

The Development of Functional Hyaluronan Hydrogels for Neural Tissue Engineering

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Abstract

Tissue engineers – in order to develop therapies for the treatment of complex neurological injuries and diseases – attempt to recreate elaborate developmental mechanisms *in vitro*. Neuronal precursor cells are excellent candidates for the study of developmental operations such as cell adhesion, differentiation, and axonal pathfinding. Hyaluronan (HA) is a common polysaccharide that is found extensively throughout the neuronal extracellular matrix (ECM), and can be functionalised and crosslinked to form stable hydrogels that support growing neuronal cells. Hyaluronan hydrogels can be modified chemically and mechanically to mimic the ECM of the developing brain, awarding control over mechanisms such as differentiation and axonal pathfinding. This thesis is concerned with the functionalisation and characterisation of HA hydrogels, ultimately in order to simulate vital properties of the developing brain. Here we show that HA hydrogels can be finely tuned mechanically (by modulating stiffness and viscosity), and chemically, by the conjugation of peptides that mimic the neural cell adhesion molecule (NCAM). NCAM mimics and novel mimics of sialylated NCAM significantly influence the differentiation of NSPCs in 2D and 3D. HA hydrogels successfully support long term culture of neural cells in 3D, and encourage the formation and extension of neurites of several cell types including human, mouse and rat neuronal precursor and stem cells. These results demonstrate for the first time that novel NCAM mimicking peptides can be conjugated to well defined hydrogel matrices that influence intricate developmental behaviours in 3D. Understanding how neural cells form functional networks is essential for the development of clinical approaches that attempt to address the injuries and diseases that affect these systems.

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“Our doubts are traitors, and make us lose the good we oft might win,
by fearing to attempt”

William Shakespeare

Vir Gerhard en Anél

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Acronyms and Symbols

Table 1: A list of acronyms used throughout this thesis

Acronym	Definition
ACAP	Adenylate Cyclase Activating Polypeptide
ACN	Acetonitrile
ADSP	Actin Depolymerisation and Severing Proteins
ANOVA	Analysis of Variance
ANPP	Actin Nucleation and Polymerisation Proteins
APLC	Agrin-Perlecan-Laminin-Slit
ATP	Adenosine Triphosphate
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CC	Cerebral Cortex
CD	Cluster of Differentiation
CNS	Central Nervous System
CREB	cAMP Response Element-binding Protein
CSF	Cerebral Spinal Fluid
CSPG	Chondroitin Sulphate Proteoglycan
CTCF	Corrected Total Cell Fluorescence
DAPI	4',6-Diamidino-2-Phenylindole
dbcAMP	Dibutyryl Cyclic Adenosine Monophosphate
DCC	Deleted in Colorectal Cancer
DCM	Dichloromethane
DIC	N,N'-Diisopropylcarbodiimide
DIPEA	N,N-Diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide

DMTMM	4-(4,6-Dimethoxy-1,3,5-Triazin-2-yl)-4-Methylmorpholinium Chloride
DPBS	Dulbecco's Phosphate Buffered Saline
DRG	Dorsal Root Ganglion/Ganglia
DS	Degree of Substitution
EC	Endothelial Cell
ECM	Extracellular Matrix
EGF	Endothelial Growth Factor
ERK	Extracellular signal Regulated Kinase
ESC	Embryonic Stem Cell
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFr	Fibroblast Growth Factor Receptor
FGL	FG Loop Peptide
FMOC	9-Fluorenylmethyloxycarbonyl Chloride
FN	Fibronectin
GAG	Glycosaminoglycan
GAP	GTPase Activating Proteins
GDNF	Glial Derived Neurotrophic Factor
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GM	Glycidyl Methacrylate
GPI	Glycosylphosphatidylinositol
GTP	Guanosine Triphosphate
HA	Hyaluronan (Hyaluronic Acid)
HA-f	Hyaluronan-Furan
HAMC	Hyaluronan-Methylcellulose
HA-t	Hyaluronan-Thiol
HCTU	2-(6-Chloro-1H-Benzotriazole-1-yl)-1,1,3,3-Tetramethylaminium-Hexafluorophosphate
HEPA	High Efficiency Particulate Air
HPLC	High Performance Liquid Chromatography
HSPG	Heparan Sulphate Proteoglycan
IFN γ	Interferon Gamma
IL	Interleukin
LEC	Liver Endothelial Cell
LV	Lateral Ventricle
LYVE	Lymphatic Vessel Endothelial Receptor

MAPK	Mitogen Activated Protein Kinase
MC	Methylcellulose
MEK	<i>see MAPK and ERK; combination of</i>
MMP	Matrix Metalloprotease
MEM	Minimum Essential Medium
MES	2-(N-Morpholino) Ethanesulfonic Acid
NF-200	Neurofilament-200
NGF	Nerve Growth Factor
NMR	Nuclear Magnetic Resonance
NRP	Neuropilin
NSPC	Neural Stem Progenitor Cell
NT	Neurotrophin
PBS	Phosphate Buffered Saline
PDL	Poly- <i>D</i> -Lysine
PEG	Polyethylene glycol
PEG-DA	Polyethylene glycol Diacrylate
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PLL	Poly- <i>L</i> -Lysine
PNS	Peripheral Nervous System
PSA	Polysialic Acid
PSAm	Polysialic Acid Mimicking Peptide
RA	Retinoic Acid
RHAMM	Receptor for Hyaluronan-Mediated Motility
RMS	Rostral Migratory Stream
RPMI	Roswell Park Memorial Institute
RSPC	Retinal Stem Progenitor Cells
SANS	Small Angle Neutron Scattering
Shh	Sonic Hedgehog
SPPS	Solid Phase Peptide Synthesis
SVZ	Subventricular Zone
TCP	Tissue Culture Polystyrene
TGF	Transforming Growth Factor
TIPS	Triisopropylsilyl
TLR	Toll-like Receptor
VEGF	Vascular Endothelial Growth Factor
VMD	Visual Molecular Dynamics
VZ	Ventricular Zone

Table 2: A list of symbols used

Symbol	Unit
$\text{\AA}$	Angstrom
Da	Dalton
$^{\circ}\text{C}$	Degrees Celsius
hrs	Hours
kg	Kilogram
l	Litre
m	Metre
$mins$	Minutes
mol	Mole
N	Newton
Pa	Pascal
$secs$	Seconds
Unit Prefixes	
c	Centi; 10^{-2}
m	Milli; 10^{-3}
μ	Micro; 10^{-6}
n	Nano; 10^{-9}
p	Pico; 10^{-12}
Physical Symbols	
ω	Angular Momentum
ρ	Density/Viscosity
G''	Loss Modulus
ξ	Meshsize
ϕ	Neutron Incident Flux
Q or q	Neutron Scattering Vector
b	Nuclear Scattering Length
R_g	Radius of Gyration
G'	Storage Modulus
γ	Strain
τ	Stress
T	Temperature
t	Time
Ψ	Wavefunction
λ	Wavelength
Mathematical Constants	
k	Spring Constant (<i>variable</i>)

Chapter 1

Introduction

1.1 Background

1.1.1 The Foundations of Neural Tissue Engineering

The nervous system has exquisitely evolved to enable us to do everything from sensing pain and feeling empathy, to calculating risk and moving muscles; it is unquestionably essential for life as we know it. Unlike many other systems in our bodies, complexity still triumphs, thereby inhibiting progress and understanding. Several of the most significant developmental neurobiological mechanisms have been uncovered and are generally accepted, although there still remain many gaps in our knowledge. Attempts at mimicking these developmental mechanisms *in vitro* are still somewhat primitive, however advances in three dimensional (3D) cell culture techniques are beginning to shed light on complex processes such as fate and pathfinding decisions made by neuronal cells.

Pioneering experiments by Ross G. Harrison in 1907 revealed that living cells – in this case “embryonic tissue known to give rise to nerve fibers” – would remain alive under the right culture conditions for up to four weeks (Harrison, 1907). These experiments dated as early as 1885, built on the foundations of Roux and others, and demonstrated the concept of *in vitro* cell and tissue culture. The last decade has witnessed the resurgence of tissue engineering and the study of regenerative medicine. With a firm understanding of the natural mechanisms governing brain development and growth, tissue engineers attempt to restore the function of tissues lost through injury or disease.

To understand why 3D cell culture is such an attractive model for various *in vitro*

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and *in vivo* applications, the limits of two dimensional (2D) cell culture will be briefly discussed. We will then examine the native neuronal extracellular matrix (ECM) in detail, the understanding of which underpins neuronal tissue engineering and regenerative medicine. The materials science and chemical engineering of artificial neuronal matrices will then be examined and the difficulties of 3D culture will be discussed exhaustively. Numerous substrate materials have been employed as suitable matrices for this application, including porous ceramics and electrospun scaffolds of various designs, but these are beyond the remit of the introduction. Instead, attention will be given to the hydrated polymeric hydrogels, or hydrogels.

Hydrogels will be defined in the context of this work as hydrated viscoelastic polymeric scaffolds consisting of crosslinked (whether permanent or otherwise) macromers that can mechanically support a cell or tissue explant. Hydrogels offer an attractive alternative to the 2D culture of any cell type, although this introduction will focus specifically on stem cells, their precursors, or terminally differentiated cells of the neuronal and glial lineages. Hydrogels are an excellent substrate within which to maintain cells: they can be modified chemically, mechanically, or otherwise, to directly influence cellular mechanisms such as adhesion, proliferation and fate, and guidance decisions. The introductory chapters will discuss in depth our current understanding of the interactions between cells and their extracellular matrices, and how hydrogels can be employed to mediate cellular mechanisms such as adhesion, proliferation, fate decisions, and guidance of somas and neurites in a three dimensional environment.

1.1 Background

1.1.2 The False Two Dimensional Security

Modern scientific method has allowed us to probe ever deeper into the biological organism, and fittingly, cell and tissue culture has entered the 3D world proper. The limitations of 2D cell culture have been exhaustively outlined by many including Lund and colleagues who suggest that once removed from their native 3D cell niche, stem cell functionality and phenotype change drastically (Lund et al., 2009). Not only will cells assume an unnatural morphology, but gene expression is directly affected, leading to different fate decisions, adhesion and neurite outgrowth (Zahir and Weaver, 2004; Birgersdotter et al., 2005; Li et al., 2007).

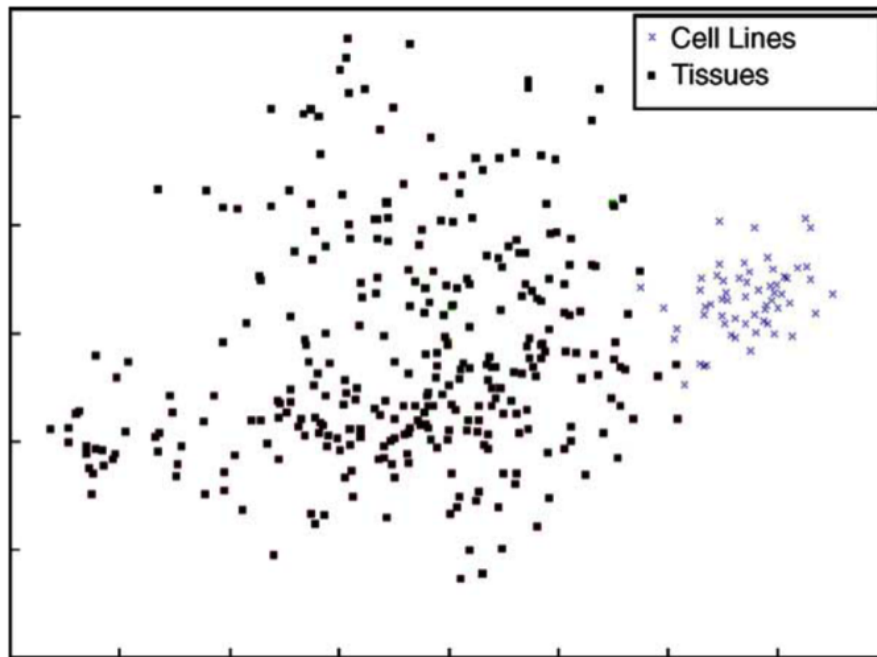


Figure 1.1: The gene expression profiles of cell lines and primary cells were compared by DNA microarray analysis. The x and y axis represent a correlation of each gene to two Eigen arrays, 1 and 2 respectively. These data suggest that cell lines cultured in 2D are genetically very different to the tissues from which they were isolated. Adapted from (Birgersdotter et al., 2005; Sandberg and Ernberg, 2005).

1.1 Background

Figure 1.1 compares the gene expression profiles of primary cells and their derived cell lines, showing a significant deviation from the normal gene expression profile. This paper suggests that cell lines cultured outside their native ECM will behave very differently to the same cells *in vivo*. It is important to note, however, that owing to the huge variation in normal gene expression of different cells, this figure should be interpreted with care. Indeed, some differences between the cell line and the primary cell are desired, and have been deliberately designed. The importance of the 3D environment was highlighted over two decades ago when ‘normal’ breast tissue cultured on 2D assumed a phenotype vastly different to the same cell type grown in its native environment (Petersen et al., 1992). Importantly, the cells reverted to their original phenotype when they were placed within their native ECM, suggesting that phenotype can surpass genotype. Tibbitt and Anseth describe this well:

“A cell can no longer be thought of as a solitary entity defined by its genome, but must be evaluated in the context of the ECM, soluble growth factors, hormones, and other small molecules that regulate organ, and ultimately organism, formation and function.”

(Tibbitt and Anseth, 2009).

Cells receive numerous mechanical and chemical signals from their native ECM, which constantly regulate their gene expression and function. These signals must be received, however, in spatiotemporal context for the cells to interpret them accurately (Lukashev and Werb, 1998). Even a simple mechanism such as adhesion is not only vital for attaching the cell to its environment, but also for many processes which depend on it; deviating from the well established *in vivo* environment

1.1 Background

too much may have deleterious downstream effects (Owen and Shoichet, 2010). These conflicting cascades are thought to be caused in part by polarisation of the cell membrane, where the concentration of receptors such as the integrins can significantly influence cell fate decisions (Zhang et al., 2005; Gieni and Hendzel, 2008).

Maintaining a cell or tissue culture in a 3D environment brings with it some additional advantages. There is a high volume available for proliferation, and thus cells are not hindered by spatial constraints such as those found in 2D; it is well known that highly proliferative cells may enter an apoptotic cell cycle when they reach confluence. The extra space in the 3D environment also allows the cell membrane to conform to a more natural morphology, also increasing the surface area of the cell available to form cell-cell contacts with adjacent somas. Indeed, most descriptions of cell morphology (for example the long spindle-like fibroblasts) were carried out on conventional cell culture surfaces, which as we have seen, are not representative of the natural cellular environment.

It is essential, therefore, that a cell is cultured in an environment that permits normal function. For example, differentiating neuronal cells develop different resting membrane potentials depending on the architecture of their ECM, whether natural or synthetic (Desai et al., 2006). Nonetheless, 2D cell culture should not be overlooked as an extremely useful tool in cell biology despite the fact that emerging evidence is drawing attention to the shortcomings of these techniques. Tissue engineers often disregard the complexity of native tissues, and may not always consider the many factors that are involved in developing and maintaining proper tissue function.

1.1 Background

1.1.3 Motivation

Neuronal tissue is inherently difficult to mimic *in vitro*, and predictable biological models of neuronal tissue are rare. Understanding complex neural processes during development and maturity is essential for the development of therapies for debilitating neurological injuries and diseases such as traumatic brain injury and stroke, and the degenerative disorders such as Alzheimer's. This thesis, therefore, does not aim to address one such injury or disease, but rather to develop models of complex developmental processes that may allow us to address biological questions related to the future treatment of the aforementioned injuries and diseases. These models include adhesion and migration, differentiation and pathfinding, or neurite guidance.

In this form, the aim may seem broad; therefore, as outlined below, this work will focus on a very specific developmental mechanism: cell fate and axonal pathfinding of subventricular zone progenitor cells, specifically the GFAP positive astrocytes that are precursors to both neurons and glia. To accomplish this, a hydrogel culture technique will be used in addition to molecular functionalisation with peptides that mimic the neural cell adhesion molecule. The neural cell adhesion molecule is essential in brain development. Mimicking the signalling cascades thought to be important in differentiation and axonal or somal pathfinding will shed light on how these processes occur naturally, and ultimately reveal how we can use this knowledge to develop therapies for neurological injuries and diseases.

1.2 Aims and Objectives

1. Synthesise and characterise a variety of hyaluronan (HA) hydrogels suitable for the three dimensional culture of neural cells and their precursors:
 - (a) Synthesise HA-furan, HA-methylcellulose and HA-thiol hydrogels;
 - (b) Characterise macroscopic properties;
 - (c) Model mass transfer events; and
 - (d) Assess the effects of long term neural cell culture and differentiation on the mechanical properties of hydrogels.
2. Evaluate the use of small angle neutron scattering as a microscopic characterisation tool:
 - (a) Investigate the microstructure of HA hydrogels by small angle neutron scattering; and
 - (b) Use computational models to identify the effect of changing hydrogel composition on their properties.
3. Surface modification of two dimensional surfaces with mimetic peptides of the neural cell adhesion molecule:
 - (a) Synthesise NCAM and PSA mimicking peptides;
 - (b) Bind these peptides to 2D surfaces such as polystyrene; and
 - (c) Delineate the effect of NCAM and PSA mimicking peptides on, specifically, cell fate and guidance decisions made by the cell.

1.3 Thesis Structure

4. Demonstrate the efficacy of long term neural cell culture in functionalised and non-functionalised hydrogels:
 - (a) Investigate viability of neurons and their precursors within hyaluronan-based hydrogels; and
 - (b) Show that functionalised hyaluronan-based hydrogels affect the behaviour of rat DRGs in 3D.

1.3 Thesis Structure

The predominant aim of this thesis is to demonstrate that hyaluronan hydrogels can be adapted to suit the requirements of neuronal cells by mimicking the neuronal extracellular matrix. *Chapter 2* will introduce the existing literature surrounding the native neuronal ECM, its structure, and what we currently know of its function. In order to accurately mimic and manipulate the behaviour of neuronal cells in later experimental chapters, we must first understand how they use their environment to develop properly. *Chapter 3* will outline past and present techniques of 3D neuronal tissue engineering. Special attention will be given to the mimicry of the native neuronal ECM, and how they can be used in combination with hydrogels or other scaffolds to influence cellular behaviour. Modern methods of characterisation will also be discussed in some depth, including rheology and small angle neutron scattering.

Chapter 4 experimentally explores the synthesis and characterisation of HA based hydrogels. The techniques of macro-characterisation, such as rheology and mass

1.3 Thesis Structure

transfer modelling are described. Importantly, this chapter investigates the effects of 3D cell culture and differentiation on specific properties of the hydrogels. The importance of controlling the micro and macro characteristics of these hydrogels is deliberated at length. *Chapter 5* investigates the use of small angle neutron scattering (SANS) as a microscopic characterisation tool. SANS is an extremely useful technique, which allows polymer chemists and tissue engineers to probe deeper into the microstructure of the hydrogels. More than one computational model is used to draw out useful information about the composition of these materials, and the effects of changing certain properties such as the macromer concentration.

Chapter 6 is dedicated to surface modification of materials with bioactive molecules that mimic NCAM. Peptide synthesis of various mimetic peptides is followed by an overview of the chemical conjugation techniques employed, and extensive bioactivity testing on several cell types. The structure of the synthesised peptides is modelled computationally, accompanied by statistical evidence. *Chapter 7* represents a brief exploration of functionalised hydrogels. The optimisation of 3D neural cell culture within HA hydrogels is first investigated, followed by a short introduction to hydrogel functionalisation. Although limited, evidence suggests that the mimetic peptides synthesised in Chapter 6 also have an effect in 3D.

Chapter 8 concludes the thesis, and briefly discusses the impact of the data herein. The conclusion and future perspectives precede any appendices, including any conference papers presented.

Chapter 2

The Central Nervous System and Neuronal Extracellular Matrix

2.1 Introduction

The central nervous system (CNS) consists of arguably the most specialised tissue types in the body. Not only does the CNS serve as a control centre for the entire peripheral nervous system, but it is involved in some extremely complex psychological processes such as problem solving and emotion. An important distinction should be made in this chapter between the different cellular components (including the neurons and the supporting glial cells), and extracellular components, which will be discussed in some depth. Tissue engineers often aim to influence a cellular component (for example a neural stem cell), by mimicking the extracellular component. This chapter will briefly discuss the anatomy of the CNS, focussing on the brain and the different tissue types within. The cell types of the brain will also be considered, including those that are of interest to neural tissue engineers.

The extracellular matrix (ECM) component of the CNS is hierarchical and, accordingly, elaborate and complex. The main focus of this chapter will be the structure and function of the neuronal ECM. The ECM is the supporting structure of the brain, which is essential during development and maturation. This scaffold-like support system guides neurons and glia, initiates differentiation of precursor cells, and regulates many cellular functions such as cell adhesion, neurite outgrowth and axonal pathfinding. Without the ECM, the brain would never develop properly, let alone function predictably when mature. This thesis is ultimately concerned with mimicking a very specific interaction between a stem cell and a functional ECM protein. Understanding native brain development, therefore, is crucial for the success of this work, and future investigations.

2.2 The Structure and Function of the Central Nervous System

The central nervous system – and especially the brain – consists of numerous anatomically distinct regions, which are not all understood in detail. Evaluating the structure and function of the entire nervous system is certainly a mammoth task, which will not be attempted here. The CNS consists of the brain and spinal chord, the latter is an important mediator of neural impulses to and from the peripheral nervous system. The spinal chord is receiving much attention in the fields of regenerative medicine and tissue engineering (Verma and Fawcett, 2005).

