in inflammatory events was noted and the strategy was considered a plausible one for management of human spinal cord injury (Wiatrak et al., 2014). Certainly it is clear with advances in the design of increasingly effective ROCK inhibitors (Guan et al., 2013), and with new developments in tissue engineering and drug delivery via nanostructures that ROCK inhibitors alone or in concert with these new technologies are likely to be widely applicable to management of inflammation in neurodegenerative conditions.

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SUPPLEMENTAL MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/journal/10.3389/fnins.2015.00050/abstract

REFERENCES


Appendix F

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Interleukin-10 conjugated electrospun polycaprolactone (PCL) nanofibre scaffolds for promoting alternatively activated (M2) macrophages around the peripheral nerve in vivo

Jason R. Potas *a,b,1, Faria Haque a,1, Francesca L. Maclean c, David R. Nisbet c

a Centre for Neuroscience, John Curtin School of Medical Research, The Australian National University, Acton, ACT 0200, Australia
b NHMRC Centre of Research Excellence in Neuroplasticity and Repair, The Australian National University, Acton, ACT 0200, Australia
c NHMRC Centre of Research Excellence in Neuroplasticity and Repair, The Australian National University, Acton, ACT 0200, Australia

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ABSTRACT

Macrophages play a key role in tissue regeneration following peripheral nerve injury by preparing the surrounding parenchyma for regeneration, however, they can be damaging if the response is excessive. Interleukin 10 (IL-10) is a cytokine that promotes macrophages toward an anti-inflammatory wound-healing (M2) phenotype. The bioactive half-life of IL-10 is dependent on the cellular microenvironment and ranges from minutes to hours in vivo. Our objective was to extend the in vivo bioavailability and bioactivity of IL-10 by attaching the protein onto nanofibrous scaffolds and demonstrating increased expression levels of M2 macrophages when placed around healthy intact peripheral nerves. IL-10 was adsorbed and covalently bound to electrospun polycaprolactone (PCL) nanofibrous scaffolds. In vivo bioavailability and bioactivity of IL-10 was confirmed by wrapping IL-10 conjugated nanofibres around the sciatic nerves of Wistar rats and quantifying M2 macrophages immunohistochemically double labelled with ED-1 and either arginase 1 or CD206. IL-10 remained immobilized to PCL scaffolds for more than 120 days when stored in phosphate buffered saline at room temperature and for up to 144 days when implanted around the sciatic nerve. IL-10 conjugated nanofibres successfully induced macrophage polarization towards the M2 activated state within the scaffold material as well as the adjacent tissue surrounding the nerve. PCL functionalized nanofibers are useful for manipulating the cellular microenvironment. Materials such as these could potentially lead to new therapeutic strategies for nerve tissue injuries as well as provide novel investigative tools for biological research.

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1. Introduction

Bioprosthetic conduits provide an attractive alternative for nerve repair and have received increasing attention over the past few years (Lu et al., 2011). An important focus for the fabrication of artificial nerve scaffolds has been to mimic the natural extracellular matrix (ECM). In the peripheral nervous system (PNS), the ECM influences nerve repair, on both physical and biochemical levels. For instance, the ECM provides binding sites for cells to anchor themselves within the extracellular matrix (Wang et al., 2012), and is involved in

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promoting and directing axonal extension (Nibet et al., 2007),
whilst also playing an important role in Schwann cell prolifera-
tion, migration and myelination (Armstrong et al., 2007).
Electrospinning has become extensively used to fabricate nano-
structured scaffolds because the fabrication process is simple and
produces fibres that are structurally analogous to naturally
occurring protein fibrils of the ECM (Li et al., 2002; Ma et al.,
2005). Scaffolds electrospun from poly(ε-caprolactone) (PCL)
offer additional advantages over other materials, including
biocompatibility, biodegradability, ease of biofunctionalisation,
proven cytocompatibility and FDA approval (Home et al., 2010;
Nisbet et al., 2008, 2009). Electrospun PCL scaffolds are therefore
an ideal material to consider for the structural support of
repairing nerve tissue.

Following peripheral nerve injury, the distal segment of the
nerve undergoes a series of cellular and molecular events that
result in the breakdown of the distal nerve segment, which is
collectively referred to as Wallerian degeneration (Rothscher.
2011). During this process, cytolytic production contributes to
an injury-induced inflammatory response (Chen et al., 2007).
Inflammatory cells, such as classically activated (M1)
macrophages, are up-regulated in response to interferon-γ that
instigates inflammatory and antireparative responses as well as
degradation of the ECM (Mantovani et al., 2004; Mosser and
Edwards, 2008; Murray and Wynn, 2011). M1 cells mediate this
by releasing additional pro-inflammatory cytokines, such as
Tumour necrosis factor (TNF)-α, interleukin (IL)-1α and IL-
1β (Shamir et al., 2002), nitric oxide, reactive oxygen species,
and metalloproteinases (Murray and Wynn, 2011) that can
promote further tissue destruction. Subsequently, the produc-
tion of the anti-inflammatory cytokines IL-4/IL-13 and IL-10,
that collectively promote a variety of "alternatively activated" (M2)
macrophage phenotypes which mediate tissue restoration
and suppression of pro-inflammatory responses (Mosser and
Edwards, 2008; Murray and Wynn, 2011; Gordon, 2003). This
signals the attenuation of the initial defensive inflammatory
response, and is necessary for initiating wound repair and
restoring tissue homeostasis (Rothscher, 2011).

IL-10 is produced by several cell types, including regulatory
T cells and M2 macrophages, in a variety of tissue types
(Mantovani et al., 2004; Mosser and Edwards, 2008; Pesce
et al., 2009) as well as the peripheral nerve (Rothscher, 2011;
Ydens et al., 2012) following injury. It has multiple modulatory
effects on the macrophage population including the suppression
of pro-inflammatory cytokines (Mantovani et al., 2004; Fe
Yde et al., 2004; Mosser and Edwards, 2008), and macrophage
towards the wound healing M2 phenotype (Mantovani et al.,
The shift in the balance from M1 to M2 populations is postulated to be essential
since it occurs in tissues that undergo wound healing
(Deonna et al., 2007; Naehr et al., 2007).

IL-10 stimulation promotes arginase-1 expression in macro-
phages (Mantovani et al., 2004; Pesce et al., 2008) which
suppresses pro-inflammatory mediators (Pesce et al., 2009;
Ydens et al., 2012; Breißen and Mosser, 2011; Edwards et al.,
2006) by shunting metabolic pathways away from the produc-
tion of nitric oxide (Mosser and Edwards, 2008; Pesce
et al., 2009; Hesse et al., 2007; Breißen and Mosser, 2011). Arginase-1
expressing macrophages have also been shown to suppress T-
cell responses causing inflammation and fibrosis (Pesce et al.,
2009) as well as producing components of the ECM (Mosser
and Edwards, 2008). In addition to arginase-1 expression, M2
macrophages also express the mannose receptor, CED56, which
has been used in conjunction with arginase-1 as a marker to
identify M2 macrophage subtypes (Eigerl et al., 2006). The
mannose receptor facilitates phagocytosis of mannose N-linked
glycoproteins that are found on a variety of microorganisms
(Allavena et al., 2004; Taylor et al., 2005).

Promoting IL-10 following nerve tissue injury at strate-
gically defined locations may therefore offer targeted therapeu-
tic benefits by capitalising on its inflammatory suppressing/
worth healing potential. To date, investigations of IL-10 have
been carried out using gene delivery techniques (Boehler et al.,
2014; Arai et al., 2000; Milligan et al., 2005) which currently
have limited therapeutic applications in humans, or direct
administration of the IL-10 protein or peptide fragments which
remains biologically active for a restricted period of up to 2.5 h
in vivo (Rachmavati et al., 2011). Extending the bioavailability
and bioactivity of IL-10 to manipulate a cell population towards
a wound healing (M2) phenotype in spatially confined regions
of interest may therefore offer novel and practical therapeutic
approaches to improve regenerative outcomes following
nerve tissue injuries.

In the present study, we immobilised rat IL-10 on the
surface of electrospun PCL submicron fibres to increase its
bioavailability and bioactivity when implanted around periph-
eral nerves. Our hypothesis is that IL-10 conjugated to the
surface of the fibres will result in a greater polarisation of
macrophages to the M2 state surrounding healthy nerves. We
confirm long-term stability of IL-10 by demonstrating storage
of immobilised protein in phosphate buffered saline (PBS) for
up to 4 months. We also demonstrate bioactivity with effective
M2 macrophage polarisation up to 14 days following implanta-
tion of IL-10 conjugated scaffolds around the sciatic nerve of
rats. Our findings demonstrate the potential for long-term
cytokine delivery that could potentially attenuate inflammato-
ry damage and thereby create a favourable in vivo milieu for
neuronal regeneration.

2. Materials and methods

2.1. Preparation of electrospun PCL scaffolds

PCL was obtained from Sigma-Aldrich (St Louis, MO, USA), molecular weight = 70,000–80,000). Polymer solutions of 15% (w/v) were made with 3% (v/v) glacial acetic
acid (Chem Supply Pty Ltd, Australia) and 2% (v/v) pyridine, obtained from Sigma-Aldrich (St Louis, MO, USA), to make up 5 mL and stirred overnight at 35°C. A custom built electrospray consisting of a syringe pump (KD-100, KD Scientific, Holliston, USA) and an adjustable
DC voltage power supply (Model RR 50-1.25R/230/DEPM, Gamma High Voltage Research, Ormond Beach, FL, USA) was used with a voltage of 20 kV and a 21 G needle. Electrospr.
ning was performed at room temperature using a flow rate of 2 mL/h and a working distance of 13 cm. The scaffolds were collected on a rotating mandrel with a
diameter of 11 cm that rotated at 300 rpm to fabricate randomly oriented fibres. The scaffolds were dried in a vacuum oven (Labec) overnight at 30°C.
(SEM) was also calculated for comparison of the predicted value (cross-hatched bar of Figs. 3 and 4) as follows:

\[
SEM = \text{square root}\left[\frac{\text{variance}_{C/n} + (\text{variance}_{D/n})}{2}\right]
\]

where SD = standard deviations and n = sample number of groups C or D.

2.6. Surgical procedure

Forty male Wistar rats (6 weeks old) were used in this study. All experimental protocols were approved by the Australian National University Animal Ethics Committee and were undertaken in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Sibling pairs were used to minimise genetic variability: one sibling was designated to the experimental group, which received IL-10 immobilised PCL scaffolds (PCL-IL10 group); and the other sibling to the control group, which received unfunctionalised PCL scaffolds (PCL group). The groups were further divided into four recovery times resulting in n = 5 for each of the following 8 groups: PCL (1 day, 3 days, 7 days and 14 days); PCL-IL10 (1 day, 3 days, 7 days and 14 days). Under general anaesthesia (-2% of isofofar, Advanced Anaesthesia Specialist), the right sciatic nerve was exposed and freed from surrounding tissues through a biceps femoris muscle-splitting incision. A 1 cm square piece of appropriate scaffold was wrapped around the nerve and the free ends of the scaffold were tied in the middle with a 5-0 silk suture in order to hold it in place. The muscle was closed with a 6-0 silk suture and the skin was closed with 4-0 nylon sutures before returning the animals to their individual home cage for recovery and post-operative antibiotics (Cephalothin sodium, 15 mg daily for up to 7 days).

2.7. Immunohistochemical staining and image acquisition

At the end of the recovery periods, rats were given a lethal intraperitoneal injection of pentobarbital sodium (60 mg/kg, Vistar, with 2% lissocaine (lidocaine)). The animals were then perfused (transcardiac) with 300 mL of saline followed by 300 mL of 4% paraformaldehyde (VWR International, USA) at 4 °C prior to collection of both left and right sciatic nerves. The harvested nerves were post-fixed in 4% paraformaldehyde for 20 min then stored in a solution of 30% sucrose (Sigma, chem-supply) and 0.1% sodium azide (Sigma, Sigma) in PBS for several days prior to embedding in Tissue Tek: (Sakura, Japan). Sections from both left and right nerves were cut frozen in the longitudinal plane at a thickness of 20 μm and mounted onto the same slide. The left nerve served as a control nerve for the scaffold-recipients right nerve.

Immunohistochemistry was performed on consecutive sections of each specimen in order to assess the macrophage populations at the scaffold implantation site. Immunostaining was performed using antibodies directed against CD68 (ED1, 1:200, MPAB:435, Merck Millipore) to label the activated macrophage/monocyte population, and then double labelled with immunostaining of either arginase-1 (Arg1, 1:200 dilution, A860176, Abcam) or mannose receptor (CD206, 1:300, A864693, Abcam) to label the M1 macrophage phenotype. Slides were dehydrated in 70% ethanol and then rehydrated by immersion in Milli-Q water (Millipore Corporation) and then in PBS. Antigen retrieval was then performed by incubating the slides in reveal IT solution (Sigma Chemical Co., St. Louis, MO, USA) for 3 h at 37 °C. Slides were rinsed three times in PBS for 5 min on a rotary shaker, blocked with 2% Triton X signal enhancer (avivogen, Carlsbad, CA) for 30 min, then incubated in primary antibodies (ED1 and either Arg1, CD206 or CD22)
diluted with 2% signal enhancer in 0.1 M PBS at 4 °C overnight. The slides were thoroughly rinsed three times in PBS the following morning prior to incubation with secondary antibody (Alexafluor 554 chicken anti-goat; A21448, Invitrogen) diluted at 1:1000 with 2% signal enhancer in 0.1 M PBS at room temperature for an hour. The slides were rinsed again in PBS three times and incubated in Alexafluor 488 goat anti-mouse/A-11001, Invitrogen) secondary antibody solution (diluted 1:1000, 2% signal enhancer, 0.1 M PBS) for another hour. They were then cover slided using an aqueous media (Aqua-Poly/Mount, 18606, Polysciences Inc.).

The sections were examined and imaged using a LED fluorescent microscope (Carl Zeiss Coherent LED light source with a LSM 510 Pa microscope) and a digital camera (AxioCamMRc 5). All acquisition settings were kept constant for each antigen/antibody staining and all images were captured at 5.0 megapixels using a 20× objective lens. Images (dimensions: 500 μm × 700 μm) were sampled from tissue regions of interest that had minimal damage and provided the clearest field of view. The two regions of interest were located at the distal edge of the scaffold; one region was exclusively within the scaffold and the other region contained the scaffold border, the nerve border and the connective tissue in between. The area of analysis included all viable tissue within the captured image (region of interest), and was traced using Image software (version 1.49 h) to define the area of cell quantification. The entire ED1+ cell population within the traced boarders was identified and the cells were quantified manually offline under the green (488 nm) channel. Arg1, CD206 or CD32 expressing macrophages were over-laying the 594 nm channel and counting the double immuno-labelled cells (yellow/orange appearance) within the identical regions as the ED1+ cells. All cell quantification was performed blind to the experimental group under analysis. Example images were taken using a Nikon A1 confocal microscope with a ×40 objective, or a ×20 objective with a digital camera (Nikon D5-R1) for bright field images.

2.8. Statistical analysis

All values presented in the figures are expressed as mean ± SEM. Where only two data sets are compared, an un-paired, two-tailed Student’s t-test was used unless otherwise specified. For a comparison of more than two data sets, a one- or two-way ANOVA followed by Fisher’s LSD post hoc test was used where appropriate, and for unbalanced data of two factors, a linear mixed model statistical test was used. For all the statistical tests performed, a confidence interval of 0.05 (a = 0.05) was used, thus p < 0.05 was considered statistically significant. For an explanation of the statistics for calculated means shown in Figs. 3 and 4, refer to the section under the heading Quantification of IL-10 above.

3. Results

In this study, PCL submicron fibres were electrospun with random fibre orientation to increase the porosity of the scaffolds. Scanning electron microscopy was used to confirm the fibrous morphology of the scaffold (Fig. 1) and demonstrated that the resultant fibres had a mean fibre diameter of 400 ± 110 nm.

3.1. IL-10 attachment

IL-10 was successfully conjugated to the surface of the PCL scaffolds. The presence of IL-10 that was adsorbed and immobilised to the PCL scaffolds was quantified using ELISA (Fig. 3). ELISA performed on the control (Group A) and amionic (Group B) scaffolds determined the background level of absorbance at 450 nm due to non-specific binding and/or adsorption of primary and/or secondary antibodies used for

Fig. 3. ELISA for quantification of adsorbed and covalently bound IL-10. ELISA was used to confirm the levels of adsorbed and immobilised IL-10 on PCL scaffolds. Baseline absorbance at 450 nm indicated by the dashed line is based on the mean absorbance of Group A (unfunctionalised PCL scaffold). Group B indicates absorbance levels of amionicyzed PCL scaffolds. Group C (soluble IL-10) represents the relative amount of IL-10 adsorbed (ads) on the surface of the matrix, but not covalently bound. Group D (immobilised IL-10) indicates the relative amount of IL-10 protein from both adsorbed and covalently (ads + covalent) bound protein. The calculated difference between Groups D and C (cross-hatched bar, calculated mean ± SEM) indicates the relative density of covalently bound IL-10 on the scaffold surface (cov). See Fig. 2 for a summary of Groups A-D. n = 12 for all groups; ***p < 0.001; ****p < 0.0001 (one-way ANOVA).

Fig. 4. IL-10 retention in biofunctionalized scaffolds. ELISA was used to confirm the immobilisation of IL-10 to the PCL scaffolds. Baseline absorbance at 450 nm indicated by the dashed line is defined by the mean absorbance of unfunctionalised PCL scaffolds (Control). Dark grey bars indicate relative IL-10 levels of functionalised scaffolds following storage in PBS for 14, 21 and 120 days at room temperature. Light grey bars indicate relative levels of IL-10 following implantation around the sciatic nerve then removal of scaffolds at 3 and 14 days. *p < 0.05, one-way ANOVA. n = 3 for all groups except control group (n = 8). Numbers indicate p-values (one-tailed Student’s t-test) compared to calculated covalent fraction (cross-hatched bar).
IL-10 detection. The scaffold architecture and surface energy also facilitate the physical adsorption of the IL-10 protein onto the scaffold surface, which was quantified by Group C (Fig. 3). Group D shows the level of both physically adsorbed and immobilised IL-10 protein. The difference between Groups D and C was calculated (cross-hatched bar, Fig. 3) and suggests that approximately two-thirds of the IL-10 is immobilised whilst the remaining one-third is physically adsorbed to the surface. Fig. 3 therefore demonstrates that both physical adsorption and covalent attachment of the cytokine to the PCL scaffold was successfully achieved.

3.2. Storage of IL-10 immobilised scaffolds

For practical applications, it is necessary to demonstrate that immobilised IL-10 can be stored prior to implantation. To test whether the protein remained conjugated to the PCL scaffolds following storage, PCL-IL-10 scaffolds were stored in PBS prior to ELISA quantification. IL-10 remained present on the scaffolds stored in PBS at room temperature for 14, 21 and 120 days (dark grey bars, Fig. 4). There was no statistical difference in IL-10 levels between the freshly prepared conjugated scaffold (Group D, Fig. 3) and those stored in PBS for 14 or 21 days (p = 0.15, one-way ANOVA), however there was a significant reduction following 4 months of storage (p = 0.001, Student's t-test). The levels of IL-10 following 120 days of storage reduced to almost half of the levels following 21 days of storage, and were significantly reduced compared to the levels of the calculated immobilised fraction (cross-hatched bar, Fig. 4). This suggests that for the PCL scaffolds conjugated with protein, the storage up to 3 weeks does not greatly affect the absorbed and/or immobilised fractions, however the storage over longer periods may result in reduced IL-10 levels in the scaffold materials.