The human brain can be deconstructed in several ways, either anatomically or functionally, the latter of which is more relevant here. In the manner of Friston *et al* (Friston et al., 2004), the human brain can be divided into 5 main functional regions:

1. Sensory, motor and plasticity
2. Vision and visual perception
3. Higher cognitive function
4. Emotion and memory
5. Language and semantics

Each region consists of varying cell types and structure. Although a lengthy review will not be presented, it is, however, vital to understand that different structures have different functions. For example the cerebral cortex consists of several layers of

2.2 The Structure and Function of the Central Nervous System

interconnected neurons, known to be involved in memory formation. Huge advances have been made in the understanding of these networks, what they connect to, and why (Hagmann et al., 2008).

2.2.1 Neural Stem Cells

In neural tissue engineering, cell choice is extremely important, and ultimately dictates function. Stem cells are a popular choice because they can be directed into many lineages, including neurons and glia. Naturally, the developing and mature brain utilises a small number of stem cell niches to regulate native neural and glial cell populations (Clarke, 2003). One such cell, known as the neural stem precursor (NSPC) or subventricular zone (SVZ) cell, migrates along a structure known as the rostral migratory stream (RMS) to the olfactory bulb (OB), where the cell predictably differentiates (James et al., 2011; Ihrie and Alvarez-Buylla, 2011). *In vitro* these cells can be isolated from the brain and differentiated into functional neurons by a common protocol (Zahir et al., 2009) that was used extensively in this thesis, and can be visualised in Figure 2.1 below. Another benefit of using the NSPC is that – due to their plasticity and multipotency – they can be differentiated into glial cells such as the astrocyte, essential for the support of neuronal cells *in vitro* and *in vivo*. The NSPC is discussed in detail in Chapter 6 and Chapter 7, where their culture and differentiation are investigated experimentally. The reader should be encouraged to also consider practicality (although certainly not the most important) as a real driver of cell and scaffold choices.

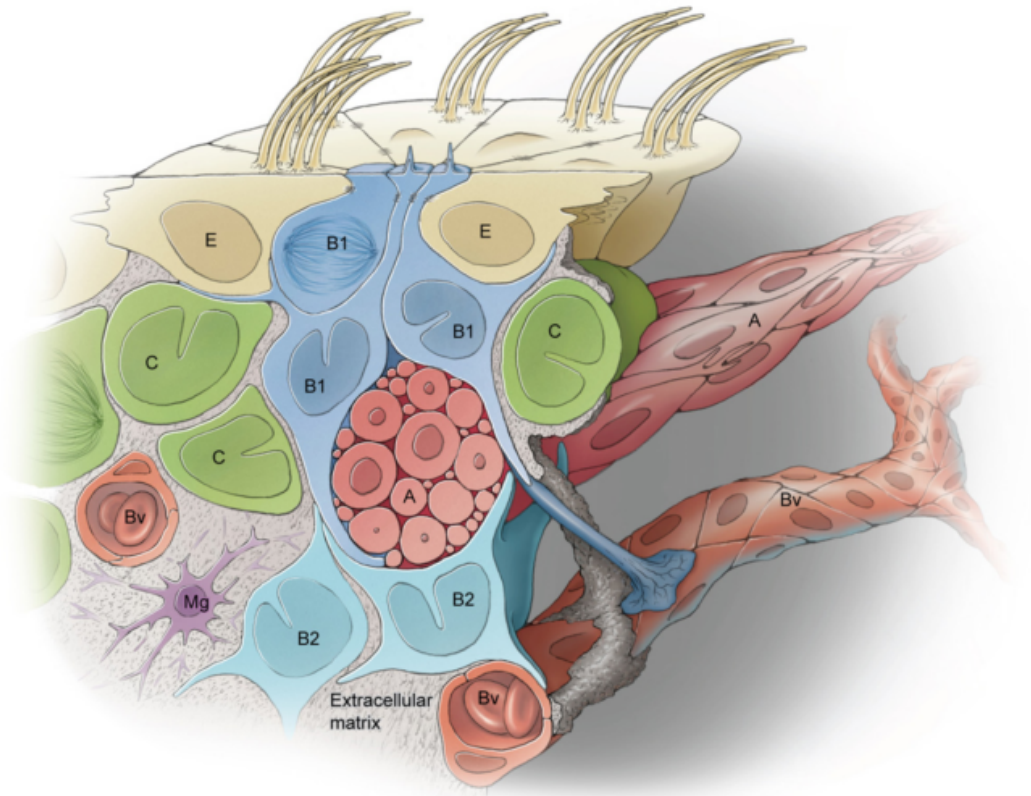


Figure 2.1: The adult subventricular zone (SVZ) niche, where the apical ventricular zone (VZ) and planar surface is shown at the top. The rest of the VZ-SVZ is shown in cross section underneath. “Ependymal cells (E, in beige) are multiciliated, and the basal bodies of these cilia are oriented in the direction of cerebrospinal fluid (CSF) flow. Ependymal cells form pinwheel-like structures around the apical processes of type B1 cells (shown in blue). Type B1 cells extend a short, nonmotile primary cilium into the ventricle. These cells maintain contact with the ventricle but disassemble the primary cilium while dividing. Type B1 cells also frequently extend a basal process with an endfoot that contacts blood vessels (Bv, shown in brown). Type B2 cells, in contrast, have astrocytic characteristics but do not contact the ventricle (shown in lighter blue). Transit-amplifying type C cells (in green) are found close to type B cells. Dividing C cells are also often found in close proximity to blood vessels (shown at left). Type B1 cells also contact their more differentiated progeny, the chains of migrating type A neuroblasts (shown in red). Type A cells migrate tangentially in chains (shown towards right of figure) that ultimately coalesce to form the rostral migratory stream taking these young neurons to the olfactory bulb for terminal differentiation. The VZ-SVZ also includes ECM (shaded) that contacts all the cell types in this region, including blood vessels and microglia (in purple)” (Ihrie and Alvarez-Buylla, 2011). Adapted from (Ihrie and Alvarez-Buylla, 2011).

2.3 The Structure and Function of the Neuronal ECM

The neuronal ECM is a highly specialised and structurally complex environment composed of many proteins, proteoglycans, and soluble factors all working in concert to facilitate an equally diverse set of functions. Each component of the ECM has a very specific role not only during the early stages of development, but also in the maintenance of mature adult tissue. In order to manipulate these cell-matrix interactions *in vitro*, it is essential that they be understood in context. Initially the structure and function of the neuronal ECM will be discussed, followed by an examination of specific cell-matrix interactions essential for complex mechanisms such as differentiation and neuronal pathfinding.

The ECM can be thought of as a scaffold-like mesh of proteoglycans and proteins; other constituents are present in smaller concentrations, such as diffusible growth factors and cues, although these are generally transient. These elements provide the majority of the structural stability of the ECM, and (through cell-matrix interactions) mediate numerous cellular functions. The composition of the ECM transforms significantly during embryogenesis, serving as an important regulator of migration and maturation of different tissue types.

Early work by Sanes and co-workers characterised the neural ECM during development, highlighting the specific roles of – among others – laminin, hyaluronan, and fibronectin in neural crest cell migration (Sanes, 1989, 1983). The structure and function of the ECM during development of neural tissue has since been studied at length (Reichardt and Prokop, 2011). It is now generally accepted that cells

2.3 The Structure and Function of the Neuronal ECM

exist in a state of homeostasis after embryogenesis, and except in a few cases such as the late developing mammary gland, remain in a pseudo-plastic environment (Engler et al., 2009; Owen and Shoichet, 2010).

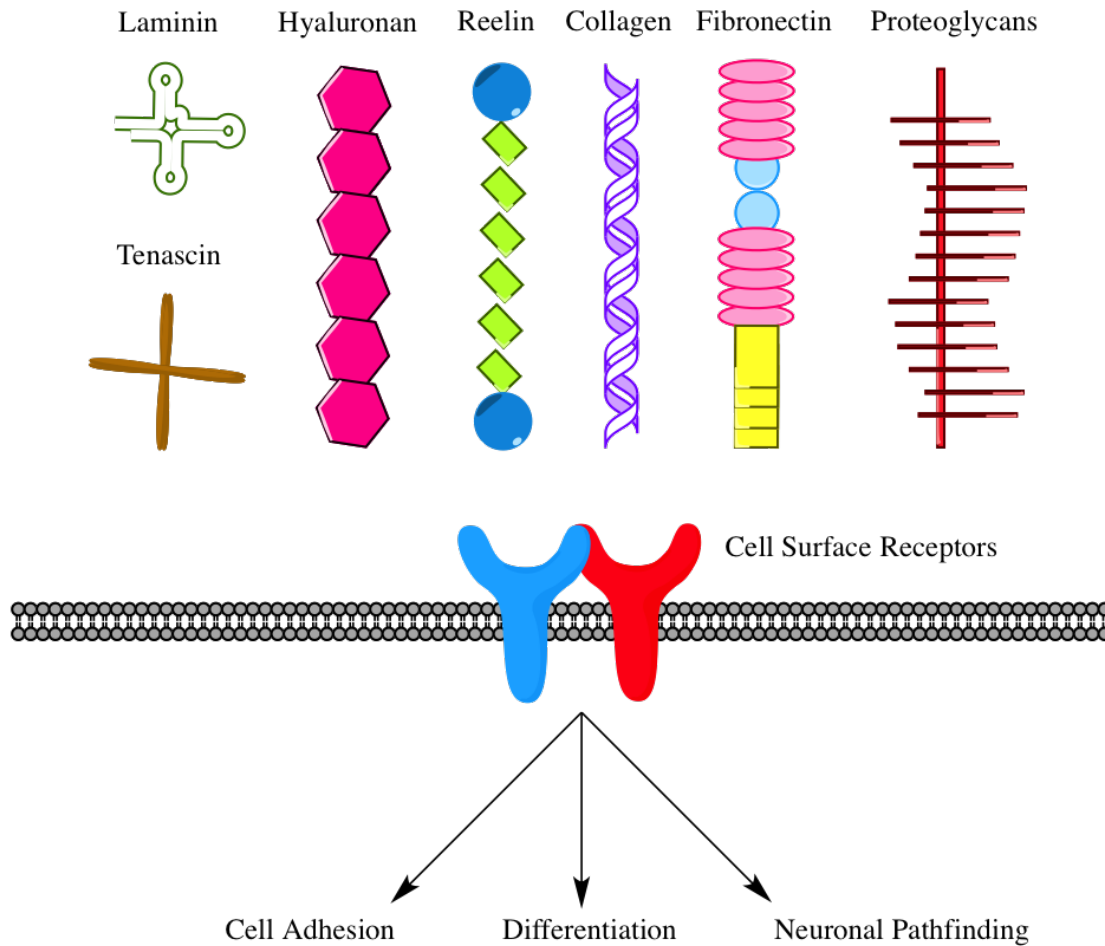


Figure 2.2: Components of the neural extracellular matrix interact with various cell surface receptors including the integrins and the cell adhesion molecules. Receptor activation leads to, amongst others, cell adhesion, stem/precursor cell differentiation and neuronal pathfinding, either by axons and dendrites, or by cell bodies. ©Phillipus J. Putter.

2.3 The Structure and Function of the Neuronal ECM

Collagen

Collagen is a trimeric protein composed of three parallel polypyrrolin-II strands. It is extremely abundant in the ECM, and can exist as one of 28 distinct types identified to date (Shoulders and Raines, 2010). Collagen is not only essential for structural stability of the neuronal ECM, but has also been implicated in adhesion, neurite outgrowth and differentiation. Many collagens have cell recognition sequences that play a significant role in integrin binding and subsequent downstream signalling cascades (Bella and Humphries, 2005).

Fibronectin

Fibronectin (FN) is a protein with three distinct regions known as fibronectin motifs (FN-I, FN-II and FN-III), which can bind homophilically to other FN motifs, or heterophilically to other neuronal ECM components, including and crucially, the cell surface receptors like the integrins. The size of individual FN molecules is highly variable due to alternative mRNA splicing, and the final tertiary conformation depends, to some extent, on the type of integrin to which it binds (Bruckner, 2010).

Laminin

This large glycoprotein is a key component of the basement membrane and is composed of an alpha, beta and gamma subunit, which bind to receptors such as the integrins, syndecans and dystroglycan (Reichardt and Prokop, 2011). Laminins are essential for development and maintenance of the neuronal ECM, and constitute a significant proportion of the basement membrane. Laminins have been found to be implicated in morphogenesis, proliferation and differentiation via their receptors, including the integrins and other non-integrin receptors (Durbeej, 2010).

2.3 The Structure and Function of the Neuronal ECM

Hyaluronan

Hyaluronan or hyaluronic acid (HA) is a non-sulphated linear repeating polysaccharide that binds to several cell surface receptors including a cluster of differentiation 44 (CD44; see Figure 2.3), the receptor for hyaluronan-mediated motility (RHAMM) and others implicated in non-neuronal functionality such as the toll-like receptor 4 (TLR4), HA receptor for endocytosis (HARE), lymphatic vessel endothelial HA receptor (LYVE-1) and liver endothelial cell clearance receptor (LEC) (Park et al., 2008; Ponta et al., 1998, 2003).

One way in which HA differs from the proteoglycans is its lack of a protein core. The role of HA in proliferation, migration and differentiation is thought to be regulated by protein kinases such as Src, focal adhesion kinase (FAK) and the MAP kinases; HA promotes the expression of some cytokines and proteins involved in ECM remodelling (Turley et al., 2002; Solis et al., 2012). The downstream mechanisms of HA–receptor activation have been extensively outlined by Solis and colleagues (Solis et al., 2012). The importance of HA and its role in tissue engineering will be discussed extensively in forthcoming chapters.

The Proteoglycans

The proteoglycans are characterised by a protein core glycosylated by glycosaminoglycan (GAG) side chains that interact with various ECM proteins, growth factors and morphogens (Nadanaka and Kitagawa, 2008). The lecticans are a group of chondroitin sulphate proteoglycans (CSPGs) which include aggrecan, neurocan, brevican and versican (Yamaguchi 2000). These proteoglycans are known modulators of adhesion, migration and neurite outgrowth. In fact, it is thought that the lecticans, specifically brevican and versican, act as bi-functional regulators of

2.3 The Structure and Function of the Neuronal ECM

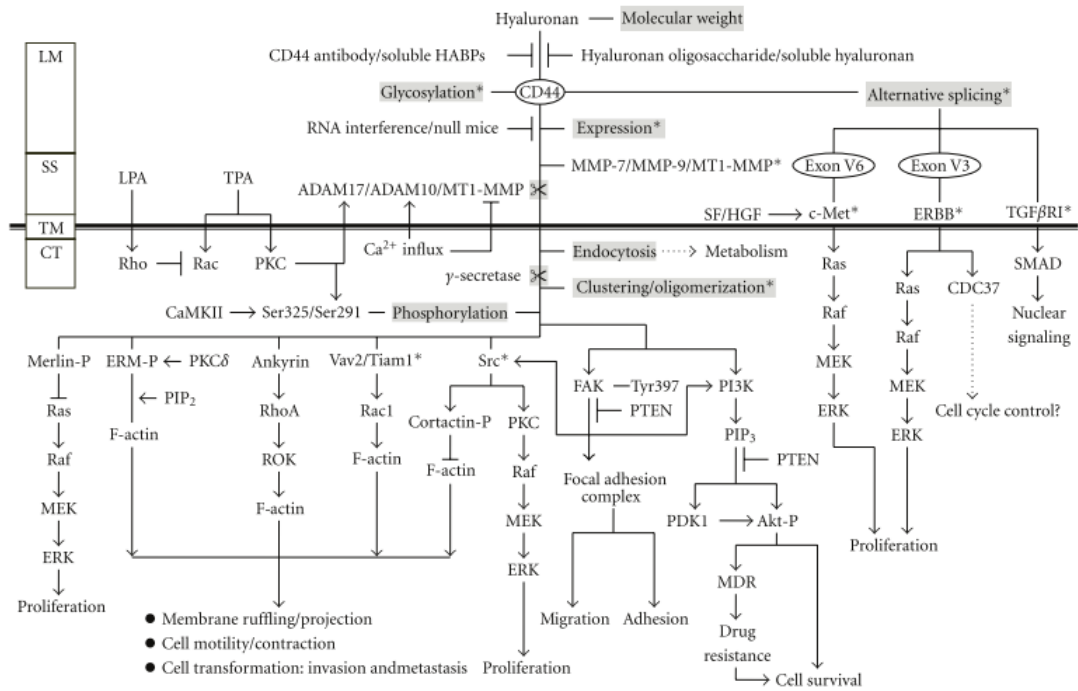


Figure 2.3: Hyaluronan can bind to receptors such as CD44 and RHAMM, mediating several important signalling cascades that ultimately influence cellular behaviours including adhesion, proliferation, migration and pathfinding. Adapted from (Solis et al., 2012).

neurite outgrowth (Yamaguchi, 2000). The CSPGs are up-regulated after certain CNS injuries, inhibiting neurite outgrowth *in vivo* (Pyka et al., 2011). The heparan sulphate proteoglycans (HSPGs) are another subset of this group of molecules, and are expressed in the neuronal ECM. They include agrin, collagen XVIII, perlecan, the syndecans and glypicans; some of these molecules have been associated with the regulation of neurogenesis, though are not necessarily essential for neuronal migration (Kirn-safran et al., 2009; Inatani et al., 2003; Jen et al., 2009).

2.3 The Structure and Function of the Neuronal ECM

Reelin

This glycoprotein consists of an N-terminal domain followed by 8 consecutive reelin repeats with an EGF-like motif at its centre (Franco and Müller, 2011). Reelin is secreted by Cajal-Retzius cells in the developing cerebral cortex, which subsequently binds to the transmembrane receptors ApoER2 and Vldlr, regulating neuronal migration of cortical neurons *in vivo* (Luque et al., 2003). A reelin mutation – also known as a reeler or reeler mutation – severely changes the pathfinding decisions of migrating neurons, leading to lamination defects and flawed neocortical development (Caviness, 1973).

Reelin may also serve as a detachment cue to some neurons, possibly facilitating cell–integrin separation by complete endocytosis of the entire integrin complex (Sanada et al., 2004). In addition to regulating neuronal cell migration and pathfinding, reelin is an important molecule during synaptogenesis, and even provides cues to enable the proper development of morphological features such as dendrites and dendritic branching (Rogers and Weeber, 2008).

The Tenascins

The tenascins are a family of five oligomeric glycoproteins that are thought to exert both inhibitory and excitatory influences on cell motility, and to mediate neuronal-glial interactions (Myers et al., 2011). Several cell types secrete tenascins, including the immature and reactive astrocytes, radial glia, granule cells, mature spinal neurons and retinal neurons (Brodkey et al., 1995; Kawano et al., 1995; Bartsch, 1996). One of the most important findings of this interesting group of glycoproteins is that the tenascins have not yet been identified in the mature brain, and instead remain in plastic regions such as the hypothalamus (Gonzalez and

2.4 Cellular Receptors

Silver, 1994). This also suggests that the tenascins may not be present in the healthy neural ECM and will therefore not be discussed at length. It should be noted, however, that the tenascins almost certainly have a role in the ‘fine-tuning’ of neural network formation and synaptogenesis, and play an important part in the development of the nervous system (Myers et al., 2011).

Additional ECM Components

There are several families of proteins that are vital for proper development and maintenance of the ECM, however these are considered minor constituents that cannot be classified within any of the categories above. For example anosmin-1 is essential for olfactory bulb formation and affects axon outgrowth and sprouting via fibronectin domains (Maccoll et al., 2002). Similarly, members of the thrombospondin family are important during brain development, as they promote proliferation, neural stem cell fate commitment and astrocyte induced neurogenesis, and are also implicated in synaptogenesis (Lu and Kipnis, 2010; Barros et al., 2011). For a review of other ECM molecules and their role in the nervous system see (Reichardt and Prokop, 2011) and (Barros et al., 2011).

2.4 Cellular Receptors

Ligands would, of course, be redundant without their counterpart: the receptors. The structure and function of cell surface receptors are numerous, extremely complex and outside the remit of this review. Nonetheless, receptors of neuronal cell types of the central nervous system must be examined in some detail. The following paragraphs will focus initially on the integrins, which play an important

2.5 Integrins in Adhesion and Pathfinding

role in contact-dependant mechanisms such as adhesion, migration and pathfinding. The function of the cell adhesion molecules will then be addressed, especially that of the neural cell adhesion molecule (NCAM). This omnipotent molecule serves as a mediator of many processes including neuronal pathfinding and stem or precursor cell differentiation.

2.5 Integrins in Adhesion and Pathfinding

It is generally accepted that the extracellular environment provides essential physical signals to neurons, which are broadly categorised by Moore and colleagues as either topographical or rigid, although this may be an oversimplified definition (Moore and Sheetz, 2011). Although other classifications could be used to evaluate the influence of typography and stiffness on function, the most common mechanisms through which cells receive these cues are the integrins. The functions of the integrins have been exploited successfully by tissue engineers, to functionalise hydrogels with peptides that improve cell adhesion, for example.