To further test whether the immobilised protein remained tethered to the scaffolds following storage and in vivo implantation, the right sciatic nerve of rats was wrapped with biofunctionalised scaffolds that were stored in PBS for up to 10 days prior to implantation. The animals were allowed to recover for an additional 3 or 14 days before the scaffolds were removed. ELISA performed on these scaffolds demonstrated that the cytokine successfully remained attached following sequential storage in PBS and implantation (light grey bars, Fig. 4). The levels of IL-10 were significantly reduced in both 3 and 14 day stored scaffolds compared to fresh scaffolds (Group D, Fig. 3) (p < 0.05, two-way ANOVA, Fisher's LSD post-hoc test).
and 14 day in vivo groups to approximately half that predicted to be immobilised (Fig. 4, compare light grey bars with crosshatched).

3.3. Effect of scaffold implantation on macrophage recruitment

To examine the effect of functionalised scaffolds on total macrophage recruitment following implantation, we quantified cells immunoreactive for ED1, a marker of activated macrophages, in the scaffold and adjacent peripheral/connector tissue (P/CT) regions of implant-recipient (see Fig. 5A–B for regions of interest for quantification), as well as contralateral nerves. There was no detectable ED1 staining in nerves or surrounding connective tissues contralateral to the scaffold implant. It was not possible to reconstruct one of the implant-recipient nerves at the 1 day recovery time point and therefore the P/CT region was omitted from further analysis at this time point (indicated in Figs. 5, 7 and 8). The mean numbers of ED1+ cells counted per region analysed of the implant-recipient side in the scaffold and P/CT regions were 39 ± 7 and 39 ± 5 respectively; the mean respective area analysed were 0.28 ± 0.12 and 0.30 ± 0.18 mm². There was no significant difference in the recruitment of ED1+ cells between the biofunctionalised (PCL–IL10) and control (PCL) groups in either the scaffold (p = 0.06, two-way ANOVA) or P/CT (p = 0.13) regions. There was, however, a temporal effect; both the scaffold and P/CT regions displayed a reduction of ED1+ cells numbers after day 3 (Fig. 5C) and day 1 (Fig. 5D) respectively. To investigate whether the functionalised scaffolds alter the proportion of ED1+ cells in either of the regions of interest, a ratio of the cell density in the scaffold/P/CT regions was calculated for each group and time point. There was no significant group effect of PCL–IL10 vs. PCL (p = 0.31, linear mixed model), time effect (p = 0.64) or the interaction of both these factors (p = 0.30), indicating that IL-10 treatment had no effect on macrophage recruitment in the scaffold or P/CT regions.

3.4. IL-10 bioavailability and activity

IL-10 is known to induce the differentiation of macrophages towards the M2 phenotype by inducing arginase-1 (Arg1). To examine the effect of functionalised scaffolds on M2 macrophage polarisation following implantation, we quantified cells immunoreactive for both Arg1 and ED1 (Arg1 + ED1+) as a proportion of total ED1+ cells in the scaffold and P/CT regions. Fig. 6A & B shows examples, and Fig. 7A & B shows group data quantification of ED1 and Arg1 immunoreactivity. In the scaffold region, approximately twice the proportion of Arg1+ ED1+ cells was found in the PCL–IL10 group for the day 1, day 7 and 14 day time points. At the 3 day time point the biofunctionalised scaffolds resulted in approximately a one third increase in the proportion of Arg1+ ED1+ cells (Fig. 7A). Although ED1 populations decreased over time (Fig. 5C), this had no significant effect on the ratio of [Arg1+ ED1+] / [ED1+] cells (Fig. 7A), indicating that time had no significant effect on M2 polarisation in the scaffold region (p = 0.08, two-way ANOVA). In the P/CT region, a similar pattern was observed, however, only the day 1 and 3 time points were statistically significant between the control and IL-10 biofunctionalised groups, whilst the latter time points failed to reach significance (Fig. 7B, day 7, p = 0.06; day 14, p = 0.05, Student’s t-test). Similar to the scaffold region, there was no effect of time on M2 polarisation observed in the P/CT region (p = 0.25, linear mixed model).

M2 macrophages also express the mannose receptor (CD206). Therefore, to confirm the proportion of activated M2 macrophages expressing the mannose receptor, we quantified cells immunoreactive for both CD206 and ED1 (CD206 + ED1+) as a proportion of total ED1+ cells in the scaffold and P/CT regions. Fig. 5C & D shows examples of ED1 and CD206 immunoreactivity, and Fig. 7C & D shows quantification of group data. CD206 as a second M2 marker showed consistent proportions of M2 in both the scaffold (Fig. 7C) and P/CT (Fig. 7D) regions as found with Arg1, i.e. confirming that approximately two thirds of ED1+ positive cells were of the M2 phenotype in IL-10 biofunctionalised scaffold recipient animals, compared to approximately one third in the control scaffold-recipients.

Arg1 produced in macrophages commits macrophages to the M2 state which switches metabolic pathways away from the M1 phenotype expression. To confirm that the scaffolds biofunctionalised with IL-10 did not affect the M1 population, we quantified cells immunoreactive for both CD32 and ED1 (CD32 + ED1+) as a proportion of total ED1+ cells in the scaffold (Fig. 8A) and P/CT (Fig. 8B) regions. IL-10 biofunctionalised scaffolds had no significant effect on the proportion of CD32 expression in ED1+ positive cells in either of the regions of interest investigated, indicating that biofunctionalised functionalised scaffolds had no effect on M1 macrophage polarisation.

4. Discussion

The capacity to manipulate resident cells at targeted locations to perform desirable functions, such as secreting factors and/or promote tissue remodelling is an exciting prospect for the development of novel regenerative therapies. Here, we report the successful conjugation of IL-10 onto the surface of PCL scaffolds and demonstrate the potential for long-term storage of the protein when conjugated. Furthermore, we show that these biofunctionalised scaffolds have the capacity to significantly alter the macrophage phenotype towards the immunosuppressive/regenerative M2 state in vivo, not only in the scaffolds where IL-10 is presented, but also in the tissue in direct contact and surrounding the nerve.

4.1. IL10 attachment

Immobilisation of IL-10 to the scaffold surface was achieved by amidolyzing the scaffold, following the N-hydroxysuccinimide (NHS) ester within the SMC cross-linker reacts with the primary amines, forming stable amide bonds. The scaffold was then incubated with IL-10 for secondary coupling of the sulphydryl-reactive maleimide with the cysteine residues of the IL-10 protein through the formation of disulphide bonds. This method has been used previously to immobilise brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) (Horne et al. 2010; Wang et al. 2012). Our data indicated that approximately one third of the IL-10 in freshly prepared samples was adsorbed, whilst the remaining two thirds of
the IL-10 was immobilised to the scaffold surface. This was calculated by subtracting the quantity of IL-10 found in Group C (PCL-IL10, Figs. 2 and 3) that lacked the protein cross-linking step, from Group D (PCL-IL10) that had both immobilised and adsorbed IL-10 on the scaffold surface. Our prediction of immobilised/adsorbed fractions assumes that the IL-10 signal is linear over the concentration range encountered. Whilst it is not possible to speculate on the accuracy of this assumption, the adsorption of IL-10 to the amine-activated PCL surface (Group C) was compared to Group D because IL-10 will react with SMCC to form covalent bonds and be immobilised to the PCL surface. If we assume no steric hindrance from the SMCC-IL10 complex, the unreacted amine groups that are present on the surface of Group D would contribute to IL-10 adsorption to the scaffold surface. However, the amine groups available for IL-10 adsorption in Group D would be reduced by the covalent bond formed between SMCC and the amine attached to the scaffold. We may therefore speculate that our measure of adsorption in Group D could be less than that shown by Group C, leaving our estimation of the immobilised fraction of two thirds somewhat conservative.

Interestingly, we found that the storage of the PCL-IL10 scaffold in PBS for 14 or 21 days resulted in a reduction of protein to approximately two thirds compared to that of freshly prepared samples, i.e. to levels not significantly different to the immobilised portion of IL-10 (Fig. 4). We therefore believe that the storage in PBS, for these periods, results in desorption of this protein, leaving only the immobilised protein on the scaffold surface. Desorption results from an IL-10 concentration gradient between the scaffold and the surrounding PBS, and is expected to occur prior to surface erosion of the scaffold.

Given its in vivo half-life of a few hours, the ability to detect significant levels of IL-10 on the scaffold and observe a biological effect after 14 days of implantation indicates that the immobilisation process permits a considerable extension of the bioavailability period. Others have used polysulfone/agarose hydrogel scaffolds filled with IL-4 to successfully induce M2 macrophages in a model of peripheral nerve injury. They were able to retain IL-4 in the hydrogel for 24 h before the cytokine diffused out (Moharram et al., 2012). Since our method employs chemical cross-linking of the protein to the nanofibre, it was expected that IL-10 would remain bioavailable for significantly longer periods which we were able to
confirm in the present study. The storage in PBS for 120 days or implantation of scaffolds for 3–14 days in vivo resulted in a reduction of IL-10 detection to about half of the calculated immobilised fraction (Fig. 4). As this reduction was greater than one third, i.e. the amount predicted to be adsorbed, these storage/implantation conditions are likely to have resulted from degradation of the protein itself and/or hydrolysis of the ester moiety in PCL, and subsequent erosion of the scaffold surface (Nisbet et al., 2000). It is not possible to determine the contribution of these factors for the additional reduction of IL-10 from the present study, however, the entire absorbed fraction is expected to be removed in the 3 and 14 day implantation groups due to deconstruction into the surrounding physiological fluid (Park et al., 2006), and therefore any additional reduction is likely to reflect degradation of the PCL surface. PCL degradation is expected not only because the material is biodegradable (Nisbet et al., 2000), but it is also consistent with the pattern of maximal macrophage recruitment observed in the scaffold region by day 3 (Fig. 5), and therefore the time when the majority of macrophage degradation of the scaffolds is expected to occur. As IL-10 has a relatively short half-life in vivo, and our PCL scaffolds are biodegradable, it is likely that a combination of both factors contributed to the reduced IL-10 following in vivo implantation of the functionalised scaffolds.

4.2. Biological activity of IL-10 functionalised scaffolds

We used immunohistochemistry to quantify macrophage polarisation. We are confident that we did not identify false positively labelled cells, as no ED1+ cells were detected on the contralateral nerve or surrounding connective tissue which was mounted onto the same slide as the scaffold-implanted nerves. Both experimental and contralateral nerves therefore underwent the identical immunohistochemistry protocols and conditions. In addition to serving as an immunohistochemistry control, our experimental procedure also demonstrates that implantation of the scaffold results in the recruitment of macrophages to the scaffold and surrounding nerve.

Arg1 is not constitutively expressed in macrophages, but rather, is tightly regulated by exogenous stimuli from the anti-inflammatory cytokines IL-4, IL-13 and IL-10 (Mantovani et al., 2004; Pesce et al., 2009; Mantovani et al., 2002). In the present study, we capitalised on the effect of IL-10 to up-regulate Arg1
as an indicator of M2 macrophage polarisation to demonstrate the biological activity of IL-10 biofunctionalised scaffolds. We also examined the expression of CD206, as a second marker for M2 macrophages, and observed a similar pattern of labelling as Arg1 over the time course investigated in both the scaffold and PN/CT regions. While others have used IL-10 to polarise macrophages using viral vectors (Boehler et al., 2014), or have mixed the M2 promoting cytokine, IL-4, with agarose inside polysulphone guidance tubes (Mokamarn et al., 2012), to our knowledge we are the first to immobilise IL-10 to PCL scaffolds and polarisation of macrophages towards the M2 phenotype in vivo.

We also quantified M1 macrophages using CD32 to confirm that IL-10 biofunctionalised scaffolds did not result in polarisation of this cell type. As IL-10 does not promote CD32 (Mantovani et al., 2004), a marker typically used to identify M1 macrophages (Mantovani et al., 2004; Vogel et al., 2013), our expectation that IL-10 biofunctionalised scaffolds did not promote M1 polarisation was satisfied. Interestingly, a reduction in CD32 expression was not evident from IL-10 biofunctionalised, compared to control scaffolds, and suggests that the IL-10 derived M2 macrophages are derived from a separate reservoir of macrophages to those that are CD32+.

Although beyond the scope of the present study, this observation is curious and warrants further investigations; it is inconsistent with the notion of switching between M1 and M2 subpopulations, but supports that proposed by Martinez and Gordon (2014) that macrophages may express mixed phenotypes (Martinez and Gordon, 2014). Moreover, it also highlights an example of how cytokine-tethered scaffolds may be useful as an investigative tool to probe the mechanisms behind macrophage polarisation.

The combination of conjugated and adsorbed IL-10 is effective in inducing macrophages to the M2 state in the vicinity of the scaffolds. IL-10 immobilised to the PCL is restricted to the site of interest and therefore macrophages are initially required to be in direct contact with the scaffold surface to encounter the protein where it is biologically active. However, the physically adsorbed component is free to diffuse from the scaffold surface into the surrounding tissue shortly after implantation (Wang et al., 2012), thereby extending a short-term bioactive halo of IL-10 beyond the surface of the scaffold surface. IL-10 desorbed from the scaffold is therefore free to promote the polarisation of macrophages beyond the scaffold surface towards the M2 state. The macrophages in contact with IL-10 can then be "switched on" to produce endogenous IL-10 (Mantovani et al., 2002) and thereby maintain protein levels in a feed-forward cycle. This schema is supported by our data where a significant increase in M2 cells was observed in the group receiving biofunctionalised scaffolds in the PN/CT located several hundred micrometres from the scaffold border (e.g. Fig. 1) as early as 1 day after implantation (Fig. 2). M2 cells normally respond in sequence after M1 macrophages have fulfilled their roles and are therefore not expected to be recruited to the PN/CT in significant numbers by day 1 (Rotthoefer, 2011). The presence of M2 cells at this early time point illustrates that M2 cells are being promoted outside their normal temporal arrangement. Furthermore, the perineural surface is insulated by connective tissue that is several hundred micrometres beyond the surface of the scaffold border and therefore not in direct contact with the scaffold surface. Several explanations could account for this: 1) macrophages could come in direct contact with the scaffold, be activated to express M2 markers, then migrate into the adjacent tissue; 2) M2 cells in the adjacent tissue could be signalled from cytokines produced by neighbouring cells; 3) IL-10 could dissociate and diffuse away from the scaffold surface; and/or 4) pieces of PCL fibres could break away from the main scaffold. Regardless of where the active signals are derived, the recipients of biofunctionalised scaffolds demonstrated a significantly elevated proportion of M2 macrophages in the scaffold and PN/CT contrary to their normal temporal arrangement. This not only confirms the bioactivity of this material in vivo, but also demonstrates that cells do not necessarily have to be in constant contact with the scaffold to alter their phenotype.

It is important to highlight that whilst anti-inflammatory in action, IL-10 may have unwanted side effects in the wrong environmental context; for example, it antagonises the tumour defensive properties of M1 macrophages and is thus potentially tumourogenic with long-term exposure (Park-Min et al., 2005). The delivery of IL-10, therefore, requires careful control in order to be of therapeutic value. Future directions may focus on the...
degradation properties of the scaffold so that the cytokine can be released from the scaffold surface into the tissue and undergo natural endogenous degradation over an appropriate time. This could be achieved by altering the chemical nature of the PCL scaffolds or the materials selected.

5. Conclusions

The ability to present a bioactive signal for extended periods of time offers a powerful tool for manipulating the cellular microenvironment. Here, we utilised IL-10 functionalised nanofibrous scaffolds to alter the properties of the perineural and surrounding connective tissue by promoting M2 macrophages into this region. IL-10 is known for promoting anti-inflammatory effects and reducing scar formation following injury or surgery. IL-10 biofunctionalised scaffolds are of practical value as they enable targeted delivery of this bioactive signal to highly restricted sites, thereby eliminating side effects beyond the region of interest. The ability to manipulate tissues adjacent to vulnerable nerves may one day eliminate the need for nerve transplantation that results in significant deficits at the donor site, thereby offering novel therapies for assisting nerve regeneration by promoting a more favourable and natural tissue microenvironment for nerve regeneration. Furthermore, the manipulation of cell populations within their natural environment to express desirable phenotypical behaviours offers a powerful investigative tool for cellular biology research.

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References


Appendix G

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Integrating Biomaterials and Stem Cells for Neural Regeneration

Francesca L. Maclean,1 Alexandra L. Rodriguez,1 Clare L. Parish,2 Richard J. Williams,2 and David R. Nisbet1

The central nervous system has a limited capacity to regenerate, and thus, traumatic injuries or diseases often have devastating consequences. Therefore, there is a distinct need to develop alternative treatments that can achieve functional recovery without side effects currently observed with some pharmacological treatments. Combining biomaterials with pluripotent stem cells (PSCs), either embryonic or induced, has the potential to revolutionize the treatment of neurodegenerative diseases and traumatic injuries. Biomaterials can mimic the extracellular matrix and present a myriad of relevant biochemical cues through rational design or further functionalization. Biomaterials such as nanofibers and hydrogels, including self-assembling peptide (SAP) hydrogels, can provide a superior cell culture environment. When these materials are then combined with PSCs, more accurate drug screening and disease modeling could be developed, and the generation of large numbers of cells with the appropriate phenotype can be achieved, for subsequent use in vitro. Biomaterials have also been shown to support endogenous cell growth after implantation, and, in particular, hydrogels and SAPs have effectively acted as cell delivery vehicles, increasing cell survival after transplantation. Few studies are yet to fully exploit the combination of PSCs and innovative biomaterials; however, initial studies with neural stem cells, for example, are promising, and, hence, such a combination for use in vitro and in vivo is an exciting new direction for the field of neural regeneration.

Introduction

Central nervous system damage

Damage to the central nervous system (CNS) is devastating because of its limited regenerative capacity. This damage can be induced by either physical trauma, such as traumatic brain injury (TBI) or spinal cord injury (SCI), or by chronic neural degeneration, as seen in Parkinson’s, Alzheimer’s, and Huntington’s diseases, as well as amyotrophic lateral sclerosis (ALS). Current treatments rely on pharmacological replacement of neurotransmitters, physical therapy, or surgical intervention. These treatment options are often associated with detrimental side effects and are usually limited to treating symptoms of the disease or injury, rather than slowing disease progression or modifying injury damage [1]. To date, there are limited therapies available that achieve the reinnervation and neuroprotection necessary for the restoration of lost neurological function to the body.

To overcome CNS injury or degeneration, a multifaceted approach is likely to be required, primarily to emulate desirable physical and biochemical characteristics of the healthy extracellular matrix (ECM). This approach can improve current cell culture environments, and improve cell transplantation outcomes in vivo by presenting a benign, supportive microenvironment for enhanced regeneration. Such requirements to provide both the physical and biochemical cues found in vivo can be fulfilled with the use of biomaterials.

The benefits of biomaterials in CNS regeneration

Many in vitro and in vivo approaches have been investigated to improve our understanding of disease and achieve neural regeneration. However, an inability to effectively replicate the biological environment has hampered their success. In the native in vivo environment, the ECM, composed of proteoglycans, proteins, and signaling molecules, provides structural and biochemical support to residing cells. It has the ability to influence cell behavior, including differentiation, proliferation, survival, and migration [2,3]. Biomaterials possess favorable properties that can be engineered to provide the necessary mechanical and biochemical signaling to cells similar to that seen in the native cellular environment [1].

In vitro, gold standard two-dimensional (2D) cell culture systems are used to model diseases and produce large number

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1Research School of Engineering, the Australian National University, Canberra, Australia.
2School of Aerospace, Mechanical and Manufacturing Engineering and Health Innovations Research Institute, RMIT University, Melbourne, Australia.
of cells for drug screening or transplantation. Currently, however, the inherent limitation of these systems is an inability to fully mimic the natural three-dimensional (3D) environment and to thus be effective when translated in vivo. Similarly, the transplantation of exogenous cells in the CNS to promote neural regeneration has become a widely researched strategy to overcome the limitations of current treatments [4]. However, issues such as long-term graft integration and cell survival have limited the application of this strategy to achieve neural regeneration. To overcome such limitations, biomaterials such as electrospun nanofibers, hydrogels, and self-assembling peptides (SAPs) are being investigated to enhance current cell culture systems to improve the accuracy of disease modeling and drug screening and to support cell transplantation in vivo.