Integrins are composed of an α and β transmembrane unit that forms a non-covalent heterodimer with a vast array of ligands in the presence of calcium ions (van der Flier and Sonnenberg, 2001). These include insoluble elements such as hyaluronan, laminin, collagen, fibronectin and vitronectin (van der Flier and Sonnenberg, 2001; Humphries et al., 2006), and soluble elements such as TGF β 1, matrix metalloproteinase-1 (MMP1) and the Von Willebrand factor (vWF) (van der Flier and Sonnenberg, 2001). Not only are integrins essential for adhesion, guidance and fate decisions, but without matrix interactions, contact-dependant cells can

2.5 Integrins in Adhesion and Pathfinding

undergo apoptotic regression (Ruoslahti et al., 1994; Stupack et al., 2001). For an in-depth review of the role of integrin-ECM interactions in the regulation of axonal outgrowth and pathfinding, see (Myers et al., 2011).

Integrin-mediated binding of neuronal precursors is essential for adhesion. For example, ganglionic eminence neural progenitor cells (geNPCs) use the $\alpha6\beta1$ integrin to bind to laminin and fibronectin in the developing forebrain (Tate et al., 2004). Adhesion is also an important prerequisite for migration and pathfinding. Cox and colleagues demonstrated that increasing or decreasing the ligand concentration (in this case fibronectin) can influence specific signalling pathways important for these mechanisms. For example, an intermediate fibronectin concentration ($3\text{--}10\text{ }\mu\text{g ml}^{-1}$) activates Cdc42, Rac1 and RhoA, which are essential for adhesion and migration. Yet at higher fibronectin concentrations ($20\text{--}40\text{ }\mu\text{g ml}^{-1}$), only RhoA is activated, inhibiting Cdc42 and Rac1 (Cox et al., 2001).

Fate decisions of neuronal precursors are also regulated to some extent by cell-matrix interactions. For example, differentiation of human NSPCs is regulated by integrins binding to matrix components such as laminin. Cells cultured on laminin functionalised surfaces produced more neurons and astrocytes in a differentiation medium supplemented with BDNF, neurotrophin-3, FBS, FGF and retinoic acid. These integrins are vital not only in adhesion, but also in migration, differentiation, and in neurite outgrowth of differentiated neurons (Flanagan et al., 2006). The importance of integrins in these mechanisms is indisputable, yet owing to the vast array of integrins, and the ligands they bind to, no general conclusions can be made about their action.

2.6 Neural Cell Adhesion Molecules and Brain Development

2.6.1 The Structure and Function of NCAM

The neural cell adhesion molecule (NCAM) is a glycoprotein belonging to the immunoglobulin (Ig) superfamily, the extracellular domain of which contains five Ig domains and two fibronectin type-III (F3) domains (see Figure 2.4). Three main isoforms of NCAM have been identified, and were designated based on the size of their intracellular domain; NCAM-120, 140 and 180. NCAM is expressed on the surface of most cells in the central and peripheral nervous system (Kochoyan et al., 2008; Kasper et al., 2000), and is known to mediate cell adhesion, neurite outgrowth, fasciculation, and the promotion of cell survival and synaptic plasticity (Hinsby et al., 2004; Kochoyan et al., 2008). This is thought to occur mainly by homophilic (i.e. NCAM–NCAM) binding interactions, which lead to the activation of various downstream mediators such as Fyn. The following paragraphs will be devoted to the homophilic interaction of NCAM and the downstream signalling cascades that lead to adhesion, differentiation and pathfinding decisions of neuronal cells. Heterophilic NCAM binding has been observed with several ligands, including heparan sulphate, chondroitin sulphate proteoglycans, the cell adhesion molecule L1, glial derived neurotrophic factor (GDNF) and its receptor, several collagens, and Tag-1/Axonin-1 (Berezin et al., 2000; Paratcha et al., 2003; Hinsby et al., 2004).

2.6 Neural Cell Adhesion Molecules and Brain Development

Adjacent transmembrane NCAM molecules bind in two main ways: homophilic NCAM binding without associated FGF receptor (FGFr), and via a direct or indirect interaction with an NCAM-FGFr complex. In addition to fibroblast growth factor (FGF), its receptor FGFr can be activated by the direct binding of NCAM (also known as a heterophilic NCAM interaction), which in turn initiates several signalling cascades including the non-receptor kinase p59, focal adhesion kinase (FAK), phospholipase C (PLC), protein kinase C (PKC), and the Ras-mitogen-activated protein kinase (Ras-MAPK). The binding of NCAM to FGFr has been extensively investigated by Kochoyan and co-workers who demonstrate that the Ig2 domain of FGFr binds to the F3 domain of NCAM (Kochoyan et al., 2008).

2.6.2 Polysialic Acid Modification of NCAM

NCAM can be modified post-translationally with polysialic acid (PSA) on the 5th extracellular Ig domain and on the first fibronectin domain (Mendiratta et al., 2006). In mammals, PSA is a linear polymer of α 2-8-linked *N*-acetylneuraminic acid (NeuNAc) and adopts a helical structure in solution (Bonfanti, 2006). The length of PSA is highly variable, ranging from 8 to 100 monomers long (Rutishauser and Landmesser, 1996). PSA-NCAM is expressed primarily in the embryo, but some data suggest a presence in more mature tissues such as the olfactory and visual system, cortex, hypothalamus, ventricles, spinal cord, and brainstem (Bonfanti, 2006); these are all areas with significant structural plasticity.

Modification of NCAM with PSA leads to two main actions: steric inhibition and ECM spatial regulation. Cells that express PSA-NCAM prevent homophilic and heterophilic NCAM interactions due to their high negative charge density, which

2.6 Neural Cell Adhesion Molecules and Brain Development

contributes to the hydrated volume of NCAM (Yang et al., 1994). The extracellular space between adjacent cells, therefore, can be regulated by PSA expression that reduces the probability of cell-cell contact (Yang et al., 1994), however quantifying these data is problematic.

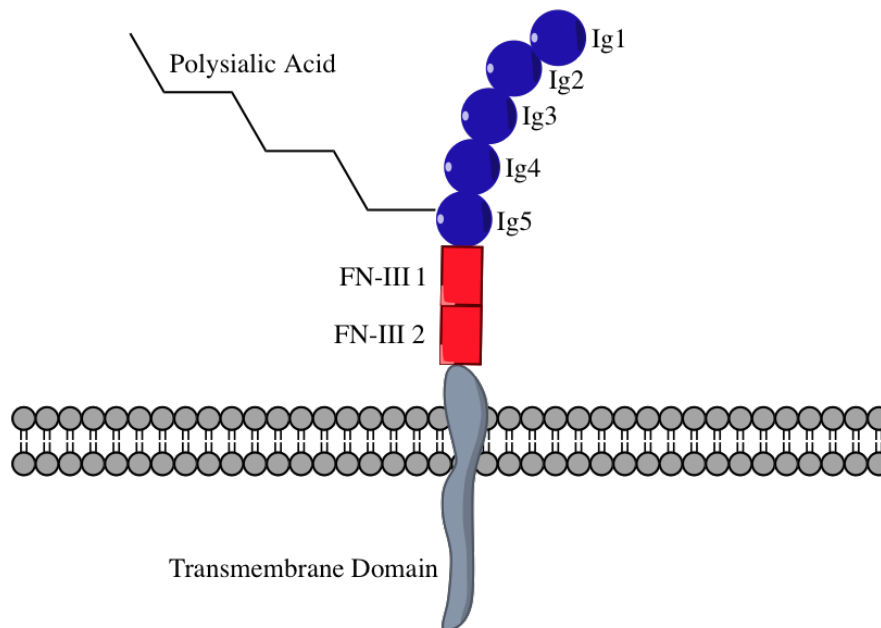


Figure 2.4: NCAM comprises a transmembrane domain of varying molecular weight, two fibronectin type III domains (FN-III) and five immunoglobulin (Ig) domains. NCAM can be modified by polysialic acid on the fifth Ig domain, which changes how a cell interacts with NCAM. ©Phillipus J. Putter.

Negative regulation notwithstanding, PSA–NCAM has been linked to a direct regulatory role in some heterophilic NCAM interactions, where PSA directly mediates interactions with molecules such as the heparan sulphate proteoglycans, which have been linked to the formation and remodelling of hippocampal synapses (Dityatev et al., 2004). Although the general action of PSA modified NCAM is thought to negatively regulate adhesion, the molecule has specific regulatory roles at particular developmental phases. Not only will PSA modulate migration and

2.6 Neural Cell Adhesion Molecules and Brain Development

growth cone progression and retraction, but it plays an important role in the regulation of myelination (Charles et al., 2000), and synaptic and morphological plasticity (Bonfanti, 2006).

Finally, PSA–NCAM has been implicated in several postnatal processes such as neurogenesis. The process most significant to this thesis relates to postnatal subventricular zone (SVZ) neurogenesis. The SVZ consists of several cell types including the neuroblasts that widely express PSA–NCAM (Rousselot et al., 1995). It is now thought that PSA plays an important role during SVZ neuroblast chain migration in the rostral migratory stream (Chazal et al., 2000).

The precise mechanism has not been elucidated, but the anti-adhesive effects of PSA–NCAM is probably not the only factor required for accurate migration (Bonfanti, 2006). PSA also plays an important role in differentiation; PSA down regulation following migration leads to subsequent cell fate changes (Petridis et al., 2004). NCAM is a powerful tool in the inventory of the developing and mature nervous system, especially after the post-translational modification by PSA; NCAM tells cells when to initiate contact-dependant signalling cascades that are essential for everything from survival to migration and differentiation. It should be noted, however, that the main role of NCAM is cell guidance in the SVZ.

The post-translational modification of NCAM may prove useful to neural tissue engineers, especially if mimetic peptides of PSA–NCAM and NCAM could be patterned in 3D. Materials such as the hydrogels are likely candidates for functionalisation, and would enable much greater control over cell migration and neurite outgrowth. Subventricular zone neuroblast migration along the RMS is a useful example; sialylated and unsialylated NCAM respectively guide migrating cells by

2.7 Guidance Proteins of the Developing Brain

inhibiting and promoting contact dependant signalling cascades. Replicating this *in vitro* may prove difficult, especially as little evidence exists to support the efficacy of mimetic PSA peptides.

2.7 Guidance Proteins of the Developing Brain

It is generally accepted that a neuronal process relies on more than one type of guidance cue to reach its target. For example, the developing cranial motor neurons are guided by diffusible factors, physical guideposts such as nerve exit points, glial cells, and muscle primordia (Guthrie, 2007b). The subsequent signalling cascade triggered by such guidance cues and their transcription factors affect the cytoskeleton in such a way as to alter the way the cell ‘behaves’, turning it towards or away from a guidance molecule (Butler and Tear, 2007). In this section, an overview of *in vivo* guidance molecules will be presented, with an emphasis on the netrins, semaphorins, slits and ephrins. For a comprehensive review of the early advances in this field, see (Baier and Bonhoeffer, 1994).

Developmental neurobiology is an extremely complex field of study, and although Section 2.7 may seem very general, it has some significant relevance in neural tissue engineering. Functionalising hydrogels with molecules that activate specific signalling pathways such as those discussed below will lead to more accurate representations of neural tissues *in vitro*. Similarly, 3D cultures of neuronal cells could benefit from the diffusion of these molecules into the culture material (for example the hydrogels), thereby directing mechanisms such as neurite outgrowth or cessation in a predicable manner. Another reason for the inclusion of this section

2.7 Guidance Proteins of the Developing Brain

is to allow the reader to consider the complexity of the *in vivo* environment in the design of smart biomaterials for cell encapsulation and culture. Any clinical application of neural tissue engineering will certainly require a careful understanding of the native cellular environment, and the chemoattractants and repellents of the developing brain are a good example of this.

2.7.1 The Netrins

The netrins are a family of attractant and repellent guidance cues that may be soluble, as in the case of Netrin-1-4, or anchored, as in the case of Netrin-G1 and G2 (Moore et al., 2007). These peptides are approximately 600 amino acids long, and have functional amino terminals similar to that of laminin (Colognato et al., 1997; Ettner et al., 1998). The roles of netrins including nervous system development have been extensively outlined by Moore *et al.*, and have been proven to have effects over large distances during development (Kennedy et al., 2006). These peptides have receptors including DCC (deleted in colorectal cancer) and UNC5A-D, which mediate attractant and repellent responses respectively (Moore et al., 2007).

2.7.2 The Semaphorins

The semaphorins consist of approximately 20 members of guidance molecules in 8 classes defined by their structural domains (Goodman et al., 1999; Kolodkin et al., 1993). These molecules have been implicated in various developmental mechanisms from organogenesis (Hinck, 2004) to complex functions such as the assembly of

2.7 Guidance Proteins of the Developing Brain

corticothalamic axonal projections (Bagnard et al., 2001). Relevant vertebrate classes include classes III (soluble), IV (transmembrane), VII (Membrane bound via GPI-1 anchor) and V (transmembrane protein significant in both vertebrates and invertebrates)(Koncina et al., 2007). The majority of semaphorin studies have focussed on semaphorin 3A, and initially demonstrated growth inhibiting effects on chick dorsal root ganglia and other models (Luo et al., 1993).

The vertebrate semaphorins can generally be considered to be inhibitory guidance molecules that encourage growth cone retraction (Pasterkamp and Verhaagen, 2006), however sema3C and 3F have been identified as chemoattractant factors that promote growth cone advancement (Bagnard et al., 1998; de Castro et al., 1999). It is important to highlight that the semaphorin family not only influence neurons, but also oligodendrocyte migration (Spassky et al., 2002; Ricard et al., 2000) and hippocampal synaptogenesis (Sahay et al., 2005).

The receptors of semaphorins can be categorised into receptors of the soluble semaphorins, such as the neuropilins (NRPs), or receptors of transmembrane semaphorins such as the plexins. The importance of other receptor molecules such as vascular endothelial growth factor receptors (VEGFRs) have been reviewed by (Koncina et al., 2007). Briefly, a semaphorin binds to a receptor or receptor-receptor complex that elicits a change in local actin dynamics through four main mechanisms: RhoGTPases, protein kinases, MAP kinases, and other pathways including MICAL and 12-HETE (Koncina et al., 2007).

2.7 Guidance Proteins of the Developing Brain

2.7.3 The Slits

The slits are a family of proteins of approximately 200kDa that include a sequence of 4 leucine rich repeats, 7-9 epidermal growth factor (EGF) repeats, an agrin-perlecan-laminin-slit (APLS) domain and a cysteine knot. Some slits have a laminin G-like (LG) module in place of the APLS domain (Chedotal 2007). One of the most important receptors of the slits are the roundabout or Robo receptors, which are members of the IgG superfamily consisting of five Ig like domains, three fibronectin type III (FN-III) repeats, a transmembrane protein and an intracellular tail with a variation of cytoplasmic motifs (Chedotal, 2007).

Like most other soluble guidance molecules discussed in this chapter, a slit–Robo complex influences intracellular actin dynamics via RhoGTPase regulation. The role of RhoGTPases in axon guidance has been widely discussed by Huber, who observes that the guanine nucleotide exchange factors (GEFs) activate RhoGTPases by converting GDP to GTP, while the GTPase activating proteins (GAPs) inactivate them (Huber et al., 2003).

More relevant to this thesis, the Robos have a role in the orientation and migration of subventricular zone (SVZ) cells along the rostral migratory stream (RMS) to the olfactory bulb. Chedotal *et al* demonstrate that Robo 2 and 3 are expressed in the SVZ and the RMS. They show that the slits are important chemorepellent factors of the septum and choroid plexus, as they orient and repel the migrating SVZ cells along the RMS to the olfactory bulb even in the adult brain (Nguyen-Ba-Charvet et al., 2004).

2.7 Guidance Proteins of the Developing Brain

2.7.4 The Ephrins

The ephrins and their receptors, the Ephs (an acronym from its expression in an erythropoietin-producing human hepatocellular carcinoma cell line) are essential for accurate neurite outgrowth and pathfinding (Guthrie, 2000; Klein, 2005). The structure of the Eph receptors are variable, but they generally consist of a 500-600 amino acid extracellular domain, a transmembrane domain of approximately 20 amino acids, and a cytoplasmic domain of 430 amino acids (Reber et al., 2007). Conversely, the ephrins consist of a 125 amino acid extracellular complex and cell attachment sites consisting of glycosylphosphatidylinositol (GPI) or PDZ interaction domains (Reber et al., 2007).

The signalling cascades following ephrin-Eph binding are complex and lie outside the scope of this chapter, however it is interesting to note that the guidance decisions of the neuron depend on signal transduction via specific mediators similar to the netrins and semaphorins. These are in turn regulated transcriptionally and post-transcriptionally (Yu and Bargmann, 2001). For example, whereas RhoA activation results in growth cone retraction (Dickson, 2001; Yuan et al., 2003), the activation of the other GTPases in this family (Cdc42, Rac1 and Pac) promote growth cone extension (Xiang et al., 2002; Kozma et al., 1997). The Ephs and ephrins play several important developmental roles, from hindbrain segmentation to topographical mapping and neurogenesis (Flanagan and Vanderhaeghen, 1998).

Significantly, the ephrins play an important role in SVZ cell migration from the lateral ventricles to the olfactory bulb. Ephrin B2/3 is expressed by SVZ astrocytes (an extremely important progenitor cell type in this region), and is essential for

2.7 Guidance Proteins of the Developing Brain

the proliferation and accurate migration of SVZ neuroblasts to the olfactory bulb (Conover et al., 2000). The effect of these ephrins on the migrating neuroblasts is thought to be repulsive in nature. It is important to note that Eph signalling has also been implicated as a proliferation suppressor in some cases (Theus et al., 2010). The ephrins have also been implicated in cell fate decisions of SVZ cells. Ephrin B1 signal blocking leads to differentiation of SVZ progenitors, however this study focusses on cortical development rather than olfactory bulb development as considered in other studies (Qiu et al., 2008).

2.7.5 Hedgehog, TGF- β /BMP and Wnt

Charron and colleagues emphasise the important guidance role of the hedgehog, TGF- β /BMP and Wnt families of morphogens. These proteins not only directly influence neuronal guidance decisions, but also control cell fate by regulating long range gradient-dependent effects such as differentiation (Charron and Tessier-Lavigne, 2007; Schnorrer and Dickson, 2004). The hedgehog proteins expressed in mammals include sonic (shh), indian (ihh) and desert (dhh), and are thought to be mediated by the Ci and Gli transcription factors (Ingham and McMahon, 2001). Of these, shh has received much attention, and experimental data points to shh and its chemoattractant effects with the aid of netrin-1 (Charron et al., 2003).

The TGF- β /BMP and Wnt proteins are expressed early in development and have different effects on developing axons. The BMPs – especially BMP-6 and 7 – exhibit chemorepellent effects on developing axons (Augsburger et al., 1999). Similarly, the TGF- β family has some influence over neuronal pathfinding, in which the unc-129 gene is required for accurate guidance (Colavita et al., 1998); the exact

2.7 Guidance Proteins of the Developing Brain

mechanism remains somewhat unclear. Finally, there exists a large body of evidence that highlights the role of the Wnt family of morphogens in axon guidance and pathfinding. Wnt5 for example, acts as a chemorepellent to certain neurons, Wnt4 controls the guidance of ascending axons to the developing brain, and Wnt3 is implicated in retinotectal topographical mapping (Charron and Tessier-Lavigne, 2007). Wnt signalling mechanisms, regulation and function have been reviewed by (Zou, 2004).

2.7.6 The Neurotrophins

It is widely established that neurotrophins such as nerve growth factor (NGF) promote neurite outgrowth (Keshmirian et al., 1989). The neurotrophins also regulate cellular behaviour such as survival, differentiation, proliferation and synaptic plasticity (Gillespie, 2003; Huang and Reichardt, 2003). These factors form part of a larger family of neurotrophic factors including glial derived neurotrophic factor (GDNF), interleukin-6 (IL-6) and hepatocyte growth factor (HGF) (Guthrie, 2007a). Whether the neurotrophic factors should be considered guidance molecules is debatable; evidence in favour includes the promotion of axon growth and orientation (Bosco and Linden, 1999; Ming et al., 1997) and common signalling pathways (Markus et al., 2002). However, although there exists substantial evidence to suggest that the neurotrophic factors can guide axons *in vitro*, there is insufficient data to support their long range effects *in vivo*, or whether these axons can orient with respect to a concentration gradient (Guthrie, 2007a).

2.8 Topographical Cues

It is generally accepted that growth cone dynamics are influenced not only by the ECM components or diffusible cues, but also by the surface topography of the substrate. Cells use these physical features as guideposts during migration and neurite pathfinding, although a recent study highlighted the importance of the size of these topographical features. Indeed, these features must be of specific dimensions relative to the dimensions of the neurite to be conducive to neurite extension (Fozdar et al., 2011). This may be due to stress transduced to the cell from these structures, in turn altering actin dynamics within the growth cone. Interestingly, topographical features may be preferred over chemical guidance cues when the neurons are exposed to both simultaneously (Gomez et al., 2007). Neurons will also respond to cell-like replicas, and use these features to ‘feel’ their way towards their target (Bruder et al., 2007).

2.9 The Role of Glial Cells in Development

Without supporting cells, a neuron in the complex and demanding developing ECM would not reach its target. We have discussed the structure and function of the ECM and the diffusible cues that support development. Glial cells are another fundamental part of the developing brain, which influence neurite outgrowth by direct signalling and stabilisation of newly formed axonal branches (Chotard and Salecker, 2004). Glial cells also provide essential topographical information; rather than migrating from an initial location to a target, a developing neuron moves between numerous intermediate targets including glial cells.