These materials have the ability to present ECM-like mechanical cues to cells, as hydrogels can be tailored to have a similar elastic modulus to the CNS tissue, whereas nanofibrous scaffolds and SAPs possess fibrillar structures on a length scale, representative of proteins found in the in vivo milieu. Chemical modification of these materials can also provide bioactive signals to cells, through growth factor presentation or the presentation of bioactive sequences, all of which have been shown to increase cell survival and proliferation, and, in some cases, direct cell differentiation [5–9].

Biomaterials and stem cells

Appropriately designed biomaterials have the ability to achieve such complex structures and have the potential to significantly improve stem cell culture systems and improve cell transplantation outcomes for neural regeneration. By functionalizing these materials with niche-specific signals, there is an opportunity for cells to be produced in vitro that possess in vivo-like morphology. These cell culture systems can then be implemented in disease models and provide large number of cells for drug screening and transplantation. In addition, by designing biomaterials as carriers that can provide physical and biochemical support to cells during transplantation, it may be possible to synthetically fabricate a milieu that is conducive to neural regeneration and can promote functional recovery post-CNS injury or disease.

Three-Dimensional Biomaterials Can Provide Superior Cell Culture Environments

Limitations of current cell culture systems

Commonly used 2D cell culture conditions are limited in their ability to provide the complex physical and biochemical cues present in the in vivo environment that are essential to cell development, survival, and function [10]. The inherent heterogeneity of traditional cell culture systems and subsequent lack of consistent spatial or temporal gradients of mechanical or biochemical signals, as well as the absence of 3D cell–cell and cell–matrix interactions, can result in significant alterations in gene and protein expression, cell morphology, and function [11–13]. These limitations can compromise the performance of these cells for further in vitro experimentation and future in vivo deployment, so it is imperative to develop systems that accurately replicate relevant in vivo environments for culture [14].

Significantly, cell culture systems that only provide insufficient 2D support to cells affect the accuracy of further in-depth investigations of cells and their behavior in vitro, including disease modeling and drug screening. As cells generated and maintained in vitro are exposed to different physical and biochemical cues than their in vivo counterparts, findings from in vitro experimentation are not always accurate representations of cellular development and behavior in their natural environment, and can, therefore, be difficult to translate into in vivo experiments [11]. Reproducibility and scalability are also limitations of cell culture systems, because of the use of human or animal tissue, including nonquantified tumor-derived materials such as Matrigel [12]. These limitations are problematic when considering the large number, and in vivo-like nature, of cells required for transplantation, disease modeling, and drug screening, and illustrate the need for a superior cell culture system that can effectively recapitulate the 3D in vivo environment for neural regeneration applications and strategies.

Electrospun nanofibers for neural differentiation and proliferation in vitro

Electrospun nanofibers have been intensely researched for applications in neural regeneration because of morphological similarities to the ECM that include similar fibrillar diameter, high surface area-to-volume ratio, and high porosity [1,15]. Such similarities highlight their suitability for use in a 3D cell culture system.

Electrospinning uses a viscous polymer solution that is electrified by a high-voltage source. At a critical voltage, the electrostatic attraction between the polymer solution and collector becomes greater than the electrostatic repulsions between charges within the solution, resulting in the formation of a fiber jet from the polymer solution. This fiber experiences whipping instabilities, during which the solvent evaporates, resulting in the deposition of polymer fibers on the collector [15,16]. Sheets of nanofibers (scaffolds) can be collected after electrospinning and easily utilized in subsequent cell culture. Polymers such as poly(e-caprolactone) (PCL), poly-l-lactide (PLLA), and poly(lactic-co-glycolic acid) (PLGA) have commonly been electrospun for applications in neural regeneration as they are biodegradable through hydrolysis of ester linkages and are approved for biomedical applications by the Australian Therapeutic Goods Administration and the US Food and Drug Administration [1]. Cell interaction with PCL nanofiber scaffolds is illustrated in Fig. 1, where the porous, nanofibrous network of the scaffolds is also evident [16]. These images are representative of cell–matrix interactions in vitro and demonstrate the suitability of nanofibrous scaffolds to act as an ECM mimic.

Electrospun nanofibers are an attractive biomaterial for a 3D cell culture system as the scaffolds can be tailored for different applications through fiber diameter, alignment, and surface modification [17]. Modification of nanofiber surfaces through chemical attachment of bioactive molecules allows for the presentation of biochemical cues necessary to create a cell culture system that effectively recapitulates the extracellular environment. For example, cell adhesion and spread were enhanced on PCL nanofiber scaffolds that were aminoalyzed with ethylenediamine as a result of the increased hydrophilicity from the presentation of amine moieties, as
shown in Fig. 2 [18]. In addition, neural stem cells (NSCs) primarily differentiated into oligodendrocytes when cultured on both control and aminolyzed PCL nanofiber scaffolds as compared to the laminin control [18], indicating that appropriately designed PCL nanofiber scaffolds have the ability to direct neural differentiation toward a specific lineage.

Neural survival, proliferation, and differentiation can also be influenced through the presentation of proteins on the surface of electrospun scaffolds. For example, covalent attachment of brain-derived neurotrophic factor (BDNF) or glial cell-derived neurotrophic factor (GDNF) to aminolyzed PCL nanofibers significantly increased cell proliferation and survival compared to presentation of these proteins in their soluble forms [5,7,8].

The effect of ECM protein coatings on nanofibers has also been investigated. Laminin-coated polyurethane nanofibers have been shown to support complex morphology of astrocytes with long cellular extensions, representative of in vivo morphology, compared to the flat morphology observed when using the 2D laminin-coated glass cover slips (Fig. 3) [13]. The expression of intermediate filament proteins (glial fibrillary acidic protein (GFAP), vimentin, and nestin) and heat-shock protein (HSP70), a marker of cellular stress, was reduced in astrocyte cultures on laminin-coated nanofibers, compared to those cultured on laminin-coated glass slips [13]. In addition, cell cultures of mouse embryonic stem cells (ESCs) on Ultra-Web™, a commercially available polyamide-based 3D nanofibrillar porous matrix, have been shown to enhance cell proliferation and self-renewal compared to tissue culture dishes [19]. Culturing primary astrocytes on 3D PCL nanofiber scaffolds resulted in decreased GFAP expression, while increasing the expression of BDNF and excitatory amino acid transporter 2 (EAAT2) compared to those cultured on 2D PCL [20]. These biochemical changes are indicative of a phenotype that is representative of astrocytes in the healthy brain and demonstrate that the morphology of the culture environment is important in developing in vivo-like cell behavior and

FIG. 2. Cultured NSCs (labeled with nuclear marker Hoechst 33,422, (A) self-aggregate into "neurosphere-like" structures on the surface of the unmodified PCL scaffolds, (B) yet readily disperse throughout the PCL scaffold after modification with ethylenediamine. Scale bar = 50 μm [18].
phenotype. Therefore, the ease in which the 3D morphology and surface chemistry of nanofibers can be altered is promising for influencing stem cell fate and thereby holds relevance for applications with regard to in vitro disease modeling, drug screening, and generation of cells suitable for in vivo clinical application.

Potential of hydrogels for improved in vitro cell culture

An exciting method for translating the benefits of electrospun scaffolds to improve current cell culture systems for in vitro application is the development of hydrogels. These are highly hydrated, water-insoluble polymer networks that contain both chemical (covalent bonds) and physical (chain entanglement and secondary forces) cross-links [1, 21]. They can be synthesized to possess numerous characteristics of the architecture and mechanics of the native cellular environment [22]. Many hydrogels have been investigated for neural regeneration applications and include biologically derived materials, such as hyaluronic acid (HA), collagen, xylotactic acid, and Matrigel, as well as synthetically derived hydrogels such as polyacrylamide and polyethylene glycol (PEG) [22, 23]. They are appealing biomaterials because of their high oxygen and nutrient permeabilities, as well as low interfacial tensions and macroporosity, facilitating cell infiltration and effective grafted-cell interface for innervation of host circuitry [1, 22–24].

It is essential that mechanical properties of biomaterials for neural regeneration are similar to that of native brain tissue to ensure that cells receive the appropriate mechanical growth cues, while also avoiding modulus mismatch and a subsequent foreign body reaction if also implemented in vivo [23, 25, 26]. This highlights another desirable characteristic of hydrogels, as their mechanical properties can be easily manipulated to reflect that of the CNS by controlling the underlying structure (eg, the number of fibers and cross-links) [1]. Although proliferation of human ESCs (hESCs) and human-induced pluripotent stem cells (hiPSCs) is insensitive to ECM stiffness, early differentiation is regulated by ECM stiffness when substrate stiffness is similar to brain tissue. It has been shown that neural differentiation of hESCs and hiPSCs is increased on hydrogels with a stiffness of 0.1 kPa as compared to substrates with stiffness of 75 kPa [27]. A downstream increase in neurites was observed when hESCs and hiPSCs were initially exposed to soft (0.7 kPa) ECMs for 5 days out of a total 19 days, indicating that mechanical signaling may also have temporal significance [27]. In addition, when neural stem/progenitor cells (NSPCs) were cultured on methylacrylamide chitosan hydrogel, it was found that cellular proliferation and neuronal differentiation were maximum when elasticity was <1.0 kPa. Oligodendrocyte maturation and myelination have been observed to be greater on scaffolds with E<1.0 kPa, whereas stiffer substrates (E>7 kPa) favored oligodendrocyte differentiation [25]. This demonstrates the varying effects that ECM stiffness can have on cell proliferation and differentiation and hence, the potential for biomaterials to create a myriad of tailored cell culture environments.