2.9 The Role of Glial Cells in Development

The relationship between the neuron and the glial cell is considered reciprocal, in that the glial cell supports neuronal guidance, and the neurons in turn regulate migration, survival and proliferation of the glial cells (Learte and Hidalgo, 2007). The lineage structure of the CNS glia remains under investigation, but it is known that these cells are not necessarily direct progeny of neural stem cells, but originate from transit amplifying cells or intermediate progenitor cells (IPCs) (Kriegstein and Alvarez-Buylla, 2009). This is shown in Figure 2.5 where the lineage structure of these important cells is outlined.

This reciprocal relationship is often overlooked. Glial cells alone have been shown to promote axonal regeneration in a rat injury model without the need for neuronal cell implantation (Ramon-Cueto et al., 2000). The role of glial cells in guidance – including the chemoattractants and repellents produced by glia, the genes involved in their development, and an exhaustive overview of the molecules involved in glial-neural interactions have been extensively reviewed (Learte and Hidalgo, 2007). In addition to migration and neurite guidance, glial cells are essential mediators of neural precursor cell mitosis, survival and homeostasis (Colón-Ramos and Shen, 2008).

Interestingly, the production of neurons during differentiation of precursor cells is also regulated by the glia to some extent, both by modulating mitosis (as described above) and neuronal cell death by engulfment to control overpopulation (Freeman, 2006). Synaptogenesis and maturation of neurons is another crucial process that is umpired considerably by the glia (Pfrieger, 2009). Most importantly, cells in the astrocyte lineage become the stem cells of the developing and mature brain. In a ground breaking experiment, the subventricular zone of a mouse was injected with

2.9 The Role of Glial Cells in Development

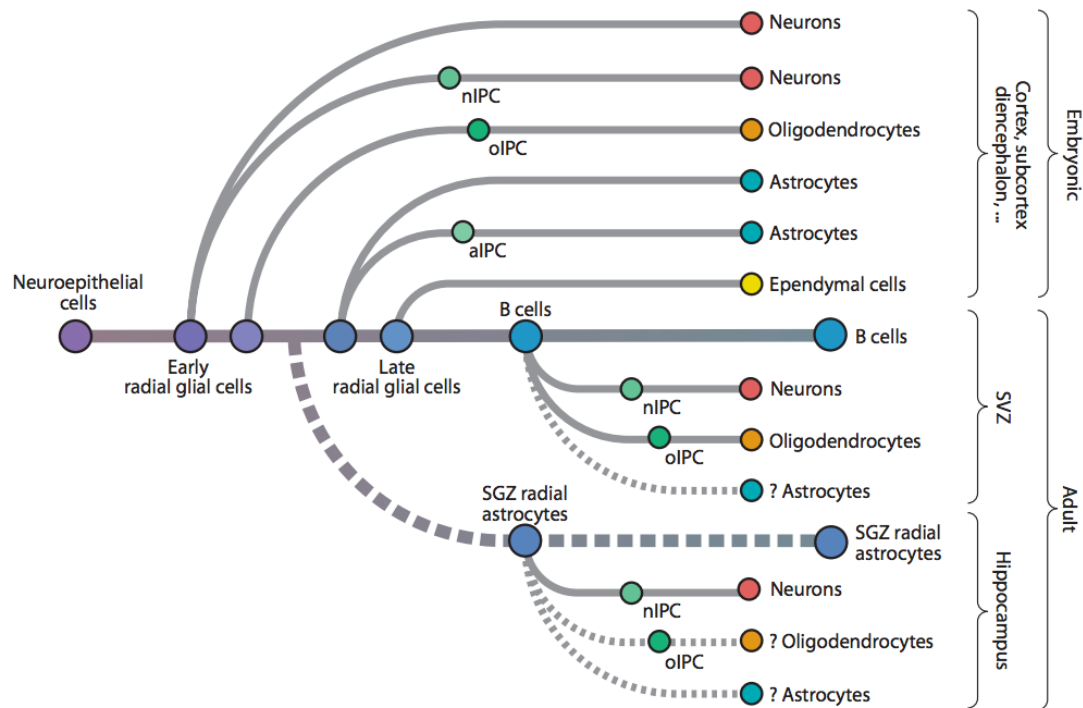


Figure 2.5: Neural stem cell lineage tree highlighting cell fate decisions made by the SVZ cells in different developmental stages. Embryonic daughter cells are shown in the upper half, whereas the lower half depicts cells derived in the adult brain. Solid and dotted lines represent experimentally confirmed and hypothetical lineage progression respectively. IPC=intermediate progenitor cell, which are indicated for both neurons (nIPC), oligodendrocytes (oIPC), and astrocytes (aIPC). NSC=neural stem cell; RG=radial glia; SGZ=subgranular zone. Adapted from (Kriegstein and Alvarez-Buylla, 2009).

a GFP labelled adenoviral vector that targeted GFAP positive astrocytes; once dissociated, it was shown that these GFAP positive astrocytes can be differentiated into neurons and glia *in vitro* (Doetsch et al., 1999). It is these astrocytes that form the basis of much of the experimental work in this thesis, and are used to form stable populations of differentiated neurons that can be used for a variety of 3D cell culture experiments.

2.10 Structure of the Growth Cone

The growth cone is the distal most part of the axon and is comprised of three general divisions: the actin rich peripheral domain, containing lamellipodia and filopodia, the myosin rich transition zone and the central domain responsible for microtubule polymerisation (Bouquet and Nothias, 2007). Neurofilaments also exist within the central domain, actively transporting vesicles and organelles into the peripheral domain. It is interesting to note that microscopically, the central domain is narrow when advancing and thicker when pausing; this is a useful tool when evaluating the current state of a growth cone (Bouquet and Nothias, 2007).

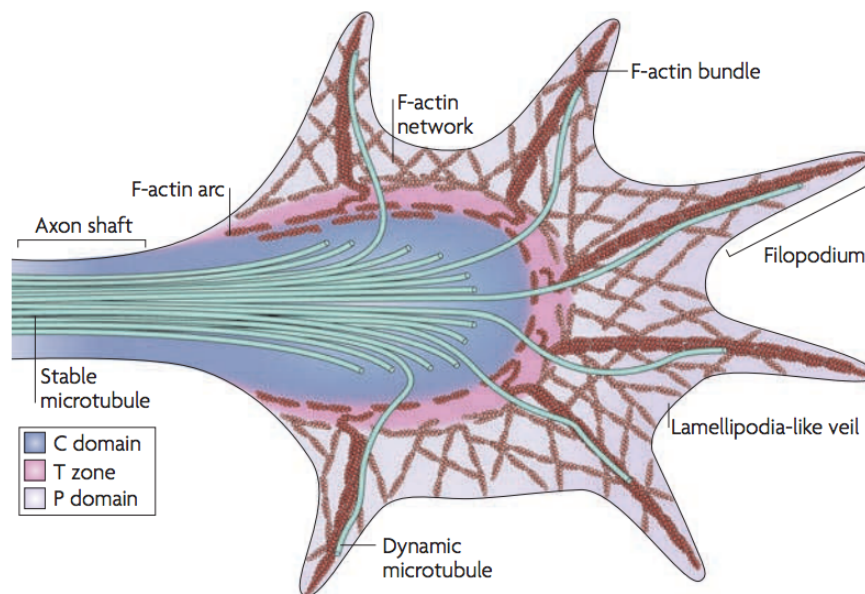


Figure 2.6: The growth cone is an extremely dynamic structure at the distal end of the axon, consisting of the exploring filopodia, each separated by the membrane-like lamellipodia. The peripheral domain consists of bundled actin filaments that form part of the filopodia, a central domain that contains bundled microtubules that enter the growth cone, and a transition zone that contains actomyosin contractile structures. Adapted from (Lowery and Van Vactor, 2009).

2.11 Growth Cone Responses to Guidance Cues

The growth cone is the part of the axon (and hence neuron) that is sensitive to various signals and cues, including the soluble growth factors and matrix bound molecules. Early hypotheses considered whether extracellular signalling molecules induced rapid changes in local protein concentrations within the growth cone and thereby directly activated specific pathways connected to the cytoskeleton of the neuron (Dickson, 2002).

An important paper by Steward coupled this change in local protein concentration to mRNA translation; either the proteins are synthesised locally, or the growth cones themselves have the ability to synthesise receptors for axonal guidance molecules (Steward, 2002). This hypothesis was similarly supported by Gallo and Letourneau in the same year, in that local protein synthesis and turnover in the distal growth cone changes the responsiveness of the axon to temporal and spatial guidance cues (Gallo and Letourneau, 2002).

2.11 Growth Cone Responses to Guidance Cues

The mechanisms by which a guidance cue initiates a response by the growth cone are relatively well understood, although some knowledge gaps do exist. For a review of recent advances, see (O'Donnell et al., 2009). Fundamentally, a local guidance cue binds to a cell receptor, and via various signalling cascades leads to polymerisation/depolymerisation cycles of cytoskeletal proteins such as actin and tubulin by altering gene expression (Condrón, 2002; Engle, 2010). These soluble cues have receptor mediated effects via integrin interactions both within and outside neuronal tissue (Nakamoto et al., 2004). It is interesting to note that

2.11 Growth Cone Responses to Guidance Cues

vascular tissues share some common guidance molecules with neuronal tissue that activate or inhibit these integrin-adhesive receptors (Serini and Bussolino, 2004). Once the signalling cascade has been activated, cytoskeletal proteins such as actin and myosin are transported to the leading edge of the growth cone as monomers where they asymmetrically accumulate and polymerise to produce a protrusion force that pulls and pushes the ECM (Brown and Bridgman, 2003).

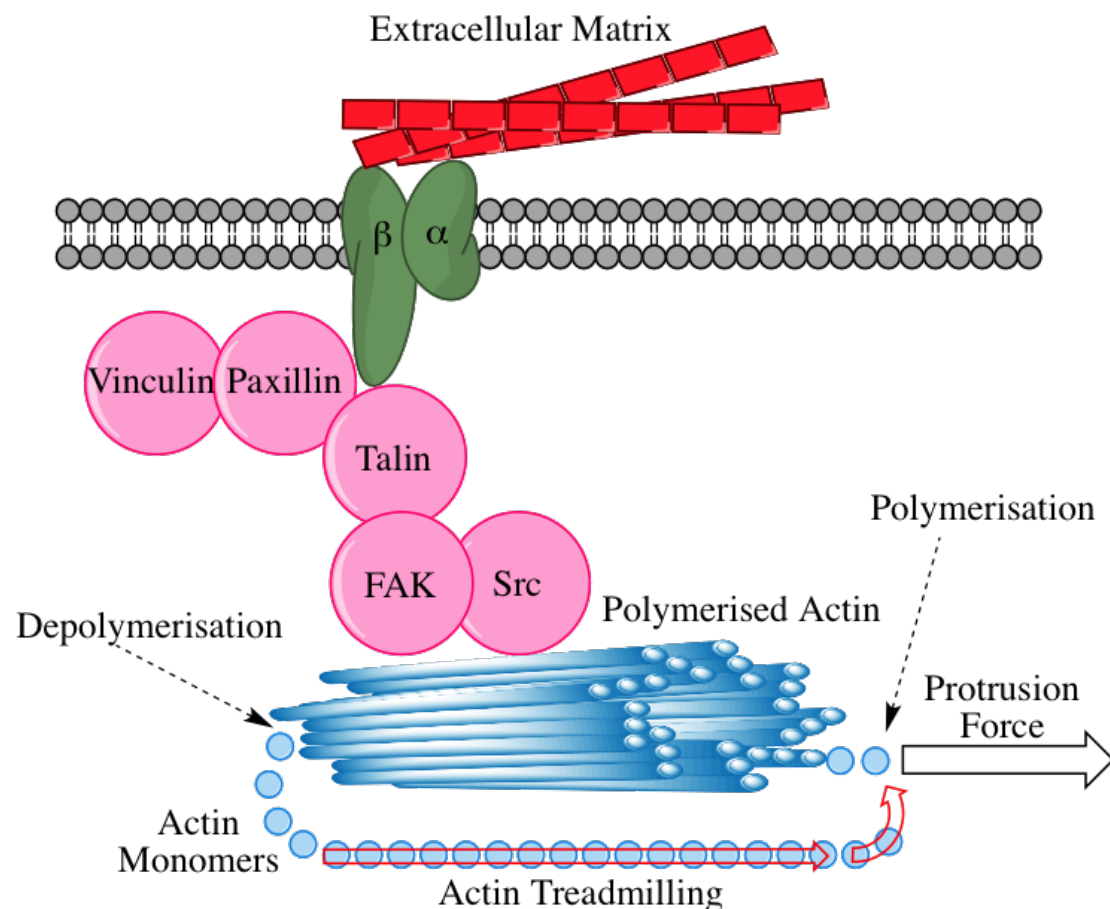


Figure 2.7: Integrins are essential mechanotransducers during pathfinding. The alpha and beta subunits bind to heterophilic ligands such as collagen, laminin and fibronectin in the ECM, eventually activating focal adhesion kinase (FAK) and the non-receptor tyrosine kinase Src. The activation of FAK and Src are thought to regulate actin treadmilling and progression or regression of the cell membrane by protrusion. ©Phillipus J. Putter.

2.12 Regulating the Dynamic Axon

Various other proteins are also synthesised in the axon, including heat shock proteins and metabolic proteins, but have been discussed in detail elsewhere (Willis et al., 2005). To maintain axonal advancement, actin filaments are transported by myosin dependent mechanisms to the transition zone where the actin filaments depolymerise; the polymerisation/depolymerisation cycle repeats indefinitely (Brown and Bridgman, 2003).

2.12 Regulating the Dynamic Axon

The process of actin turnover is regulated by two groups of proteins: the actin nucleation and polymerisation proteins (ANPP) and the actin depolymerisation and severing proteins (ADSP) (Bouquet and Nothias, 2007). The Rho family of GTPases have been identified as mediators of this complex mechanism, and as we will see, are essential for cellular motility of both neuronal cells and other cell lineages.

Repulsive and attractive guidance cues produce specific signalling cascades that activate or deactivate members of the Rho-GTPase family. Rho, Rac and Cdc42 are switched on or off by oscillating between an active GTP-bound state, and an inactive GDP-bound state (Huber et al., 2003). For example, the repulsive semaphorin family of proteins activate Rho, but inhibit Rac, thereby mediating different cellular responses that include neurite outgrowth or regression (Hu et al., 2001). The RhoGTPases are in turn modulated by three families of molecules: the GTPase activating proteins (GAPs) which facilitate GTP hydrolysis (Moon and Zheng, 2003), the guanine nucleotide exchange factors (GEFs) which facilitate

2.13 Remodelling of the Neuronal Extracellular Matrix

GDP-GTP exchange (Schmidt and Hall, 2002) and the GDIs (guanine dissociation inhibitors), which inhibit GDP dissociation and keep the GTPases in an inactive state (Bouquet and Nothias, 2007).

Our knowledge of the dynamic axon and the important signalling cascades such as the RhoGTPases may be important in neural tissue engineering. For example, functionalising 3D substrate materials with molecules that activate these cascades would allow for greater control of axonal and dendritic growth and guidance. It is important to remember the complexity of the *in vivo* environment, so that we can use this to our advantage when developing neuronal tissues *in vitro*.

2.13 Remodelling of the Neuronal Extracellular Matrix

The neuronal ECM is not an inert substrate; rather, the cells constantly remodel the ECM to suit their requirements. For example, remodelling may decrease local stiffness, decrease the concentration of matrix adhesion points, and therefore also liberate ECM fragments that may provide important signals to the respective cell (Owen and Shoichet, 2010). Remodelling occurs in part by the action of proteolytic enzymes produced by the cells, which break down the ECM components discussed in previous sections. There are various proteases that enzymatically facilitate this action, including the matrix metalloproteinases (MMPs), serine proteases (e.g. plasmin and plasminogen activator) and the cysteine proteases (e.g. cathepsins), that are in turn regulated by integrin signalling mechanisms (Daley et al., 2008).

2.13 Remodelling of the Neuronal Extracellular Matrix

Not only do these proteins breakdown the ECM physically, but they also convert the ECM components into signalling molecules. It should be noted that remodelling also occurs by non-enzymatic means, facilitated by receptor signal transduction proper, and by the regulation of transcription/translation of ECM proteins. For a complete review of the mechanisms underpinning proteolytic and non-proteolytic remodelling of the ECM in the context of tissue engineering and regenerative medicine, see (Daley et al., 2008). Hydrogels used in cell culture can be engineered with enzymatically cleavable peptide sequences that allow the encapsulated cells to remodel their environment. This is especially significant when studying the development of various tissues and mechanisms such as angiogenesis and neurogenesis, where the tissue is especially plastic (Aizawa and Shoichet, 2012).

2.14 Summary

Chapter 2 exhaustively reviewed the native neuronal ECM. It is important to note, however, that this review was tailored towards the application of neural tissue engineering. To that end, the structure and function was discussed in some detail, including the ligands and receptors that are important during the development of the brain. It is generally accepted in the the field that in order to produce accurate mimics of the brain (whether embryonic or otherwise), we must first understand exactly how these tissues develop *in vivo*. Indeed, reproducing complex mechanisms such as neural differentiation and axonal or dendritic pathfinding *in vitro* is a non-trivial exercise in practice, and must be approached carefully.

Chapter 3 will investigate this proposition further, specifically focussing on how tissue engineers use their knowledge of the native ECM to produce functional materials that mimic the developing and mature brain. The importance of carefully characterising all the components of the developing brain cannot be understated. It is, after all, the developing brain that we are attempting to recreate, albeit extremely primitively. This knowledge also forms the basis of many of the scientific questions that remain unanswered. For example, how exactly does NCAM facilitate migration of the SVZ cells to the olfactory bulb? How do the ECM proteins (laminin, fibronectin etc.) facilitate cell fate decisions made by neuronal precursor cells such as the SVZs? With this knowledge, we can recreate developmental environments *in vitro*, allowing us to observe important biochemical mechanisms without the use of animal models.

Chapter 3

Mimicking the Neuronal

Extracellular Matrix: Scaffold

Materials and Three Dimensional

Neural Cell Culture

3.1 Introduction

Three dimensional neuronal cell and tissue culture can be defined as the growth or maintenance of living neural and glial cells or tissues within a substrate not defined by a single growth plane. As the following chapter will reveal, 3D cell culture provides significantly more control over certain cellular functions. Chapter 2 reviewed the complexity of the *in vivo* environment and how neuronal cells interpret extrinsic cues for guidance and pathfinding. In Chapter 3, this knowledge will be applied to tissue engineering and biomimetics. The advances in 3D cell culture and the modification of biomaterials with a variety of peptides, proteins and bioactive molecules will be deliberated at length. Special consideration will be given to hydrogels, how they are synthesised, characterised, and modified to influence cellular behaviour. Not only will these advanced materials lead modern cell culture, but also encourage the development of cell transplantation devices, drug delivery systems and tissue engineering.

Biomaterials have long been used to influence the way cells behave. They can be used to modify viability, adhesion, cell fate decisions and very specific signalling cascades leading to complex processes such as neurite outgrowth and apoptosis. Functionalising these materials to elicit specific cellular responses has been the focus of a great many research groups. A recurring theme in this thesis is the mimicry of the brain during development and maturation; the importance of functionalising biomaterials accurately for 3D cell culture, therefore, cannot be emphasised enough. We will begin by exploring the hydrogels and how they can be characterised. This will be followed by an extensive review of the advances of 3D neuronal cell culture.

3.2 Scaffold Materials

The false two dimensional security discussed in Chapter 1 has led to the evolution of 3D cell culture techniques. Although this section will focus mainly on hydrated polymers such as the hydrogels, it is important to discuss a selection of the myriad materials and processing techniques that have been employed for 3D cell culture.

Several classes of materials have been considered for 3D cell culture, including the metals, ceramics and arguably the most versatile, the polymers; a non-exhaustive list is provided in Table 3.1. Metals have a long history in biomaterials, although their impact on 3D cell culture remains generally limited to load bearing applications where cellular integration is important (Alvarez and Nakajima, 2009). Some disadvantages to note is the difficulty of surface functionalisation (important for cell culture), toxicity, and processing (Matassi et al., 2013). Similarly, few cell types will remain stable on a material with such a high stiffness. Metals are, however, excellent in load bearing applications where we currently do not have a suitable alternative. For a review of the use of metals in bone tissue engineering see (Alvarez and Nakajima, 2009). Ceramics have also shown some promise in 3D cell culture (Goralczyk et al., 2009), but as with the metals, the disadvantages often outweigh the benefits when compared to the polymers. Notwithstanding, some advances have been made in load bearing applications such as bone and cartilage engineering, where – as with the metals – structural integrity is extremely important (Leukers et al., 2005).

Polymers are extremely popular as biomaterials for cell culture, and their strength

Table 3.1: Several scaffold types have been considered for three dimensional cell culture (including neuronal cells), which are tabulated below. For an accurate description of the cell type and source, the reader is referred to the accompanying reference. It should be noted that these data are not exhaustive, and serves merely as an indication of the range of materials currently in use.

	Scaffold Composition	Cell Type Cultured	Reference
Natural	Chitosan	Adipose Derived Stem Cells	(Malafaya et al., 2005)
	Coral	Rabbit Mesenchymal Stem Cell	(Chen et al., 2002)
	Silk Fibroin/Chitosan	Hepatocytes	(She et al., 2009)
Synthetic	Calcium Metaphosphate	Rat Mesenchymal Stem Cells	(Lee et al., 2001)
	Hydroxyapatite	Goat Mesenchymal Stem Cells	(Kruyt et al., 2003)
	Nylon	Hippocampal Neurons	(Yoo et al., 2011)
	Poly (caprolactone)	K-562, HL-60, Kasumi-6	(Blanco et al., 2010)
	Poly (glycolic acid)	Bovine Chondrocytes	(Vunjak et al., 1998)
	Poly (hydroxymethylglycolide-co- ϵ -caprolactone)	Endothelial Cells	(Seyednejad et al., 2012)
	Poly (<i>L</i> -lactic acid)	Dorsal Root Ganglia	(Wang et al., 2009)
	Poly (<i>L</i> -lactic-co-glycolic-acid)	K-562, HL-60, Kasumi-6	(Blanco et al., 2010)
	Poly (styrene)	K-562, HL-60, Kasumi-6	(Blanco et al., 2010)
	Poly (urethane)	K-562, HL-60, Kasumi-6	(Blanco et al., 2010)
	Titanium	Rat Mesenchymal Stem Cells	(Holtorf et al., 2005)

3.2 Scaffold Materials

lies in biomimicry. Put simply, the human body consists of a vast set of tissues and a respective ECM, all with different chemical and physical properties; the polymers have to date provided the best platform from which to mimic these vastly differing properties. In an excellent review, Carletti and colleagues discuss the advantages and disadvantages of several of the most popular biomaterials used as 3D culture scaffolds (Carletti et al., 2011). These include the synthetic poly (lactic acid) and poly (glycolic acid) (and the copolymer poly (lactic-co-glycolic acid)), (Li et al., 2010), polycaprolactone (PCL) (Hutmacher et al., 2001), polyurethane (Mauth et al., 2009), and polyester (Gümüşderelioğlu and Türkoğlu, 2002) to name a few. In addition to the numerous polymers, many processing techniques can be employed to produce different macro and microstructures. As discussed previously, cells have the ability to sense their environment so the processing method must be selected carefully. Some of these techniques include solvent casting/particulate leaching, freeze drying (lyophilisation), phase inversion, electrospinning, stereolithography, selective laser sintering, 3D printing and shape deposition manufacturing, all discussed by Carletti *et al* (Carletti et al., 2011).

3.2.1 The Hydrogels

Materials often considered for their near-physiological properties are the hydrogels. These versatile hydrated polymer matrices are largely simple to prepare and crosslink, support chemotrophic/chemotropic factor immobilisation and have easily modifiable mechanical properties. A recent review by Aurand *et al* extensively discusses the use of hydrogels for neural tissue engineering. The review outlines important considerations with regard to hydrogel design, including polymerisation,

3.3 Three Dimensional Neural Cell Culture

degradation, mechanical properties and physical architecture (Aurand et al., 2012). Hydrogels are also becoming increasingly popular due to the fact that they can be chemically modified with peptides and growth factors that promote various cellular behaviours such as adhesion and differentiation.

Both natural and synthetic materials have been considered for 3D neuronal cell culture. Synthetic materials are free from animal products, which reduces the risk of disease transmission, a characteristic that also bypasses some ethical arguments against the use of animal products in medical devices. Attempts at conjugating functional motifs that mimic the natural ECM – such as RGDS and IKVAV – to self-assembling peptide hydrogels have been extremely successful, and will be discussed in some depth in the following chapter (Gelain et al., 2006). Natural materials (such as collagen, hyaluronan, and chitosan) are advantageous over synthetic materials in that they tend to be naturally biomimetic, cheap, readily available, and non-toxic.

3.3 Three Dimensional Neural Cell Culture

Categorising 3D neural (neurons only) and neuronal (neurons and glia) culture studies depends on the context of the review. In this case, a historical overview will be presented, followed by an in-depth look at specific aspects of hydrogel culture such as mechanical behaviour and functionalisation. One of the first papers to demonstrate the efficacy of 3D neuronal culture was in 1978 by Paul Letourneau at Stanford. Letourneau’s work highlighted the advantages of 3D culture to study neurite outgrowth of chick dorsal root ganglia, initially using a concentration

3.3 Three Dimensional Neural Cell Culture

gradient of NGF (Letourneau, 1978). This study also highlighted the advantages of using hydrogels to maintain a concentration gradient of a chemotrophic factor, and paved the way for many cell and drug encapsulation studies.

Letourneau's experiments were initially succeeded by relatively few studies. For example, in 1993 Boisseau and co-workers used alginate as a cell affinity column, seeding N18 neuroblastoma cells to assess the impact of encapsulation on differentiation (Boisseau et al., 1993). This paper was important as a proof of concept, and demonstrated that neuronal cells were not detrimentally affected by encapsulation. The following decade was relatively slow to adopt the new technique, and it was only until Krewson and colleagues showed that encapsulated PC12 cells remained viable and responded to a slow release of NGF, that the potential of encapsulation was truly realised (Krewson et al., 1994).

Several papers in the 1990s were influential in the development of the field, especially of Bellamkonda, who encapsulated chick DRGs within a well-characterised agarose hydrogel (Bellamkonda et al., 1995). The paper also highlighted the importance of characterising the substrate well; without understanding the material, conclusions about its effect on the cell cannot be made certainly.

In 2000, collagen was first used as a hydrogel scaffold for neuronal precursor cell encapsulation, demonstrating the cells' ability to proliferate and differentiate into neurons and astrocytes (O'Connor et al., 2000). Collagen proved popular as a hydrogel for neuronal cell culture, and was used several times for the culture of cortical neurons (O'Shaughnessy et al., 2003). The same group later demonstrated that the same neural precursors could not only differentiate into a neuronal lineage, but also showed that the cells could form functional neural networks as highlighted

3.3 Three Dimensional Neural Cell Culture

by Figure 3.1 (Ma et al., 2004).

By 2005 hydrogels were becoming a real asset in 3D neuronal cell culture. Several groups reported on the functionality of neural precursors, or differentiated neurons and glia. Mahoney and Anseth, for example, evaluated the functionality of differentiated neural precursor cells in PEG hydrogels that were finely tuned to control the degree of neurite outgrowth (Mahoney and Anseth, 2006). Stabenfeldt *et al.*, in the same year, encapsulated rat cortical neurons in a laminin-1-methylcellulose hydrogel, highlighting the usefulness of laminin in promoting adhesion, although viability within the hydrogels remained relatively low overall (Stabenfeldt et al., 2006). This paper importantly demonstrated that functionalising hydrogels with matrix components such as laminin could enhance neuronal cell adhesion.

Similar studies also showed that functionalisation of hydrogels can enhance neurite outgrowth of encapsulated DRGs (Novikova et al., 2006) and differentiated neurons and glia (Frampton et al., 2011). During this period, composite hydrogels were also being investigated, introducing another level of control. For example, Cullen and co-workers considered the effect of collagen IV–agarose hydrogels on the viability and neurite outgrowth of primary rat cortical neurons (Cullen et al., 2007).

Towards the end of the last decade, bioreactors were becoming commonplace in tissue engineering laboratories. These devices have become more complex in recent years, but fundamentally they consist of a reactor space (which could be of many geometries), a way of moving culture medium through this space (usually with a syringe pump system) and in some cases a way of agitating the culture medium. Microfluidic devices work by similar methods, although the volumes of fluid used are significantly smaller, as is the reactor space (typically in the order of several

3.3 Three Dimensional Neural Cell Culture

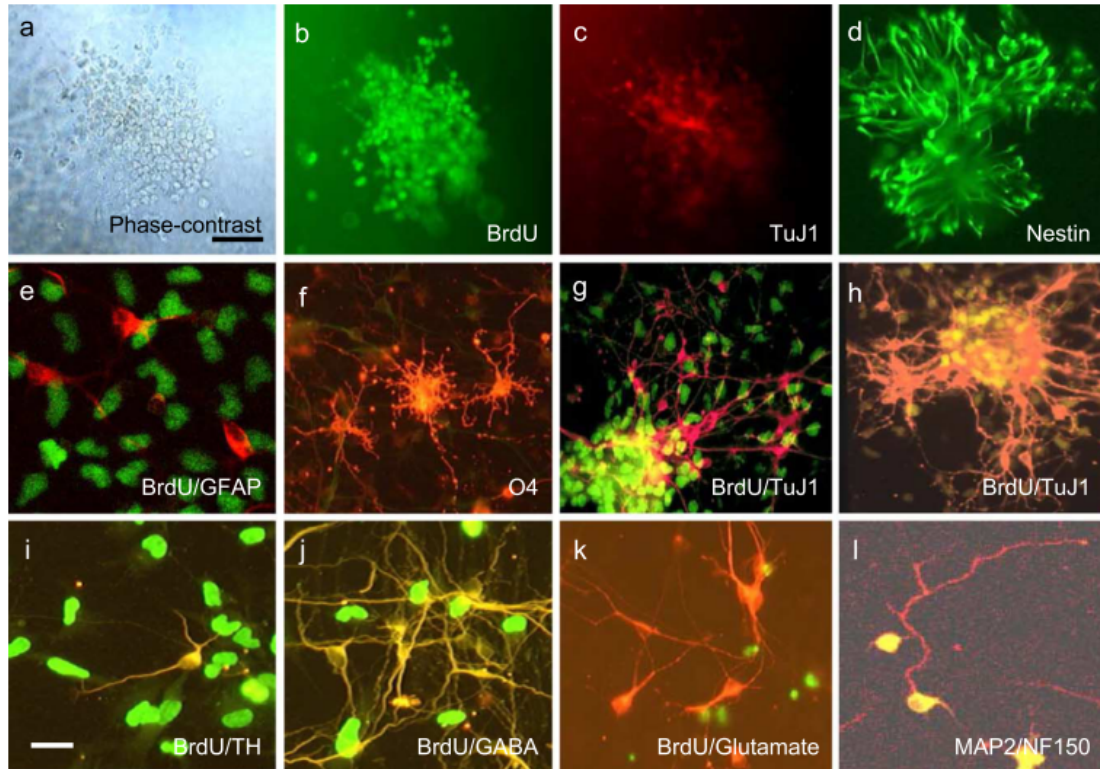


Figure 3.1: Encapsulated cortical and subcortical progenitor cells can be differentiated into neurons and glia. (a–d) After 5 days, cells actively proliferate (BrdU^+ , b and nestin^+ , d), but neuronal cells are few (TuJ1^+ , c). After day 14 (e–l), individual GFAP positive daughter cells are visible (e), as are the oligodendrocytes (O4^+ , f). Cells migrate from cell clusters (BrdU^+ , g, h). Some neurons are TH^+ (i) and BrdU^+ while others are either GABA and BrdU^{++} (j) or glutamate $^+$ and BrdU^+ (k). Neurons stain positive for both dendrites (MAP2^+) and axons (NF150^+ , l). Scale bar (a–d) $150\mu\text{m}$ and (e–l) $50\mu\text{m}$. Adapted from (Ma et al., 2004).

hundred microns). Bioreactors and microfluidic devices could be used to layer neurons and other cell types within hydrogels, introducing spatial complexity which could be finely controlled (Kunze et al., 2011); this study was the first instance of multilayered cortical neurons cultured within agarose hydrogels.

The last decade has rapidly advanced our knowledge of 3D neuronal cell culture in hydrogels. Although many gaps exist, some excellent studies have highlighted

3.4 Hydrogels and their Key Properties

the promise of carefully controlled microenvironments that can be functionalised to precisely influence cellular behaviour. Future studies will certainly have to consider the molecular interactions between a cell and its environment. Rather than concluding that a matrix interaction results in a response, such as differentiation, the specific signalling cascades must be investigated and understood. This is especially important in clinical applications where a material will potentially be delivered into a complex *in vivo* environment. For a review of the role of hydrogels in 3D culture – although not neuronal specific – see (Tibbitt and Anseth, 2009). The reader is referred to Table 3.2 for an overview of the hydrogels used in 3D neural cell culture.

Hydrogels are useful tools, although the subtext of this evaluation must be interpreted liberally. During development, cells own a vast set of developmental tools that allow them to form functional tissues without the influence of their substrate; these two factors should not be thought of as existing in isolation. For example, 3D embryonic stem cell aggregates differentiating along a retinal cell lineage form optic cup-like structures in the absence of a lens or surface ectodermal tissues (Eiraku et al., 2011). This finding suggests therefore, that although external forces are important during *in vivo* development, cells can in fact form tissue structures somewhat unaided *in vitro* if ‘prompted’ in the right way.

3.4 Hydrogels and their Key Properties

It is now widely accepted that the mechanical properties of the ECM can dramatically influence cellular behaviour, including cell adhesion, differentiation, and

Table 3.2: Hydrogels previously used in three dimensional neural cell culture, including the specific hydrogel composition and cell type used. For an accurate description of the cell type and source, the reader is referred to the accompanying reference.

Hydrogel Composition		Cell Type Cultured	Reference
Natural	Agarose	Dorsal Root Ganglia	(Luo and Shoichet, 2004a)
	Agarose–Collagen	Primary Cortical Neurons	(Cullen et al., 2007)
	Alginate	Hippocampal Neurons	(Frampton et al., 2011)
	Collagen	Dorsal Root Ganglia	(Willits and Skornia, 2004)
	Fibrin	Neural Precursor Cells	(Liu et al., 2010)
	Hyaluronan	Striatal Neurons	(Nakaji-Hirabayashi et al., 2009)
	Hyaluronan–Collagen	Neural Stem Cells	(Zhang et al., 2008)
	Hyaluronan–Methylcellulose	Neural Stem Cells	(Tam et al., 2012)
	Matrigel ^a	Neural Precursor Cells	(Uemura et al., 2010)
	Methacrylamide–Chitosan	Neural Precursor Cells	(Leipzig et al., 2010)
Synthetic	Methacrylated–Hyaluronan	Neural Precursor Cells	(Seidlits et al., 2010)
	Mebiol Gel ^b	Neural Stem Cells	(Yang et al., 2009)
	Poly (ethyl acrylate–co–Methacrylic acid	Neural Stem Cells	(Veiga et al., 2010)
	Poly (ethylene glycol)	Neural Precursor Cells	(Lampe et al., 2010)
	Poly (ethylene glycolated) Fibrinogen	Dorsal Root Ganglia	(Sarig-Nadir et al., 2009)
	Poly (glycolic acid)	Neural Stem Cells	(Park et al., 2002)
	RADA16	Neural Stem Cells	(Gelain et al., 2006)

^aMatrigel is a commercial product consisting mainly of laminin, collagen type IV, heparan sulphate proteoglycans and entactin.

^bMebiol gel is a thermo-responsive polymer consisting of conjugates of poly (ethylene glycol) and poly (isopropyl acrylamide).

3.4 Hydrogels and their Key Properties

pathfinding. A recent paper by Leipzig and Shoichet demonstrate that differentiating neuronal precursor cells prefer soft substrates, whereas oligodendrocytes preferentially differentiate on stiffer substrates (Leipzig and Shoichet, 2009). Matrix stiffness has also been linked to neurite extension. Willits and Skornia determined that in very stiff collagen hydrogels, neurites do not advance through the substrate as easily as within softer substrates (Willits and Skornia, 2004). It has also been established that specific cell functions are affected by substrate stiffness. For example, neural stem precursor cell (NSPC) proliferation favours a stiff substrate, whereas differentiation favours a softer substrate (Leipzig and Shoichet, 2009).

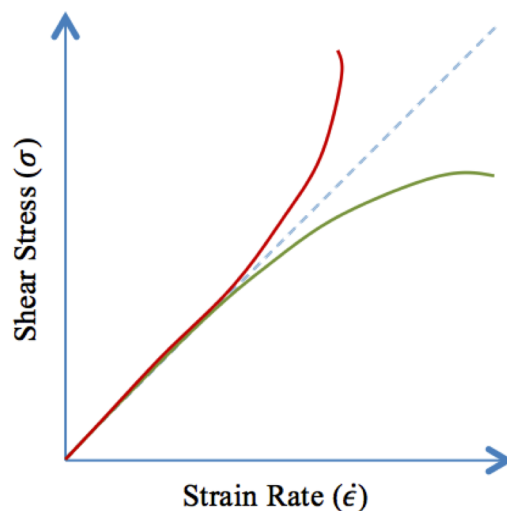


Figure 3.2: The viscosity – represented as the gradients of the above curves – of a Newtonian fluid does not change with increasing shear stress (blue dotted line). Pseudo-plastic materials demonstrate shear-thinning behaviour (green), where the viscosity decreases with shear stress. Dilatant fluids demonstrate shear thickening (red), where the viscosity increases with shear stress. ©Phillipus J. Putter.

Hydrogels are generally regarded as viscoelastic materials that have both viscous and elastic properties. Viscoelasticity of hydrogels is important, as it means that we can control how solid or liquid the material is. Viscosity can be calculated

3.4 Hydrogels and their Key Properties

from a stress/strain curve, which also yields information about the strain rate dependance of different materials (Figure 3.2). Modification of the mechanical properties of hydrogels is essential to the work in this thesis and will be explored extensively. The viscous component is governed by Newtonian fluid mechanics, where the viscosity η of a material is defined by the proportionality of stress σ with the velocity gradient, or strain rate $\dot{\epsilon}$ of the liquid, and ϵ is the strain

$$\sigma = \eta \dot{\epsilon} = \eta \frac{d\epsilon}{dt} \quad (3.1)$$

It can be seen from 3.1 that the shear stress response of a Newtonian fluid is linear with respect to strain rate, and viscosity is independent; as $\dot{\epsilon}$ increases or decreases, η remains the same.

The elastic component of a viscoelastic material is generally governed by Hookean solid mechanics, where the force F of an extended spring is a function of its displacement, x , and its spring constant, k

$$F = -kx \quad (3.2)$$

The spring constant of a material can be found by measuring the relationship between the force exerted on a material and its extension. As discussed above, viscoelastic materials such as hydrogels display properties of both viscous fluids and elastic solids. The response of viscoelastic materials to stress can be better described by rheology; the study of the flow of matter.

3.4 Hydrogels and their Key Properties

3.4.1 Rheology

The response of a material to stress is an important parameter to consider when designing scaffolds for cell encapsulation. Rheological techniques can be used to understand how these materials respond to stress, and can yield useful information including the stiffness, or creep behaviour of the material. As discussed above, hydrogels are viscoelastic, and their response to stress is a function of both Hookean and Newtonian mechanics. In a typical rheological study (see Figure 3.4), a hydrogel is subject to sinusoidal shear strain γ given in Equation (3.3) – where γ_0 is the shear strain amplitude, ω is the oscillation frequency and t is time.

$$\gamma = \gamma_0 \sin(\omega t) \quad (3.3)$$

Due to the viscoelastic nature of these hydrogels, having both Hookean and Newtonian components, the hydrogel responds to this strain by resisting deformation, as shown in Equation (3.4)

$$\omega = G'(\omega)\gamma_0 \sin(\omega t) + G''(\omega)\gamma_0 \cos(\omega t) \quad (3.4)$$

where G' is the storage modulus or Hookean component in which energy is stored within the material, and G'' is known as the loss modulus or Newtonian component, where energy is lost as viscous heat. The combined modulus, or complex modulus, G^* , is given by

$$G^*(\omega) = \sqrt{(G')^2 + (G'')^2} \quad (3.5)$$

3.4 Hydrogels and their Key Properties

It is evident from equation (3.4) that G' is modified by a function of $\sin$, and G'' is modified by a function of $\cos$, which suggests that G' lags behind G'' by a phase angle δ ; i.e. the viscous component responds to stress slower than the elastic component. The ratio of storage and loss moduli is known as the phase angle, $\tan \delta$, where

$$\tan \delta = \frac{G''}{G'} \quad (3.6)$$

The phase angle can be used to assess the extent to which materials are solid or liquid, based on how much the material can store or lose energy. Equations (3.3)–(3.6) were adapted from a paper by Ambrosio (Ambrosio et al., 1999). This is the theory behind the spring and dashpot model of viscoelasticity (Grodzinsky et al., 2007).

Experimentally, a hydrogel behaves much more like an elastic solid. When a hydrogel is crosslinked, there is a very distinct point at which the storage modulus exceeds the loss modulus; these are manifestations of the elastic and viscous components of the hydrogel respectively. After this point, the viscosity of the polymer is almost negligible compared to the elasticity (see figure 3.10b). This behaviour has also been documented by Ghosh *et al.*, who discuss briefly the importance of the G'/G'' cross over point (Ghosh et al., 2005).

3.4 Hydrogels and their Key Properties

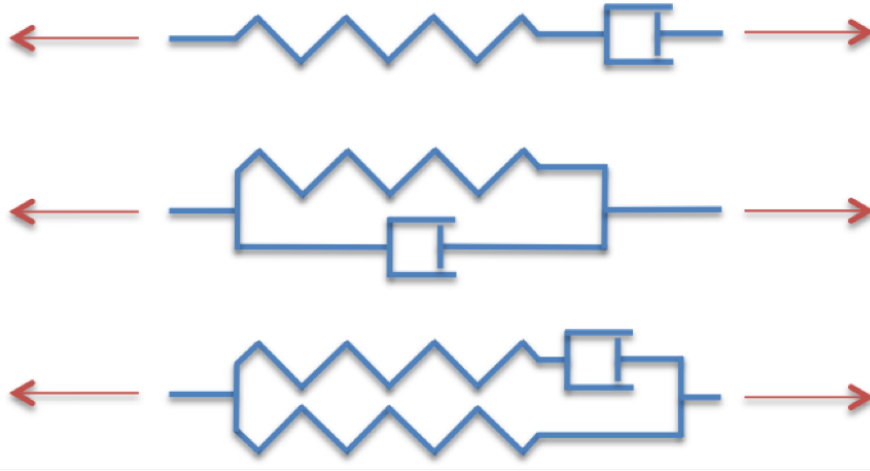


Figure 3.3: From top to bottom, the Maxwellian, Voight, and Kelvin, are variations of viscoelasticity models. As a force is applied (represented by the red arrows), the spring extends by the function $f = kx$. This is followed by the dashpot, which, as a representation of viscosity, follows $\omega = \eta\dot{\epsilon} = \eta\frac{dn}{dt}$. In both the Voight and Kelvin models, the extension of the spring is slowed by the viscous dashpot, which dissipates non-recoverable energy as as viscous heat. ©Phillipus J. Putter.