The significance of the mechanical properties of a potential scaffold highlights the suitability of hydrogels in cell culture systems, as their mechanical properties can easily be tuned through the regulation of cross-linking density [1]. However, limited biodegradation, shrinkage of hydrogels after cross-linking, and potentially toxic monomers and cross-linking agents are shortcomings that hinder the clinical application of synthetic hydrogels in neural regeneration [28]. Conventional hydrogels, although porous, lack the inherent fibrous morphology found in the ECM, which is an essential aspect in creating superior, 3D cell culture environments. As such, an alternative approach has been investigated to develop more sophisticated hydrogel structures that comprise fully degradable small molecules and possess fibrous morphology, namely SAPs.

FIG. 3. Cell morphology of enhanced green fluorescent protein (EGFP)-expressing astrocytes cultured on: (A) laminin-coated cover slips (2D) and (B) laminin-coated polyurethane nanofibers (3D) as revealed by 3D reconstruction of confocal images. Scale bar = 10 μm [13].

2D, two-dimensional; 3D, three-dimensional.
SAPs provide tailored bioactivity in vitro

SAPs are an alternative hydrogel scaffold that can be used in 3D cell culture applications, as they have the capacity to self-assemble and form complex nanostructures from simple peptide building blocks without the limitations already described. The noncovalent self-assembly of peptide-based molecules enables the presentation of chemically functional peptide sequences at the surface of ordered nanostuctures such as tubes, rods, and sheets, which can influence cell survival, proliferation, migration, and differentiation [29]. In addition, self-assembly can be controlled by pH, ionic strength, temperature, or enzymatic triggers [29]. Peptide amphiphiles (PAs), aromatic N-terminally capped peptides, and proline-containing oligopeptides are examples of SAP systems that have been investigated for neural regeneration applications, as they mimic the ECM through the presentation of biochemical and physicochemical cues [28–30].

Specific peptide sequences can be introduced to SAP systems to impart bioactivity and influence cell behavior. For example, the laminin-based epitope, isoleucine-lysine-valine-alanine-valine (IKVAV), known to promote neurite growth [31], has been incorporated into a variety of SAP systems, including RADA16 [32–34]. RADA16 is a class of commercially available SAPs where self-assembly occurs due to complementary charge and hydrophobic interactions. Containing a 16 amino acid sequence with alternating charge, RADA16 self-assembles to form stable β-sheet structures [29,32]. The presentation of a bioactive laminin epitope, IKVAV, in a RADA16 scaffold has been found to promote neuronal differentiation, with an increased expression of the neuronal marker, MAP2, as well as a significantly higher percentage of neurons than astrocytes on the IKVAV-containing scaffold [9].

In addition, when encapsulated with NSCs, the bioactive SAPs ac-(RADA)2-GG-SKPPGTTSS-COH2, ac-(RADA)2-GG-PFSSTKT-COH2, and ac-(RADA)2-GG-RGD-COH2 were found to have greater cell viability over 5 months than Matrigel and Collagen I [35]. This was attributed to the beneficial effects of the specific sequences SKPPGTTSS, PFSSTKT, and RGDs on neural cell growth, migration, adhesion, and differentiation [35]. It was also found that after 3 months in culture, NSCs encapsulated in the peptide hydrogels differentiated into approximately 27% neurons, 25% astrocytes, and 26% oligodendrocytes [35]. Poor long-term cell survival on Matrigel suggests that the beneficial effect on cultured cells is predominantly because of nonquantified biological molecules present in Matrigel, and this is supported by the poor response when cultured on Collagen I. Although routinely used in neural cultures [36], there is a biological incompatibility between Collagen I and neural cells, as Collagen I is not found in brain tissue [35]. This highlights the insufficiencies of current 2D cell culture systems and presents bioactive SAPs as a potentially biocompatible, nonimmunogenic alternative that can provide a suitable environment for stem cell proliferation and differentiation in vitro.

Fluorenylmethylxycarbonyl chloride (Fmoc–SAPs have also been investigated for biological applications [29]. These SAPs use a minimalist approach to forming complex structures through noncovalent interactions known as π–π self-assembly [37]. They are advantageous because of their ease of synthesis and ability to form a nanofibrous scaffold at physiological pH (Fig. 4) with the capacity to present bioactive epitopes at high density on the surface of the nanofiber [33]. Recently, the fibronectin cell adhesion sequence, arginine-glycine-aspartate (RGD), was incorporated into this SAP system by flanking the RGD sequence with structure-inducing aromatic residues to promote its self-assembly at physiological pH [38]. Human mammary fibroblasts were cultured on both an RGD Fmoc–SAP and a structurally and chemically analogous scrambled sequence, DGR, to assess the bioactivity of the sequence within the peptide sequence once assembled. On the DGR Fmoc–SAP, fibroblasts displayed reduced viability with apoptosis occurring until day 4 of the culture. In contrast, cell viability was maintained on the RGD Fmoc–SAP, suggesting that the cells recognized and interacted with the adhesion molecule [38]. In addition, morphological differences between cells cultured on the two different sequences were observed [38].

The mechanical and morphological properties of Fmoc–FRGDF can be tuned through the gelation mechanism, illustrating that these SAPs can be tailored for applications with particular mechanical and morphological requirements [39]. Gelation of Fmoc–FRGDF using glucono-δ-lactone resulted in the formation of nanofibers with minimal entanglement, and hence, a weaker hydrogel, than those formed using a hydrochloric acid (HCl)-based pH-switch method. The HCl pH-switch method allowed the gel stiffness to be

**FIG. 4.** Transmission electron microscope images of nanoscale structures formed by Fmoc-based self-assembling peptides. (A) Fmoc- FRGDF (B) Fmoc-DIKVAV, scale bar = 200 nm (author contribution). Fmoc, fluorenylmethylxycarbonyl chloride.
tuned, dependent on PBS concentration, without altering the self-assembly mechanism involving the formation of π-β sheets and antiparallel β sheet structures [39]. The influence of the gelation mechanism over the mechanical and morphological properties of SAPs diversifies the potential application of this sophisticated class of materials, holding great promise for tailoring SAP stiffness according to the desired differentiation pathway.

Therefore, through their nanofibrous structure, presentation of peptides that are similar to the ECM proteins, and tailored mechanical properties, SAPs can provide a cell culture system that effectively mimics various aspects of the in vivo environment, including relevant biochemical and topographical cues [29,35]. Combined with stem cells, this class of biomaterials could allow for the culture of cells in an in vitro environment morphologically representative of that found in vivo. In addition, the ability of SAPs to be tailored for tissue-specific applications through specific peptide sequences or stiffness could enable efficient differentiation of cells into a specific lineage, critical for the ultimate success of cell transplantation in neural regeneration, as well as necessary for accurate disease modeling and drug screening [40].

Biomaterials are ideal for developing new cell culture systems as their properties can be engineered to represent those of the native ECM. The provision of the ECM cues and a 3D environment essential to cell development can be exploited to create cell culture systems to produce high-quality cells for use in drug screening and enhance current disease models. In addition, they can facilitate directed cell differentiation, which is of use when designing disease-specific cell transplantation strategies that could also employ the use of biomaterials in vivo.

Applications of 3D biomaterial cell culture systems

Biomaterials have exhibited properties that are desirable for developing an alternative 3D cell culture system to produce large numbers of cells representative of those found in vivo, for a variety of applications. One shortcoming for the current in vitro cell culture and disease model systems is that cellular oxygenation, as well as nutrient and waste removal, occurs through diffusion, as the systems are not connected to the blood stream [41]. Integrating biomaterials and PSCs under dynamic cell culture conditions (such as those found in bioreactors) can provide critical mass transport to and from cells, circumventing potential mass diffusion limitations currently associated with 2D cell culture [11]. Using biomaterials in conjunction with bioreactors would improve the accuracy, and therefore efficacy, of the current in vitro disease models, as the in vivo environment would be better represented with the incorporation of physical and biochemical cues from the material and environmental control of the bioreactor.

Large scale cell generation and drug screening. 2D cell culture systems have been identified as a limitation for the provision of sufficient cell numbers for applications such as cell replacement therapy (CRT) and drug screening because of their limited scalability and reproducibility [12]. Predictive toxicity assays during preclinical testing are essential for the development of new drugs [42]; however, the prediction of the pharmacokinetic bioavailability and indirect toxicity of such drugs are still limited in current culture systems [11]. As such, a large number of cells, which possess reliable disease pathology, as well as a superior culture environment, are required for improved drug screening technologies.

Biomaterials can be incorporated into culture systems to generate large cell numbers and provide a culture environment more representative of in vivo environment. Long-term culture of hESCs and hiPSCs in a thermoreversible hydrogel, poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG), resulted in a large cell yield, high-expansion rate, and high yield. It was also demonstrated that this 3D system supported the differentiation of hiPSCs into the three germ layers as well as directed differentiation into NPCs and midbrain dopaminergic neurons. Combining such a biomaterial with a bioreactor to provide the optimal environment for cell proliferation and differentiation could yield more than 10^7 cells from 5 L of hydrogel [12]. Such cell numbers can be used for applications including clinical studies employing cell transplantation, or high-throughput drug screening, potentially saving the time, money, and resources currently expended on unsuccessful clinical trials.

Disease modeling. The ability of stem cells, particularly iPSCs and ESCs, to recapitulate disease phenotype in vitro is promising for disease modeling. However, there are limitations associated with cells grown in 2D culture in accurately representing the behavior of those cells found in vivo. For example, gene expression patterns of ESCs possessing Mus musculus, Mus spretus, or Mus terricolor mitochondrial DNA haplotypes were different when cultured on electrospun 3D PCL scaffolds and 2D culture dishes. In addition, there was an 18–24-fold increase in gene expression in a 3D environment [43]. This indicates that there is a significant impact on cell behavior, dependent on the culture conditions. Therefore, it would be advantageous to implement biomaterials in future disease modeling experiments in an attempt to recapitulate the native cellular environment, and obtain meaningful results that can be used to understand disease pathology and the effect of potential therapeutic drugs.

In addition, encouraging iPSCs out of their embryonic state into populations of mature adult cells that behave reliably is a key challenge of their use in disease modeling [44]. This is of particular importance in modeling diseases such as ALS, Alzheimer’s, and Parkinson’s, where disease manifestation occurs in mature neurons, with synaptic properties typical of those in an adult brain. In conjunction with the development of appropriate differentiation protocols, it has been suggested that it may be necessary to develop culture conditions to allow for the full maturation of cells, as disease phenotypes may not be revealed in immature cells [44]. As such, 3D biomaterials can provide a platform on which to direct differentiation through the presentation of growth factors and cytokines and enable prolonged culturing systems not sustainable on 2D culture ware.

Biomaterials to Enhance Cell Transplantation Outcomes

Limitations of current in vivo treatment strategies

Injury to the CNS, such as trauma or stroke, can result in the formation of cystic cavities that impede cellular replacement and differentiation because of the absence of appropriate
structural support [45]. In addition, current pharmacological treatment for neurodegenerative diseases does not slow disease progression, and in some cases can actually exacerbate symptoms or induce unwanted side effects [1]. Therefore, there is a need for the development of treatments to address disease progression and/or the injury response of the CNS. In addition to the limitations associated with the generation of suitable cells in 2D cell culture for use in CRT, there are also limitations associated with long-term graft–host integration. Populations of pure multipotent NSCs have previously shown poor survival in most CNS regions, as a result of the host failing to provide a sufficient environment to support the survival of NSCs [46]. Also, in the treatment of necrotic brain injuries, such as stroke, TBI, and cerebral palsy, often a void needs to be filled to facilitate regeneration. Biomaterials not only easily fill a void but also provide the physical and biochemical support required to recapitulate the stem cell niche when cells are transplanted.

**Electrospun nanofibers provide physical and chemical cues in vivo**

PCL nanofiber scaffolds have been shown to be biocompatible materials that can integrate with host brain circuitry, a requirement for the development of scaffolds for cell transplantation. Typically, the nanofiber scaffolds are rolled up on themselves into a “cylinder” of nanofibers, as shown in Fig. 5D, when implanted into the brain, as shown in Fig. 6. When randomly and partially aligned PCL nanofiber scaffolds were implanted into the caudate putamen of the adult rat brain, neurite infiltration was found to be dependent on fiber alignment [47]. Randomly aligned scaffolds were found to facilitate neurite infiltration, whereas neurites remained at the interface between partially aligned scaffolds and adjacent brain parenchyma (Fig. 5). This was attributed to the reduced interfiber distance of the partially aligned scaffolds inhibiting neurite infiltration. However, it was found that the partially aligned nanofibers provided a uniform perpendicular contact guidance to neurites, highlighting the potential for guided growth in vivo using partially aligned scaffolds for the development of disease-specific treatment strategies [47].

Current methods of growth factor delivery such as direct injection are suboptimal, because despite achieving targeted delivery, a steep growth factor concentration gradient is created upon injection, which can alter or destroy tissue around the injection site [48]. In addition, as growth factors are readily degraded in their natural physiological environment, when they are delivered in vivo, degradation and diffusion to nontarget tissue limit their therapeutic value [7]. A more stable method to deliver growth factors is to use slow-releasing microspheres. Microspheres made of polylactic-co-glycolic acid have been found to release GDNF upon degradation at the site of implantation, which can last for several months [49]. However, this delivery method is still uncontrolled, with a nonconstant rate of growth factor release over the first few weeks. Therefore, it is desirable to control the behavior of growth factors by their stable

**FIG. 5.** Neurite interaction with PCL electrospun scaffolds 60 days after implantation: (A) PCL scaffold with random fiber alignment that has been sectioned parallel to the implantation, illustrating the extent of neurite infiltration within the scaffold. (B) Partially aligned fibers sectioned perpendicular to the scaffold. (C-C’) Higher magnification images of the implanted aligned nanofibers. (D) A schematic showing that the nanofiber scaffold is rolled up on itself for implantation, as well as the neurite interaction with the partially aligned PCL nanofibers. White dotted lines demarcate the implanted scaffold and endogenous tissue [47].
FIG. 6. Micrographs showing the implanted nanofiber scaffold, rolled up on itself for implantation into the rat striatum, with migrated cells labeled for Hoechst. (A) Unfunctionalized PCL scaffold, (B) PCL scaffold in the presence of a GFP+ cell graft, (C) PCL scaffold functionalized with GDNF and in the presence of a GFP+ cell graft, and (A’–C’) higher magnification images of A–C [12]. GDNF, glial cell-derived neurotrophic factor.

presentation within multifunctional nanofibrous biomaterials. The influence of prolonged, stable protein presentation in vivo has been investigated by implanting NPCs together with functionalized (GDNF-tethered) PCL scaffolds into rat brains [8]. Cell viability significantly increased in the presence of immobilized GDNF compared to implantation of cells alone, demonstrating the ability of GDNF to maintain its trophic effects following tethering and implantation. Furthermore, there was a significant increase in immature neurons within the grafts exposed to immobilized GDNF as compared to the control cell graft and unfunctionalized PCL. This suggests that tethered GDNF is capable of continually supporting immature neurons within the graft, accounting for the increase in grafted NPCs [8].

The implantation of PCL with tethered GDNF was also found to improve stem cell graft outcomes in the intact and injured brain. Functionalized scaffolds enhanced the yield of neurons and oligodendrocytes within the graft, decreased reactive astrocytes, and increased the penetration of neuronal processes into the scaffold [8]. This demonstrates that the immobilization of GDNF on PCL scaffolds provides functional trophic cues capable of influencing cellular proliferation, differentiation, and neurite growth in vivo [8]. Therefore, neurotrophic factors are of great importance to improve transplanted cell integration, and the ability of electrospun nanofibrous scaffolds to be readily functionalized to control cell behavior is also highlighted.

The modification of the conventional electrospinning setup can also allow the fabrication of cylindrical nanofiber scaffolds that replicate the spinal cord. The implantation of PLGA nanofiber cylindrical grafts, fabricated using water vortex electrospinning, into a transsected spinal cord rat model resulted in better locomotive and sensory scores than the control group that did not receive scaffold implantation [50]. This can be attributed to the physical support provided by the nanofiber graft for axonal growth at the interface of the spinal cord and scaffold, as well along the scaffold nanofibers [50]. This scaffold could be enhanced by further chemical functionalization, as previously discussed, to potentially improve the functional recovery of injured spinal cords.

Electrospun nanofibrous scaffolds have the ability to provide physical and chemical cues in vivo to promote tissue repair, and cylindrical nanofiber grafts have great potential for the treatment of SCI with further development of material choice and scaffold functionalization. However, geometric limitations imposed by the fabrication mechanism make them unable to fill a void effectively, an important requirement of treatment strategies in certain acquired brain injuries. In this regard, treatment of neural injuries, neurodegenerative or traumatic, should be targeting the use of void filling nanofibrous hydrogels.

Hydrogels to support transplanted cell growth in vivo

Hydrogels are promising biomaterial for implementation in neural regeneration strategies as they have clearly demonstrated their suitability to provide physical support to transplanted cells and the ability to be functionalized with multiple growth factors. Incorporating stem cells with functionalized hydrogels provides the potential for preferential differentiation in vivo and the development of application-specific cell/biomaterial systems.

The physical and chemical diversities that are possible with hydrogels make them desirable candidates for cell transplantation applications. Implantation of xylloglucan, a
thermally gelling hydrogel, functionalized with poly-L-lysine (xyloglucan-graft-PDL), into the caudate putamen of adult rats was found to regulate the inflammatory response, with homeostatic levels of microglia and astrocytes achieved after 60 days [51]. The amount of PDL immobilized to xyloglucan was found to also affect the amount of astrocyte and neurite scaffold infiltration. This was attributed to the positive charge on PDL enhancing cell adhesion, as well as the potential secretion of laminin and other neurotrophins by astrocytes, which can provide cytotrophic support to, and attract, neurons. Both 100% xyloglucan and the xyloglucan-graft-PDL were not phagocytosed by microglia, indicating that they did not elicit an inflammatory response, and have the potential to support nerve regeneration [51]. Xyloglucan has also been incorporated as composite material, when it was loaded with short electrospun nanofibers functionalized with GDNF, to support engraftment of dopaminergic progenitors in a mouse Parkinson’s model [52]. Survival, innervation, and graft volume of the transplanted fetal cells were achieved when xyloglucan was blended with GDNF, as well as combined with short PLLA fibers possessing immobilized GDNF. Such a composite biomaterial that incorporates dual growth factor delivery methods significantly contributes to the development of an optimized biomaterial to deliver physical, biochemical, and enhance cell graft survival and innervation. In addition, when seeded with mesenchymal stem cells and implanted into hemisected rat spinal cords, hydroxypropylmethacrylate hydrogels, either polymerized in the presence of a solid porogen (HPMA-SP), with the fibronectin sequence RGD attached (HPMA-SP-RGD), or prepared by heterogeneous separation (HPMA-HS-RGD), as well as the HEMA-MOEATCA (hydroxy ethyl methacrylate [2-(methacryloyloxyethyl) trimethylammonium chloride] hydrogel, all promoted blood vessel and axon growth inside the scaffold, dependent on their physical and chemical properties [53]. It was found that blood vessel growth was promoted when RGD was present in the hydrogel, whereas axon growth inside the scaffold was encouraged when it possessed the web-like architecture of HPMA-SP-RGD [53]. These systems have illustrated that they are biocompatible in vivo, and demonstrate that altering the surface chemistry and physical architecture of hydrogels can influence cell infiltration and growth. Hyaluronic-methyl cellulose (HMAC) is of relevance for neural regeneration applications as it gels at physiological temperatures and has been used as a drug delivery vehicle in the CNS. Encapsulating HMAC with epidermal growth factor (EGF) or poly(ethylene glycol)-modified EGF (PEG-EGF) was found to enhance the proliferation of NSPCs in the subventricular zone, in both uninjured and stroke injured brains [54]. In addition, when rat NSPCs mixed in platelet-derived growth factor-A immobilized to HMAC (HMAC-PDGF-A) were transplanted into a rat SCI model, the differentiation of NSPCs to oligodendrocytes was enhanced as compared to NSPCs transplanted in culture media [6]. Greater cell survival was observed in rats receiving NSPC/HAMC-PDGF-A, which was attributed to the presence of HA, as it is a key component of the ECM, and its interaction with cells through CD44 and RHAMM receptors influences cell survival and migration, which could account for the observed in vivo NSPC survival enhancement [6]. In addition, HA and MC have antioxidant properties that can reduce the flux of free radicals at the cell surface, reducing cell damage and enhancing NSPC survival [6]. Rats injected with NSPC/HAMC-PDGF-A exhibited more neurons alongside the lesion site than those that received NSPC/media; with 33% more spared neurons in the NSPC/HAMC-PDGF-A transplants (Fig. 7).