3.4.2 Relating Bulk Properties to Microstructure

Rheological methods provide an excellent insight into the macroscopic characteristics of hydrogels. Clearly this does not yield much information about the microstructure, as rheology measures the *overall*, or *bulk* response of the material. Relating these bulk properties with the microstructure may enable tissue engineers to tailor a scaffold to a specific cell or tissue type. Anseth and co-workers in an excellent review evaluate the mechanical properties of hydrogels, with extensive reference to mathematical models and their experimental determination (Anseth et al., 1996). The author draws special attention to the contribution of microscopic features of the material to bulk mechanical behaviour.

3.4 Hydrogels and their Key Properties

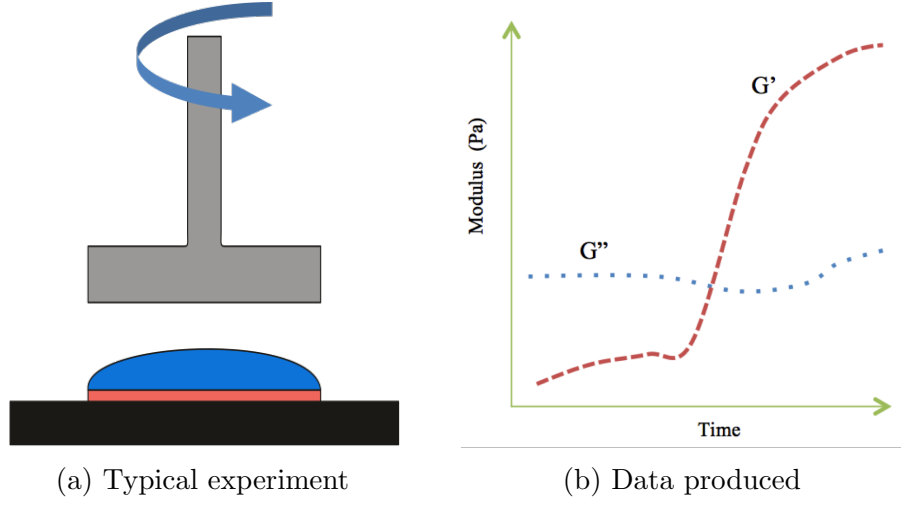


Figure 3.4: (a) In a typical rheology experiment, a plate oscillates, thereby deforming the material. As a result, the material resists the force exerted upon it. (b) Viscoelastic polymers have a viscous and elastic component, related to the amount of energy the hydrogel loses and stores respectively. Before a hydrogel crosslinks, the loss modulus (G'') that corresponds to viscosity dominates. During crosslinking, there is a distinct point in time where the storage modulus (G') (related to the elasticity of the hydrogel) exceeds the loss modulus. ©Phillipus J. Putter.

For example, the shear stress τ , is related to the molecular weight between crosslinks, $\bar{M}_c$, and the molecular weight of the polymer chains prior to crosslinking, $\bar{M}_n$:

$$\tau = \frac{\rho RT}{\bar{M}_c} \frac{\bar{r}_0^2}{\bar{r}_f^2} \left(1 - \frac{2\bar{M}_c}{\bar{M}_n} \right) \left(\lambda - \frac{1}{\lambda^2} \right) \quad (3.7)$$

where ρ is the density of the polymer, T is temperature, R is pressure, λ is the extension ratio, and the quantity $\frac{\bar{r}_0^2}{\bar{r}_f^2}$ is referred to as the front factor, or the ratio of the end-to end distance of chains in a network (Anseth et al., 1996).

3.4 Hydrogels and their Key Properties

The same author also correlates G' directly to crosslinking density, although a more useful derivation is provided by D'Erricot *et al.*:

$$G' \cong \frac{RTc}{M_e} \left(1 - \frac{M_e}{M_n} \right) \quad (3.8)$$

where c is the polymer concentration expressed in g/vol , M_n is the average molecular weight of polymer chains, and M_e is the average molecular weight of the polymer segments between two crosslinking points (D'Erricot et al., 2008). Although this equation provides a useful relationship between modulus and crosslinking density, it is limited, and omits any function of time or more importantly, cell density. We know that encapsulating a cell within a material will alter the mechanical properties of the material, but all of the functions discussed in this section do not provide any indication of what would happen to the micro-(or macro-) structure of the hydrogel if cells were added.

We have seen in this section that the mechanical properties of hydrogels can be modified predictably, but why is this important to tissue engineers? Put simply, controlling the mechanical properties of these materials provides greater control over cellular function. Durotaxis is the study of mechanically-mediated cell movement, synonymous with mechanotaxis. It has been extensively demonstrated that cells can migrate along a gradient of stiffness by mechanotransduction of extracellular forces into the cell. Cells also appear to spread and migrate preferentially on certain moduli (Jiang et al., 2006).

The exact mechanisms of durotaxis have not been completely elucidated, but it is believed that cell surface receptors such as the integrins (see Section 2.5) and

3.5 Microstructural Characterisation Tools

focal adhesion kinases (FAK) are key mediators that pass on signals via several intermediates into the cell (Jiang et al., 2006; Wang et al., 2001). Mathematically this cell-matrix interaction can be modelled to highlight the optimum stiffness for durotaxis, based on the formation and contraction of actin fibre bundles (Harland et al., 2011). There are significant gaps in our knowledge of durotaxis, for example exactly how does a change in stiffness change the expression of specific genes? What are the molecular mediators inside the cell that lead to these changes, and which genes are involved? What is certain is that this mechanism can be utilised to guide and direct neurons in 2D and 3D.

3.5 Microstructural Characterisation Tools

Characterising the microstructure of any material is essential if we are to understand how this structure will affect how a cell behaves. There are few techniques available, which are limited mostly due to cost and infrastructure. For example the technique discussed in this thesis –small angle neutron scattering – requires a multimillion pound particle accelerator and neutron detector; only a handful of these facilities exist in Europe. Other techniques include small angle light scattering (Singh et al., 2011), x-ray diffraction (Ricciardi et al., 2004) and scanning electron microscopy Miller and Peppast (1986). Small angle neutron scattering (SANS) was chosen firstly for its versatility in characterising the microstructure of many materials, but also due to the availability of facilities. Although including other techniques would improve the strength of the data, that the amount of useful data generated by SANS was more than enough for a very thorough discussion.

3.5 Microstructural Characterisation Tools

3.5.1 Neutron Scattering and Microstructure

Neutron scattering is an extremely useful technique that can be used to characterise the properties of numerous materials, including the hydrogels. Although not exhaustive, this brief overview of the background of neutron scattering principles should allow the reader with little or no knowledge of particle physics to understand the key concepts of SANS. It is important to note that the majority of the following background information, including the mathematical functions, were adapted from (Jackson, 2008).

A neutron has both particle-like and wave-like properties, defined by quantum mechanics as the particle-wave duality. In practice, this extremely useful property means that neutrons can also be reflected, refracted, and diffracted (the property measured in SANS), similar to a wave; not only do neutrons behave like waves, these wave-like properties can be used to measure the characteristics of a vast set of materials on the atomic scale. A neutron beam z away from the sample nucleus, with wavenumber $k = \frac{2\pi}{\lambda}$, can be represented by a plane wave with wavefunction

$$\Psi_i = e^{ikz} \tag{3.9}$$

Once refracted by the nucleus of an atom in the sample, the scattered wave at distance r away from the nucleus, and nuclear scattering length b , can be given

3.5 Microstructural Characterisation Tools

by

$$\Psi_s = -\frac{b}{r}e^{irk} \quad (3.10)$$

This concept is graphically represented in Figure 3.5. The refracted neutrons, here represented as a plane wave, are elastically refracted (i.e. no energy is exchanged between the neutron and the nucleus) into a measurable direction and angle, described by Equation 3.10 above.

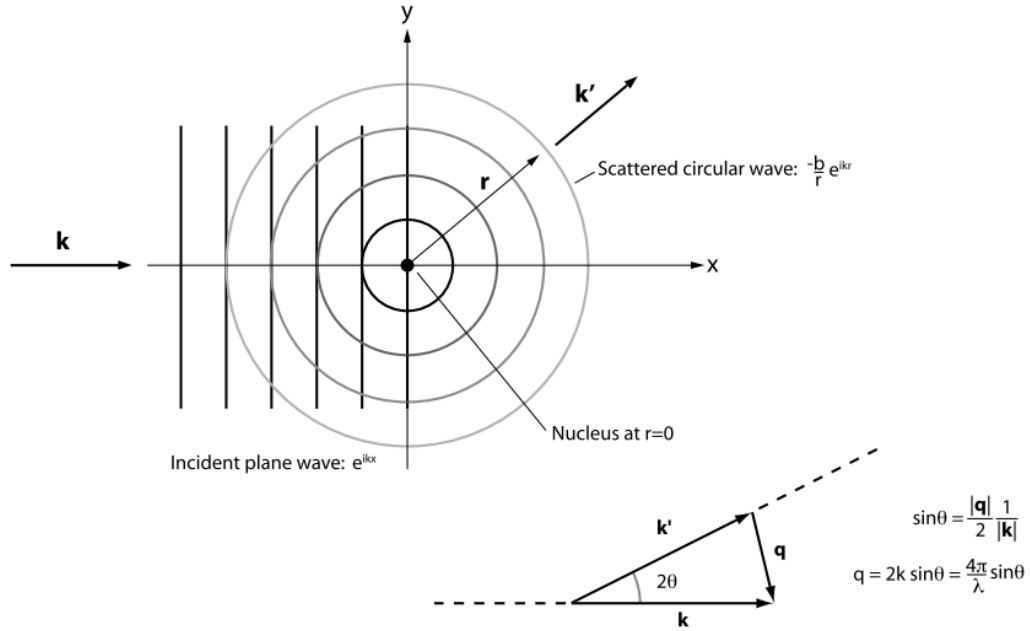


Figure 3.5: Elastic interaction of incident neutrons with a fixed nucleus is simpler to visualise in two dimensions. Here we see the incident plane wave (Equation 3.9) interact with a nucleus at (0,0). The nucleus subsequently refracts the plane wave, which can be described by Equation 3.10. Adapted from (Jackson, 2008).

In reality, however, materials are more complex. Firstly, we must define the refracted plane wave for a three dimensional atomic structure. This can be represented by

3.5 Microstructural Characterisation Tools

the following equation, where $q = k_i - k_s$ and k_i and k_s are the wave vectors of the incident and refracted neutrons respectively

$$\Psi_s = \sum_i \left(\frac{b_i}{r} \right) e^{irk} e^{i\mathbf{q} \cdot \mathbf{r}} \quad (3.11)$$

A neutron detector, therefore, counts the number of neutrons scattered into a known area $d\Omega$, of known direction (θ, ϕ) , known as the differential cross section, and is given by

$$\frac{d\sigma}{d\Omega} = \frac{\text{neutrons scattered per second into } d\Omega \text{ in direction } (\theta, \phi)}{\phi d\Omega} \quad (3.12)$$

where ϕ is the number of incident neutrons per unit area per second, or the *incident flux*. This is the directly measurable quantity in any SANS experiment, and is highlighted in Figure 3.6.

For a collection of nuclei, rather than a single fixed nucleus, the differential cross section found in equation 3.12 becomes

$$\frac{d\sigma}{d\Omega}(\mathbf{q}) = \frac{1}{N} \left| \sum_i^N b_i e^{i\mathbf{q} \cdot \mathbf{r}} \right|^2 \quad (3.13)$$

The wavefunction of the above equation can be given by Equation 3.11. In this form, the differential cross-section is more useful; it is now a function of the scattering vector $\mathbf{q}$.

Characterising HA hydrogels is far more involved than characterising a single atom, or even a collection of atoms. Thus, a more accurate way of representing

3.5 Microstructural Characterisation Tools

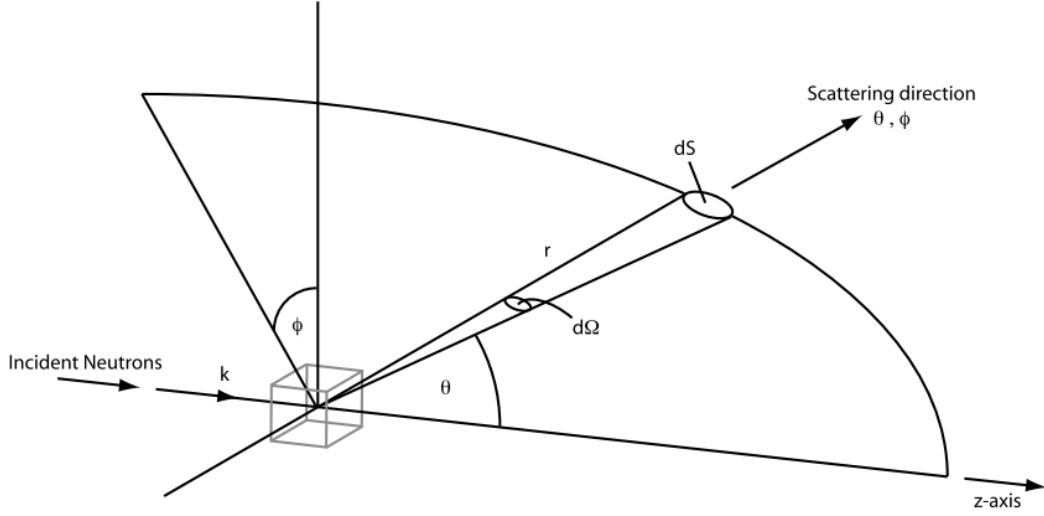


Figure 3.6: The geometric concepts of neutron scattering, showing the scattering cross section $\frac{d\sigma}{d\Omega}$ into which the neutron is scattered. The reader is referred to 3.12 for the mathematical background. Adapted from (Jackson, 2008).

the interaction of a neutron beam with HA, which is a complex system, is by considering the scattering length density ρ of a sample, with scattering length b_i , and n atoms contained within a volume $\bar{V}$

$$\rho = \frac{\sum_i^n b_i}{\bar{V}} \quad (3.14)$$

Taking account of inhomogeneities, where the scattering length density of the inhomogeneities is given by $\rho(r)$ and $\Sigma = \frac{\sigma}{\bar{V}}$ is the microscopic cross section

$$\frac{s\Sigma}{d\Omega}(q) = \frac{N}{V} \frac{d\sigma}{d\Omega}(q) = \frac{1}{V} \left| \int_V \rho(r) e^{iq \cdot r} dr \right|^2 \quad (3.15)$$

Equation 3.15 is known as the Rayleigh-Gans Equation, and considers the homogeneity of a sample in a normalised sample volume by integrating the scattering

3.6 Mass Transfer in Hydrogels

length distribution across the whole sample. This method was adopted in this thesis. The reader is referred to Chapter 5 for specific details of how the models alluded to above can be implemented to yield useful information about the properties of HA hydrogels.

3.6 Mass Transfer in Hydrogels

Mass transfer is a very important aspect of 3D cell culture, and ultimately impacts on the health of an encapsulated cell population. Mass transfer here is defined as the movement of a solute such as glucose through a 3D environment (in this case a scaffold such as a hydrogel). Understanding how molecules essential for healthy cell function move through a material, can allow tissue engineers to develop more efficient culture systems. Additionally, by modelling the mass transfer of molecules such as glucose or oxygen, we can predict the viability of encapsulated cells after implantation during *in vivo* investigations. Mass transfer is also gaining significant traction in the relatively new field of drug delivery, where many groups are attempting to predict the release kinetics of pharmacological agents within 3D scaffolds (Dash et al., 2010; Fu and Kao, 2010).

Modelling the movement of a diffusible solute through a material is inherently complex, and many assumptions are made which are not reflected in reality. For example gradient shapes - a particular pattern produced by diffusible cues in 3D - can influence cellular behaviour in a very unpredictable way. A quantitative analysis by Pujic *et al* shows that the shape and concentration of a guidance cue can vary sometimes unexpectedly with the molecular weight of the molecule in

3.6 Mass Transfer in Hydrogels

question (Pujic et al., 2008). For this reason, a thorough understanding of how a guidance cue behaves in a 3D matrix is essential.

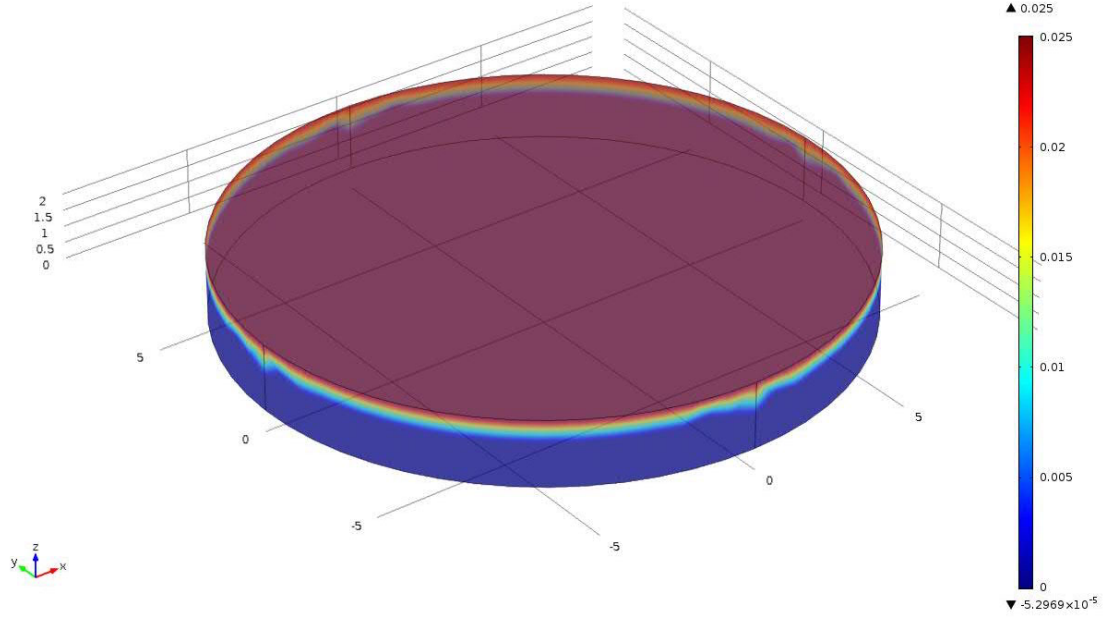


Figure 3.7: A typical finite element computational model of a diffusible cue interacting with a homogeneous hydrogel at an arbitrary time t . Each axis represents a spatial constraint, in this case 0.5mm between adjacent lines on the z -axis, and 5mm between adjacent lines on the x - and y -axis. The colour gradient represents a concentration gradient between $0.025 \times 10^{-5} \text{ Mol m}^{-3}$ (red) and $5.297 \times 10^{-5} \text{ Mol m}^{-3}$ (blue). These data can be used to assess the probability of a diffusible cue interacting with an encapsulated cell. ©Phillipus J. Putter.

Many studies have mathematically evaluated how molecules behave in 3D, for example the effects of hydration and swelling (Barba et al., 2009), or movement through two-phase hydrogels (Hickey et al., 2006). It is generally accepted that for diffusion to be predictable, molecule size and hydrogel mechanical properties must be optimised. He *et al* briefly outline the factors influencing the movement of a chemoattractant through a hydrogel, and highlight some basic mathematical models to predict release kinetics (He et al., 2010; Coviello et al., 2005). Even though

3.7 Functionalisation of Hydrogels

many papers successfully predict the movement of many solutes, they inherently lack complexity that is present *in vivo*. For example, these studies do not consider the effect of encapsulated cells on the diffusion kinetics. For example, will the cell consume the solute? If so, what happens to the concentration gradient with respect to cell density? Figure 3.7 presents an example of a mass transfer study, but as discussed, does not yield any data that are truly relevant to the complex *in vivo* environment. The work presented in this thesis by no means focusses on modelling mass transfer events, but rather recognises the importance of understanding how a solute will get to an encapsulated cells. Diffusion studies such as those presented in Chapter 4 and depicted in Figure 3.7 yield data about, but clearly not limited to:

1. How long it takes for a solute such as glucose or oxygen to reach an encapsulated cell;
2. How long it takes for a waste product such as carbon dioxide to leave the local cellular environment; and the
3. Geometric constraints of scaffolds that allow healthy cell function (i.e. how *big* can a scaffold be before cells die).

3.7 Functionalisation of Hydrogels

The true power of hydrogel culture lies in their ability to be functionalised with molecules that directly interact with a cell or tissue. Biomimicry, here defined as the engineering of artificial cellular environments to more closely resemble the

3.7 Functionalisation of Hydrogels

native cellular niche, is quickly emerging as a field in itself. Tissue engineers are beginning to understand that the complexity of *in vivo* tissue requires a more holistic approach to cell culture. Materials must be adapted with proteins, peptides, and other bioactive molecules to mimic a native tissue during development or adulthood.

Novel experiments have shown that functionalised materials can be tailored to specific applications such as differentiating neural stem cells, or encouraging neurite outgrowth or synaptogenesis of terminally differentiated cells. Recent advances have also enabled tissue engineers to specifically pattern these molecules in 3D, thereby mimicking *in vivo* molecular patterning. Several techniques have been employed to functionalise biomaterials, based usually on a form of covalent interaction between the polymer, and a functional group on the biomolecule. For a thorough text on this topic specifically, that of Greg Hermanson is highly recommended (Hermanson, 2008). In it, Hermanson reviews numerous bioconjugation techniques, including zero-length crosslinkers, cleavable reagents, silane coupling, biotinylation, dendrimers and dendrons, and homo/hetero/trifunctional crosslinkers to name a few (Hermanson, 2008). The techniques employed in this thesis are based on thiol reactions with maleimides, and were chosen mostly for practicality, although the application of this reaction to all peptide based molecules was an attractive feature.

Shoichet and colleagues show that VEGF can be patterned in an agarose hydrogel (figure 3.8). Following cell encapsulation, the data show that neural stem cells can sense these molecular gradients and migrate towards higher concentrations of the growth factor (Aizawa and Shoichet, 2012). The following paragraphs will

3.7 Functionalisation of Hydrogels

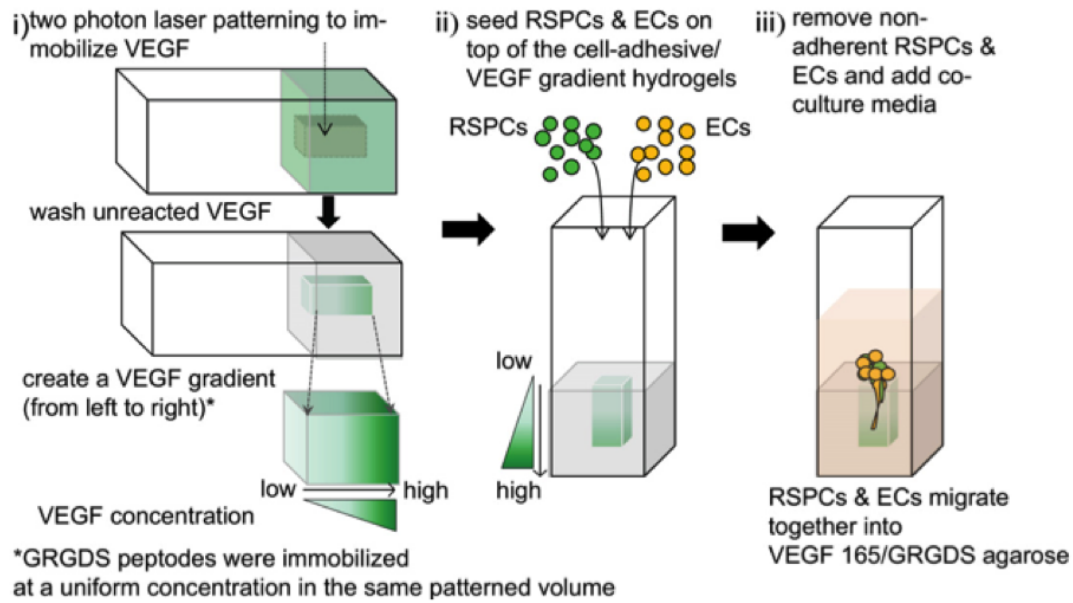


Figure 3.8: A two-photon optical technique empowers tissue engineers to very specifically pattern large proteins within agarose hydrogels, in this case forming a stable and covalently bound molecular gradient of VEGF. Encapsulated retinal stem precursor cells (RSPCs) and endothelial cells (ECs) predictably migrate towards a higher VEGF concentration. Adapted from (Aizawa and Shoichet, 2012).

introduce biomimicry, specifically how neurons or their precursors interact with mimetic peptides of NCAM, reviewed in Section 2.6. The same group demonstrated that immobilised GRGDS (a common peptide sequence that binds to the integrins and mediates cell adhesion) could be used to very finely control the migration of neurites into an agarose hydrogel (Luo and Shoichet, 2004b).

3.7.1 Peptide Mimics of NCAM

Of considerable interest to tissue engineers and developmental neurobiologists is the FG loop (FGL) peptide. This 15aa long peptide is present in the FG loop region on the second fibronectin type 3 module (FN-III) of NCAM. It is essential for

3.7 Functionalisation of Hydrogels

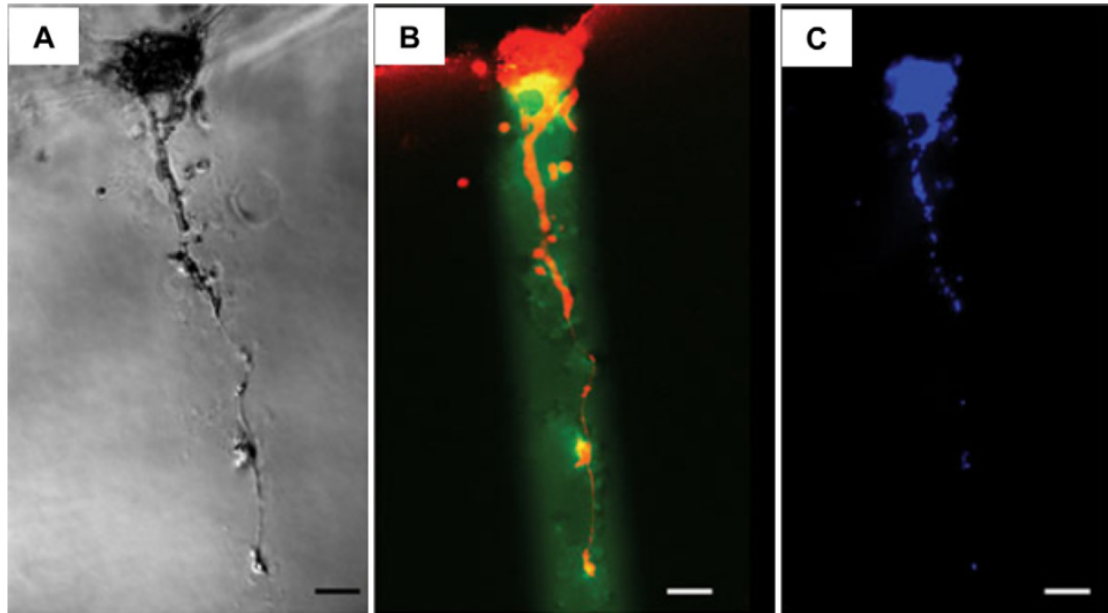


Figure 3.9: Cells from a dorsal root ganglion migrate and extend neurites into agarose channels modified with the adhesive peptide sequence GRGDS. (A) Optical micrograph, (B) fluorescent micrograph showing the modified agarose channel (green) and the cells (red). (C) Nuclei of the cells can be visualised with DAPI (blue). Adapted from (Luo and Shoichet, 2004b).

NCAM-mediated cell adhesion, and heterophilic interactions with several ligands (Kiselyov et al., 2003; Kasper et al., 2000; Sandig et al., 1996). FGL – with the amino acid sequence *EVYVVAENQQGKSKA* – can be synthesised as a tetrameric dendrimer composed of four monomers of FGL attached to a lysine backbone (Neiiendam et al., 2004). Monomeric FGL was also shown to interact directly with FGFr, although much less sensitively when compared to the dendrimeric form (Kiselyov et al., 2003). The FGL sequence directly corresponds to the E681–A695 of the second F3 module of NCAM (Kochoyan et al., 2008), and can therefore mimic a heterophilic NCAM interaction with FGFr.

3.7 Functionalisation of Hydrogels

3.7.2 The Molecular Mechanisms of FGL Binding

FGL binds to the 2nd and 3rd fibronectin Ig module of FGFr via the A686ENQ–QGKS693 binding motif. This motif is similar to the common Walker motif, G/AXXXXGKT/S, where X is any amino acid (Berezin and Bock, 2004; Walker et al., 1982). Adenosine triphosphate (ATP) has emerged as a competitive inhibitor of this binding mechanism, where the ATP binding site on the FGL Walker motif overlaps with that of FGFr (Kiselyov et al., 2003; Berezin and Bock, 2004). ATP has therefore been highlighted as a possible regulator of NCAM–FGFr interactions, by inhibiting NCAM and FGL mediated neurite outgrowth (Kiselyov et al., 2003; Skladchikova et al., 1999). As with all peptides, their efficacy will depend on conformation. An important point to consider with respect to 3D functionalisation is whether, once bound to the polymer, the cell will recognise the sequence. The microstructure of the material becomes very important, and should not inhibit cell–peptide contact.

Upon binding, FGL phosphorylates FGFr, analogous to the heterophilic NCAM–FGFr interaction; FGL can bind to, and activate, both FGFr1 (Kiselyov et al., 2003) and FGFr2 (Christensen et al., 2006). FGL, therefore, does not need to interact with an FGFr–NCAM receptor complex, but can bind directly to FGFr.

The subsequent signalling cascade is complex and has not been fully elucidated, but it is thought that FGL mediated phosphorylation of FGFr initiates the downstream activation of, among others, Raf1, MEK1/2, PKC and PI3K (Klementiev et al., 2007). The same group also demonstrate that FGL inhibits the action of GSK3 β , thought to be an important mediator in chronic stress and ageing (Klementiev

3.7 Functionalisation of Hydrogels

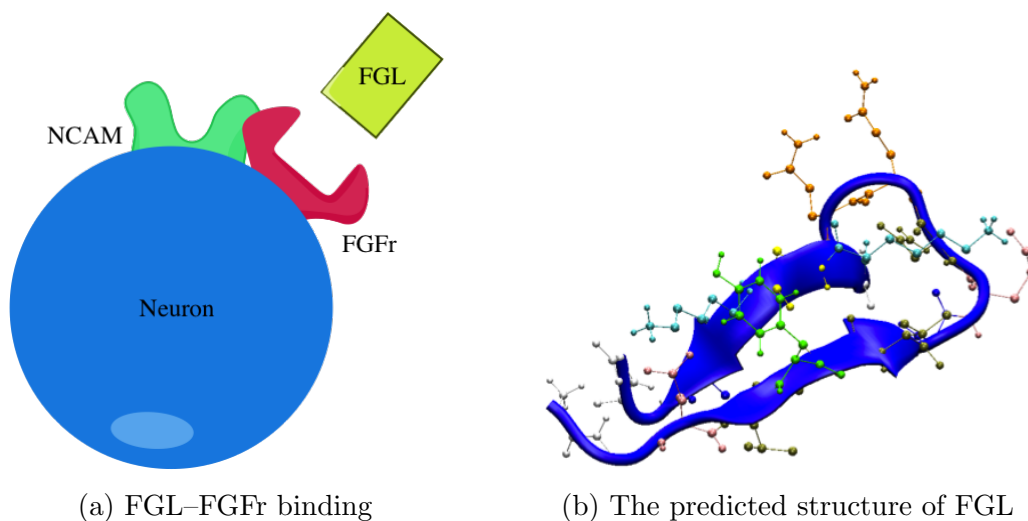


Figure 3.10: (a) The NCAM mimetic FGL binds to FGFr in a manner similar to the heterophilic NCAM–FGFr interaction. Downstream signalling leads to several important mechanisms, including adhesion and neurite outgrowth and pathfinding. (b) The structure of FGL is important for cell–receptor binding, probably via the looped region that exposes important amino acid residues. ©Phillipus J. Putter.

et al., 2007; Borcel et al., 2008). Evidence of FGFr internalisation and recycling is extremely important, and suggests that signal amplification may play an important role in downstream signalling cascades (Francavilla et al., 2009).

Few experimental data exist that convincingly describe what happens when FGL binds to FGFr. Notwithstanding, it has been implicated in the phosphorylation of Erk1/2 and Akt (part of the Ras–MAPK and PI3K pathways respectively) (Neiendam et al., 2004), Src (which is important in migration) (Francavilla et al., 2009), calcium/calmodulin dependent kinase II and IV (which have a role in cognitive function), (Aonurm-Helm et al., 2010), and PLC γ , FRS2 α and ShcA (which are important in neurite outgrowth) (Chen et al., 2010). The latter group also demonstrated that PI3K and Erk1/2 have a role in cell survival and neurite outgrowth (Chen et al., 2010).

3.7 Functionalisation of Hydrogels

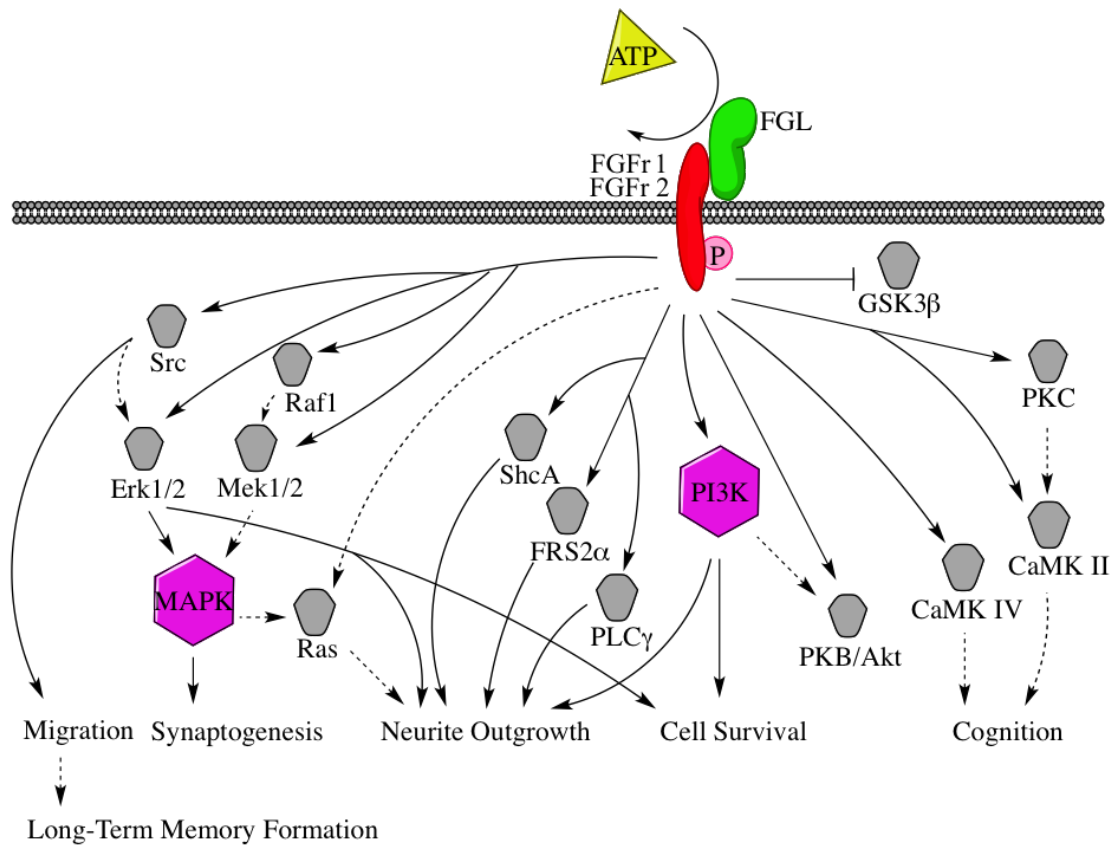


Figure 3.11: A comprehensive delineation of FGL signalling via FGFr. The FGL peptide binds to both FGFr1 and FGFr2, triggering the phosphorylation of several downstream signalling partners. The cellular response to FGL includes migration, synaptogenesis, neurite outgrowth, and cell survival. Solid lines have been confirmed experimentally whereas dotted lines represent hypothesised interactions based on homophilic and heterophilic NCAM data. ©Phillipus J. Putter.

Synaptogenesis and the role of FGL therein have been investigated by Cambon and colleagues. The authors implicate MAPK as a possible mediator (Cambon et al., 2004; Li et al., 2002). MAPK is also believed to affect long term memory formation – mediated by the cyclic AMP response element binding protein (CREB) – and neurite outgrowth, by activating Ras. It is important to note that the effect of FGL on this pathway has not yet been fully evaluated (Jessen et al., 2001; Aonurm-Helm et al., 2008). Recently, FGL has been implicated in the activation of several genes,

3.7 Functionalisation of Hydrogels

including neurofilament, adenylate cyclase activating polypeptide-1 (ACAP1) and the gene for RNA binding protein p45AUF1 (Pedersen et al., 2008).

We have seen that FGL binds to the second and third Ig domains of FGFr, mimicking a heterophilic NCAM interaction. For completeness, it should be noted that two NCAM mimetic peptides – C3 (Ditlevsen et al., 2003) and P2 (Pedersen et al., 2008)–have been identified, which bind to the N-terminal Ig module of NCAM and mimic a homophilic NCAM interaction in contrast to FGL. Insufficient evidence exists linking these sequences to any useful application to date.

3.7.3 FGL influences Cellular Behaviour *In Vitro* and *In Vivo*

FGL has three possible actions on neural cells, although more are being discovered: differentiation, migration and neurite outgrowth/pathfinding. For example, FGL can modulate cell migration by internalising and recycling FGFr (Francavilla et al., 2009). Various forms have been shown to influence the differentiation of hippocampal, dopaminergic, and granular neurons by activating FGFr (Kiselyov et al., 2003; Neiiendam et al., 2004). This mechanism is thought to be similar to that of NCAM and FGFr, which can also lead to neuronal differentiation. FGL was also shown to influence synaptogenesis, although little experimental data exists to explain this finding on a molecular level (Cambon et al., 2004). *In vivo* FGL has been implicated in the modulation of the transcriptional response following traumatic brain injury; FGL seems to increase the expression of factors important for cellular stability and functionality, and reduce those which facilitate

3.7 Functionalisation of Hydrogels

degeneration, including oxidative stress related to neural cell death (Pedersen et al., 2008; Skibo et al., 2005). Several groups are pursuing FGL as a therapeutic, although success has been limited.

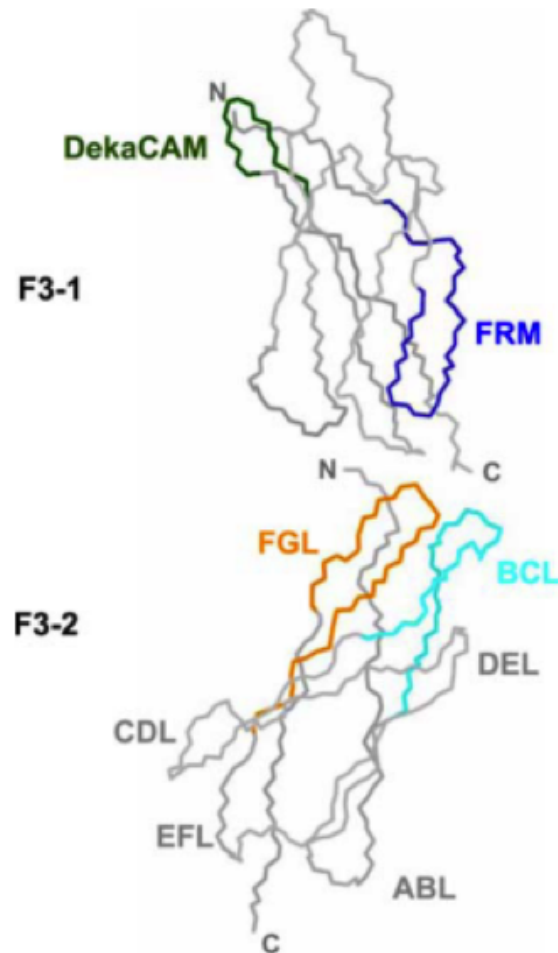


Figure 3.12: The first and second FN-III modules of NCAM, showing the loop regions of FGL, FRM, and BCL. It is interesting to note that all peptides form a loop structure that is thought to interact directly with the cell surface receptor FGFR. Adapted from (Jacobsen et al., 2008).

3.7 Functionalisation of Hydrogels

3.7.4 FGL and Biomaterial Functionalisation

Two peptides have been identified with similar effects on FGFr: FRM (a peptide from the first FN-III module of NCAM), and BCL, a sequence encompassing the B and C beta strands derived from the second FN-III module of NCAM (see figure 3.12). Both sequences have been shown to promote neurite outgrowth and cell survival via FGFr dependant interactions (Jacobsen et al., 2008). Little is known about these sequences, but they are believed to contribute to the complex heterophilic FGFr–NCAM interaction alongside FGL, which are essential for adhesion and migration as outlined above (Anderson et al., 2005; Carafoli et al., 2008). Cyclisation of the mimetic peptides (in this case FRM) may also increase the potency of the peptide, suggesting that a loop-like structure could be important for proper FGFr function (Anderson et al., 2005). For a thorough review of the synthetic NCAM derived peptides, including FGL, FRM and BCL, the reader is referred to (Hansen et al., 2010).

Few studies have attempted to conjugate FGL to a polymeric scaffold. Zou *et al* recently enriched the RADA–16 peptide sequence with FGL. The authors demonstrate that a 1:1 blend of RADA–16:FGL–RADA forms a hydrogel onto which rat dorsal root ganglia can adhere and extend neurites (Zou et al., 2010). Zhang and colleagues functionalised a peptide amphiphile with the FGL sequence, producing FGL–PA. It should be noted, however, that the source of the experimental data is questionable, although they appear to demonstrate that a biocompatible hydrogel of FGL-PA can be formed (Zhang et al., 2009).

3.7 Functionalisation of Hydrogels

There is a significant lack of data to support the efficacy of FGL on cellular behaviour in a 3D environment. This is surprising when considering the attention given to this molecule in 2D. FGL has some extremely interesting effects, that will certainly be of use to clinicians, but many gaps in our knowledge remain. For example, will the conformation of FGL be different in 3D than in 2D, and will cells be able to bind to the sequence accurately?