NSPC/HAMC-PDGF-A transplants also resulted in an increase of 23% more host oligodendrocytes, demonstrating the benefit to host tissue achieved using NSPC/HAMC-PDGF-A transplants. This was attributed to the sustained presence of PDGF-A, as well as the presence of the HA component that has been shown to block NMDA-induced neuronal cell death. These studies demonstrate that HAMC is a promising hydrogel for both growth factor delivery and cell transplantation in the CNS. Further investigation of this material and its regenerative capacity when delivering various growth factors in

FIG. 7. Photomicrographs illustrating NeuN staining of transplanted neural stem/progenitor cell in the presence of (A) the functionalized hydrogel HMAC-PDGF-A or (B) culture media. Note the increased survival and integration of transplanted cells in the presence of the functionalized hydrogel HMAC-PDGF-A [6]. HMAC-PDGF-A, platelet-derived growth factor-A immobilized to HMAC.
combination with PSCs to support and direct differentiation, as well as to encourage regeneration, is an exciting prospect. Fibrin-based hydrogels have also been utilized to deliver stem cells and growth factors in a dorsal hemisection subacute SCI in rats [55]. Embryonic neural stem/progenitor cells (ENSPCs) were encapsulated in fibrin hydrogels that were either unmodified, mixed with neurotrophic factor-3 (NT-3) and PDGF, or covalently attached to a heparin binding delivery system (HBDS), which then bound to the heparin binding growth factors NT-3 and PDGF. Poor cell survival was reported for ENSPCs transplanted without a fibrin scaffold, emphasizing the importance of physical support for transplanted cells. Cell numbers showed a 10-fold increase at 2 weeks when ENSPCs were transplanted in the fibrin hydrogel with the growth factors (with or without HBDS), whereas only a 2-fold increase was observed for the cells encapsulated in unmodified fibrin or transplanted without fibrin. In addition, there was an increased number of ENSPC-derived NeuN+ mature neurons when the ENSPCs were encapsulated in the fibrin/HBDS/NT-3/PDGF scaffold. This could be attributed to the HBDS limiting diffusion of the growth factors, allowing NT-3 and PDGF to influence cell behavior for a longer period of time.

Self-assembling peptides

SAPs hold great promise for in vivo applications, since their assembly is triggered by physiological changes in pH and temperature, allowing them to be injected directly into an injury site in a minimally invasive manner [56]. Importantly, the structure of SAPs can influence cell behavior, as was demonstrated when the IKVAV peptide and the IKVAV PA (self-assemblies in vivo, Fig. 8) were injected into a clip compression model of SCI in mice. Functional recovery was not promoted with the injection of the IKVAV peptide alone, demonstrating that behavioral improvements are attributable to not only the IKVAV sequence but also the nanorough structure of the self-assembled peptide [57].

At 9 weeks, the IKVAV PA–injected group had significant functional recovery compared to the control groups, indicating its ability to facilitate long-term functional recovery. At 5 and 11 weeks after SCI, the progression of astrogliosis at the lesion site in the IKVAV PA–injected group was suppressed, whereas process extension in astrocytes or the initial hypertrophy that may be essential for repairing the blood–brain barrier and restoring homeostasis was not altered [57]. It was also observed that IKVAV PA injection increased the oligodendroglial cell numbers while concurrently reducing apoptotic cell death and reduction of glial scar formation. This is significant as the IKVAV PA allows the initial functions of astrogliosis that are beneficial to CNS regeneration, while limiting the progression of gliosis that restricts axon outgrowth [57]. In addition, IKVAV PA was found to have degraded after 4 weeks, so the degradation time of IKVAV PA and other SAPs could be advantageous to provide temporal chemical cues to cells in vivo.

The physical support provided by SAPs is also an important feature to consider when developing scaffolds for CRT. RADA16 or RADA16-IKVAV with encapsulated NSCs (RADA16/NSCs and RADA16-IKVAV/NSCs, respectively) self-assembled into a 3D nanofibrous hydrogel immediately after in situ injection into a cavity in a rat brain [30]. As compared to NSCs suspended in the cavity without the peptide hydrogel, cell survival was enhanced when NSCs were transplanted within the peptide hydrogel. NPCs and neurons were present 3 weeks after the transplantation of RADA16-IKVAV/NSCs, whereas few NPCs and neurons were present when RADA16-IKVAV was transplanted without NSCs. This suggests that NSCs are integral to the repair and regeneration of tissue. In addition, reactive astrocytes were significantly reduced around the boundary of the implanted RADA16-IKVAV/NSCs, as compared to the RADA16/NSCs, demonstrating that the functionalization of RADA16-IKVAV could reduce glial scarring through the suppression of astrocytic differentiation [45].

The peptide K2(QL)6K2 (QL6) is another SAP that has shown potential for the functional recovery of rat SCI. Injection of the QL6 SAP 24 h after clip compression resulted in a reduction of astrogliosis and apoptosis, and improved Basso, Beattie, and Bresnahan (BBB) locomotor scores as compared to the control group that did not receive any SAP. The biocompatible QL6 provided the physical and chemical support necessary for the suppression of an inflammatory response, as well as axon survival and sprouting. However, it was noted that there was a lack of neurons in the grafted SAP [58], so using SAPs to support cell grafts would be promising for these materials. NPCs delivered using Fmoc–SAPs such as Fmoc–PRGDF, Fmoc–D1YSGRF, and Fmoc–D1KVAV resulted in a limited foreign body response and innervated the host brain tissue after 28 days. This study also highlights the potential for tissue-specific SAP development, as YGSR and IKVAV are laminin epitopes, whereas RGDS is derived from fibronectin. Therefore, these SAP sequences can be tailored for the desired tissue application, which could potentially

FIG. 8. (A) Schematic of individual PA molecules assembled into a bundle of nanofibers interwoven to produce the IKVAV PA. (B) Scanning electron micrograph image shows the network of nanofibers in vitro. Scale bar = 200 nm [57]. IKVAV, isoleucine-lysine-valine-alanine-valine; PA, peptide–amphiphile.
influence the differentiation of stem cells in vivo. A possible extension of this treatment strategy would be to combine it with PSCs such as ESCs or iPSCs to enhance the cell growth in the scaffold, and hence, across the lesion site. However, although SAPs have been thoroughly characterized for their material properties, the biological characterization of SAPs is limited and warrants further investigation.

**Future Perspectives**

Hydrogels, SAPs, and electrospray nanofibrous scaffolds hold great promise for the development of 3D culture systems and in vivo applications in the quest to improve the development of new therapies in the treatment of brain injuries. Here we have described their benefits of improved in vitro culturing for the purpose of disease modeling, drug development, and cell transplantation. These systems illustrate that the provision of a microenvironment with structural composition and elasticity of the native tissue, as well as high water content, improves implanted cell survival in vivo, and thus, are promising scaffolds. The ability of bioengineered scaffolds to provide an environment representative of the ECM, allow for cellular infiltration, and control of cellular behavior through functionalization suggests that they are ideal biomaterials to combine with cells to further the field of neural regeneration, both in vitro and in vivo. In addition to NSCs and NSPCs, PSCs such as ESCs and iPSCs are promising cell sources because of their unrestricted capacity for differentiation and proliferation. However, minimal research has been conducted to investigate the performance of these biomaterials as scaffolds to facilitate ESC or iPSC differentiation and proliferation in vitro, or neural regeneration using ESC or iPSC grafts in vivo. Therefore, to fully understand and exploit the behavior of stem cells for neural regeneration, it is suggested that biomaterials including hydrogels, SAPs, and electrospray nanofibers should be combined with such cell sources to significantly enhance current cell culture systems and in vivo neural regeneration strategies.

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**References**


20. Lau CL, M Kovačević, TS Tingleff, JS Forsythe, HS Cate, D Merlo, C Cederfur, FL Maclean, CL Parish and MK Horne. (2014). 3D electrospun scaffolds promote a cyto-
21. Liang Y, P Walczak and JW Buhe. (2013). The survival of engrailed neural stem cells within hyaluronic acid hydro-
22. Tibbitt MW and KS Anseth. (2009). Hydrogels as extracel-
25. Leipzig ND and MS Shoichet. (2009). The effect of sub-
dimensional neural tissue cultures in functionalized self-
43. Kelly RDW, AE Rodda, A Dickinson, A Mahmud, CM Netzer, W Lee, JS Forsythe, JM Polo, IA Trounce, et al. (2013). Mitochondrial DNA haplotypes define gene expression patterns in pluripotent and differentiating embry-
onic stem cells. Stem Cells 31:703–716.
47. Nisbet DR, AE Rodda, MK Horne, JS Forsythe and DI Finkelstein. (2009). Neurite infiltration and cellular re-
sponse to electroporus polycaprolactone scaffolds implanted into the brain. Biomaterials 30:4573–4580.
50. Zaman F, M Armani-Tehrani, M Latifi, M Shokrgozar and A Zamini. (2014). Promotion of spinal cord axon regen-
51. Nisbet DR, AE Rodda, MK Horne, JS Forsythe and DI Finkel-
stein. (2010). Implantation of functionalized thermally

Address correspondence to:
Dr. David R. Nisbet
Research School of Engineering
The Australian National University
Canberra ACT 0200
Australia

E-mail: david.nisbet@anu.edu.au

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