3.8 Summary

In the last decade, tissue engineering has progressed into a much more specific and targeted discipline. Hydrogels provide a versatile starting block for three dimensional cell culture of neurons and glia, which can be modified chemically and mechanically to suit a range of applications.

Of particular importance is the functionalisation of hydrogels with molecules that activate very specific signalling cascades. These cascades could ultimately influence complex cellular functions such as migration, differentiation and neurite outgrowth. FGL, and other mimetic peptides of NCAM, can provide a platform from which countless biological questions can be addressed. Utilising functional hydrogels for 3D neural and glial cell culture will enable the mimicry of complex tissues with structural and functional hierarchy; after all, the brain exists in a state of structural and functional hierarchy during development and maturation. It should be noted however, that characterisation of these functional matrices is essential if the application is to have any significant standing in clinical medicine. Clinical approval for a functional hydrogel by the FDA (or any other regulatory body) will require extensive data proving the safety and efficacy *in vivo*.

Lastly, modern techniques (based on very precise optical methods) are beginning to provide an additional level of control. With the aid of some intelligent chemistry, even large molecules and proteins can be immobilised in very specific patterns in 3D. In the context of brain biomimicry this is an important step; the embryonic brain relies on patterns of molecules and proteins in order to develop properly.

Chapter 4

Synthesis and Characterisation of Hyaluronan Hydrogels

4.1 Introduction

Polymers can offer exceptional control over the microenvironment in which an encapsulated cell resides. Chapter 3 extensively outlined the use of hydrogels in 3D neuronal cell culture, from mechanical behaviour to functionalisation. Chapter 4 introduces the first of four experimental chapters in this thesis, where the synthesis and characterisation of HA hydrogels are thoroughly investigated.

Hyaluronan is a versatile linear non-sulfated glycosaminoglycan, and is a major ECM constituent of most tissues. This polysaccharide has been implicated in several physiological mechanisms, including inflammation, tissue regeneration, embryonic development, and cellular proliferation (Solis et al., 2012). HA can also be functionalised to form covalently crosslinked hydrogels (Nimmo et al., 2011). Although several polymers were considered, HA was chosen not only for its presence in the neural ECM, but practically it is inexpensive, readily available and simple to crosslink and functionalise.

Characterisation of a potential scaffold is of significant importance to tissue engineers who aim to mimic the native environment closely. Chapter 4 importantly aims to demonstrate the highly adaptable nature of HA hydrogels. As we will see, a variety of properties such as stiffness, porosity, and diffusion can be optimised for a specific application, from neurite outgrowth, to migration and differentiation. We show here for the first time that fully crosslinked HA hydrogel stiffness is stabilised by neuronal cells. Advanced characterisation tools are also used to elucidate the effect of polymer concentration on crosslinking HA hydrogels.

4.2 Materials and Methods

4.2.1 Synthesising and Crosslinking Hyaluronan–Thiol Hydrogels

HA was purchased pre-functionalised with a thiol group (HA-t; Glycosan). The lyophilised powder was initially reconstituted to a working concentration of 1% w/v with sterile degassed H₂O as per manufacturer’s recommendations, and placed on a rocker at room temperature to dissolve fully. Similarly, poly (ethylene glycol) (PEG) was purchased pre-functionalised with acrylate groups, producing PEG-diacrylate (PEG-DA; Glycosan). The lyophilised powder was reconstituted to a final concentration of 3400g mol^{-1} with degassed H₂O as per the manufacturer’s recommendations. PEG-DA was inverted several times to allow the solid to fully dissolve.

Once both constituents were fully dissolved, they were combined in sterile conditions in a volumetric ratio of 4:1 HA:PEG-DA (i.e. 0.5ml PEG-DA was added to 2ml HA-t) and carefully mixed by pipetting, avoiding bubble formation. Any bubbles were centrifuged briefly prior to use. This 4:1 ratio produces a 1% w/v hydrogel. Depending on application, hydrogels were prepared in well plates, or on coverslips, and allowed to fully crosslink for 35mins at 37°C in a humid incubator with a sterile dH₂O border to reduce evaporation. This reaction is highlighted in Figure 4.1.

4.2 Materials and Methods

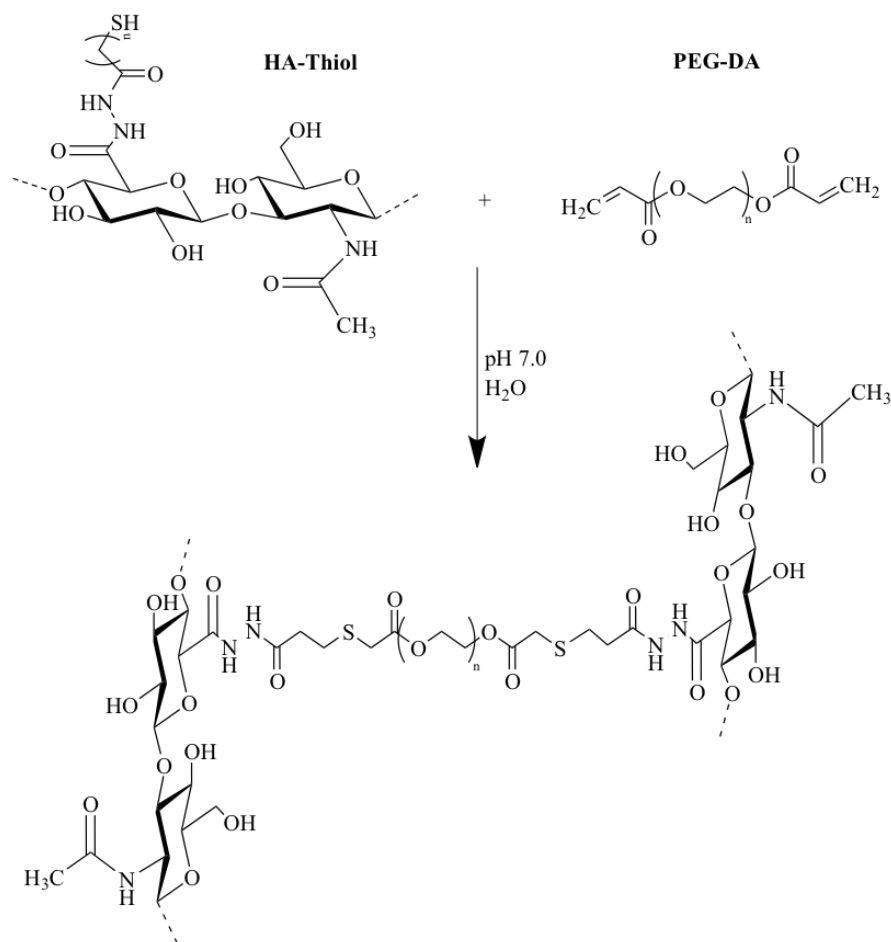


Figure 4.1: Hyaluronan is functionalised with a thiol group that subsequently reacts by a Michael-Type reaction with poly (ethylene glycol)-diacrylate (PEG-DA). The reaction is complete after approximately 30mins, forming a stable hydrogel at pH 7.0.

4.2.2 Synthesising Hyaluronan–Furan Hydrogels

To utilise the Diels-Alder click reaction, sodium hyaluronan (Lifecore Biomedical) of 245kDa was dissolved by continuous stirring in 0.1M 2-(N-morpholino) ethanesulfonic acid (MES; Sigma) at room temperature and pH5.5 for 2hrs.

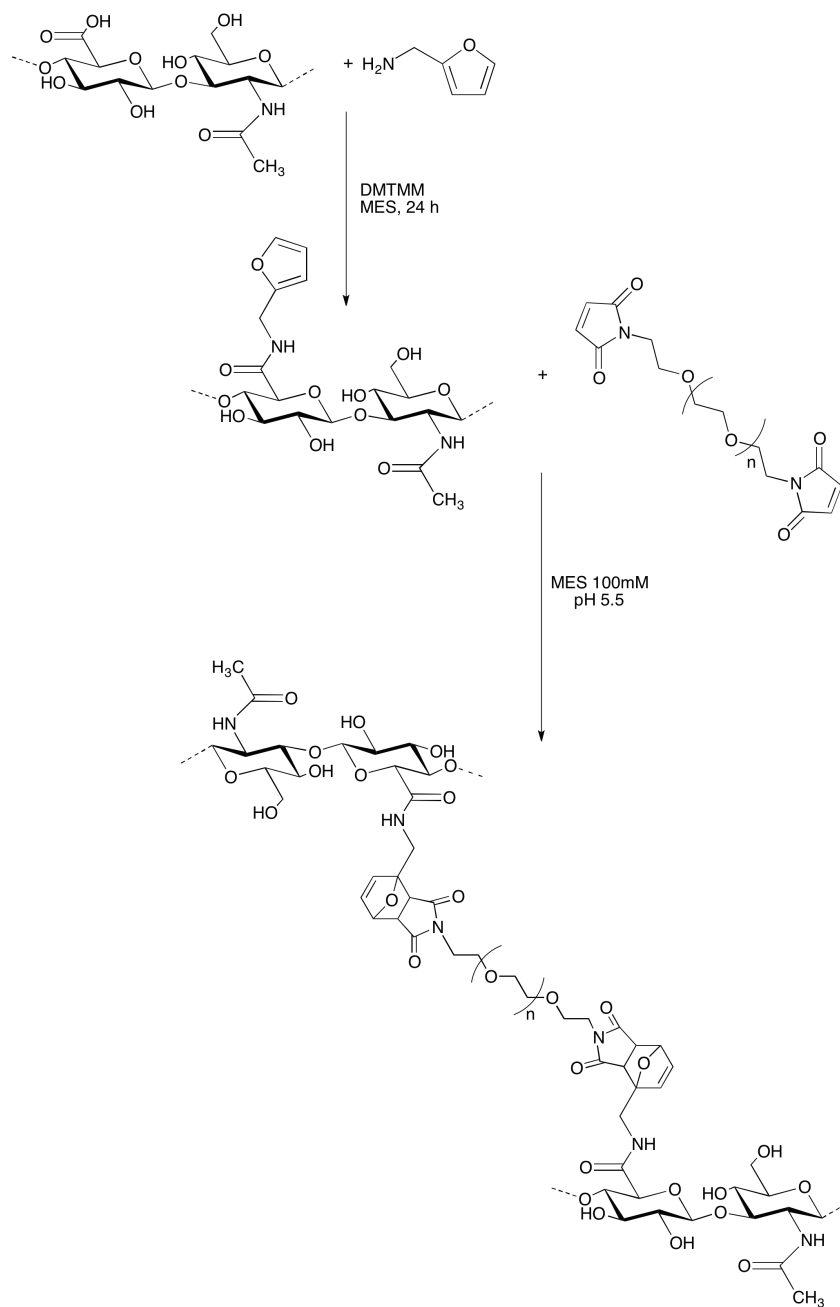


Figure 4.2: Hyaluronan was functionalised with furfurylamine before crosslinking with bis-maleimido-PEG at pH5.5. A stable hydrogel is formed, although a significant neutralisation step is required prior to cell culture. Additionally, the lengthy crosslinking time rendered this hydrogel unsuitable for direct encapsulation studies, as the cells would not remain viable without culture medium.

4.2 Materials and Methods

Once dissolved, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM; Sigma) was added in a 1 molar ratio of DMTMM relative to -COOH groups on HA. To this, furfurylamine (Acros Organics) was added drop wise at a 0.5 molar ratio relative to -COOH groups on HA. Reagents were stirred in a round bottom flask at room temperature for 24hrs. After 24hrs, HA-f was dialysed by a 12-14kDa MW cutoff membrane (Spectrum Labs) in 0.1M NaCl (Sigma) for 24hrs, followed by 48hrs in pure dH₂O; water was changed twice a day. After 72hrs, the polymer was passed through a 0.22 μ m syringe filter, frozen in liquid N₂ and lyophilised until completely dry.

Proton nuclear magnetic resonance (NMR) was used to assess the degree of substitution (DS) of HA with HA that has been functionalised with a furan group. Briefly, HA-f was dissolved thoroughly in deuterium oxide (D₂O) at a concentration of 1mg ml⁻¹, and sterile filtered if this step was not performed earlier. Tubes were cleaned and loaded into a Varian Mercury 500MHz spectrometer (Agilent), detecting ¹H protons at 298K. To calculate the DS, the ratio of the areas under the proton peaks at 6.26, 6.46 and 7.65 ppm (furan protons) to the peak at 1.9 ppm (methyl group protons of HA) were compared. Raw data is represented as a ¹H spectrum with chemical shift in parts per million (ppm) on the *x*-axis (Figure 4.3). As an example, in the following figure we average the furan integral $\frac{(0.26+0.25+0.35)}{3} = 0.2867$ and divide the methyl group integral of N-acetyl glucosamine by 3 (as there are 3 hydrogen protons) yielding $\frac{3.00}{3} = 1.00$. The DS can now be calculated as a ratio of these integrals respectively: $\frac{0.2867}{1.0} = 0.2867$. Thus the degree of substitution for this reaction is **28.7%**. In this case, the DS is very low and would either be reacted further or blended with another macromer with a higher DS.

4.2 Materials and Methods

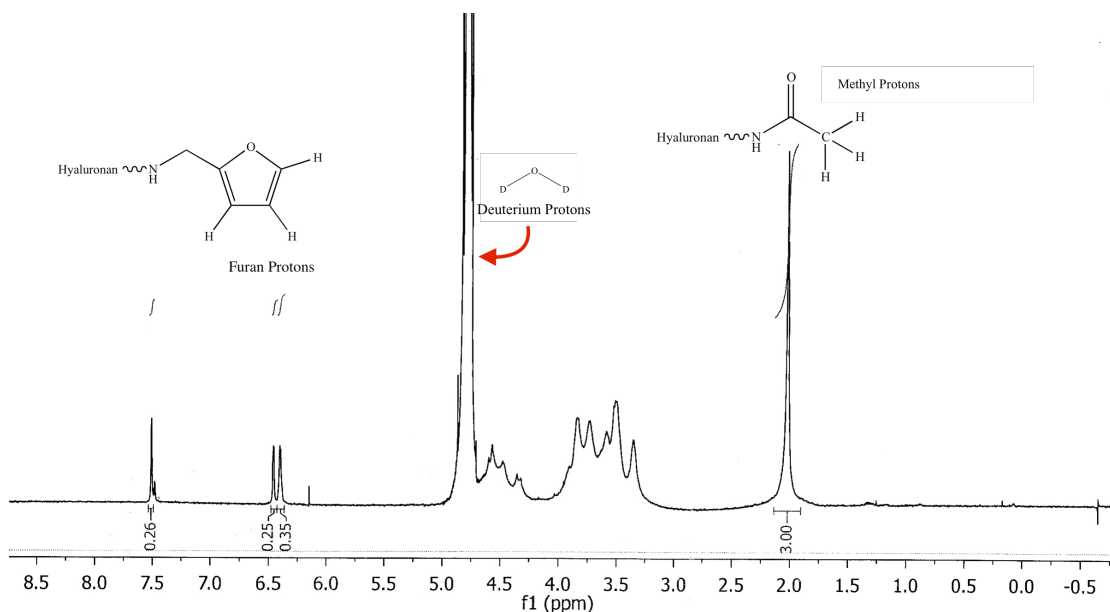


Figure 4.3: Nuclear magnetic resonance (NMR) was used to calculate the degree of substitution of HA and furan. This calculation hints at the efficiency of the functionalisation of HA, and hence how much crosslinker to add to form the hydrogel. Hydrogen peaks at 6.26, 6.46 and 7.65ppm refer to furan protons, whereas the peak at 1.9ppm refers to the hydrogen protons of of the methyl group on HA.

4.2.3 Hyaluronan–Furan Crosslinking and Hydrogel Formation

To form a sterile hydrogel, HA-f was crosslinked by bis-maleimido-poly (ethylene glycol) ((MI)₂-PEG; RAPP Polymere GmbH) at a 1 molar ratio of (MI)₂-PEG to furan. Hydrogels were plated in 96, 48 or 24 well plates, and allowed to fully crosslink for 24hrs at 37°C in a humid incubator, with a sterile dH₂O border to reduce evaporation. After 24hrs, hydrogels were washed in sterile dH₂O (3x30mins) and complete medium to neutralise prior to cell culture. Hydrogels must be neutralised to prevent cell death or apoptosis, which was observed in early experiments. For an overview of this reaction, the reader is referred to Figure 4.2.

4.2 Materials and Methods

4.2.4 Synthesising Hyaluronan–Methylcellulose Hydrogels

Sodium hyaluronate (HA) of 245kDa (Lifecore biomedical) was dissolved in pure dH₂O by continuous stirring for 2hrs at room temperature. After 2hrs, the solution was filtered through a 0.22 μ m syringe filter, frozen in liquid N₂, and lyophilised until completely dry. Methylcellulose (MC; Shin-Etsu, Japan) was similarly dissolved in dH₂O by continuous stirring for 2hrs at room temperature. After 2hrs the macromer solution was filtered by a 0.22 μ m syringe filter, frozen in liquid N₂ and lyophilised until completely dry. It should be noted that the lyophilisation tubes were capped with a 0.22 μ m filter to keep the contents as sterile as possible.

4.2.5 Hyaluronan–Methylcellulose Hydrogel Formation

To form the hydrogel, HA and MC were both weighed on an analytical balance in a Class II HEPA filtered hood. Although the ratio of HA to MC can be varied depending on application, a 50%w/w of each component was generally acceptable in most cases (i.e. half HA and half MC by mass). Complete medium (depending on cell type) was added to form a HAMC solution of 6mg ml⁻¹, or 0.60%w/w, which was gently agitated overnight at 4°C.

The dissolved solutions were mixed vigorously on a speed mixer for 20secs at 3500RPM. Tubes were centrifuged briefly to remove any bubbles, and cooled on ice to minimise any structural damage caused during the mixing process. This process was repeated four times in total. HAMC hydrogels were diluted to a final concentration of 0.75% w/v with complete medium for routine culture and plated (along with cell type; see following Chapters) in 96, 48 or 24 well plates. Hydrogels

4.2 Materials and Methods

were allowed to crosslink – with the cell or explant – fully at 37°C in a humid incubator with a sterile dH₂O border to reduce evaporation. For cell culture see following methods. Although Figure 4.4 suggests that there may be a physical crosslink between adjacent oxygen atoms, in reality the majority of the stability of this hydrogel will be a result of hydrogen bonding.

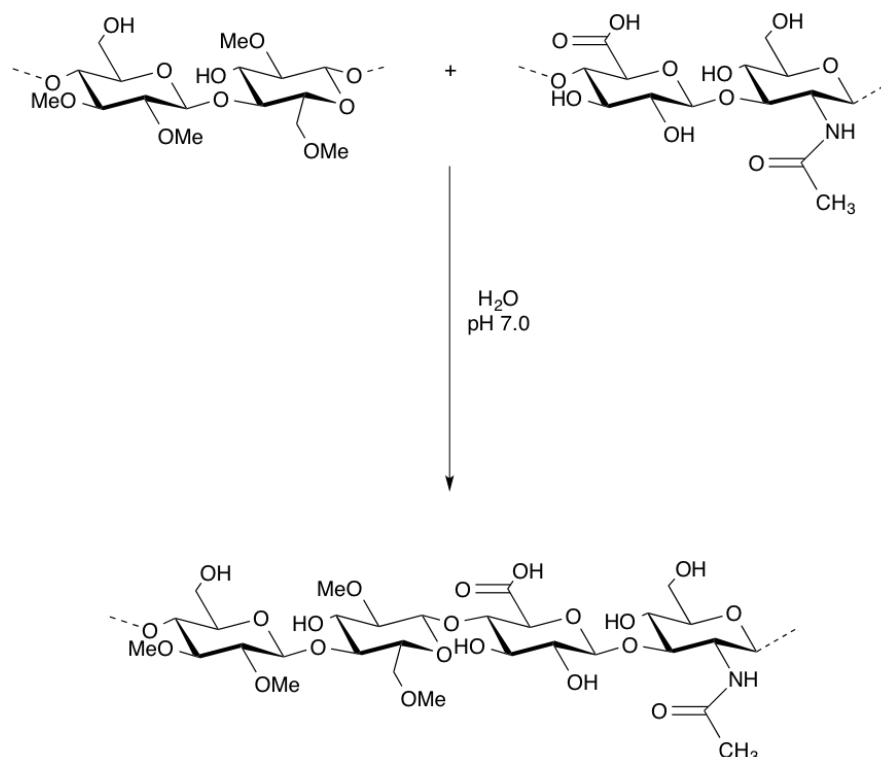


Figure 4.4: Hyaluronan and methylcellulose can be blended to form a physically crosslinked hydrogel. This process requires no additional reagents, which simplifies the reaction greatly, but produces a very weak hydrogel.

4.2 Materials and Methods

4.2.6 Rheology

Assessment of Gelation Time

To determine the gelation time of HA-t hydrogels, the above solutions were analysed with a Physica MCR 301 rheometer (Anton Paar), with an 8mm or 25mm parallel plate at 37°C. Crosslinker and macromer solutions were blended by gentle pipetting, and solutions of various concentrations ranging from 0.25-1.00% w/v were transferred to a pre-warmed rheometer base plate. A time sweep was performed at a constant strain of 5% and oscillations of 1Hz, and terminated after 30mins. The crossover point of the storage and loss moduli were interpreted as the point of gelation, as used by Ghosh and others (Ghosh et al., 2005). To facilitate analysis, a graph of time against storage and loss moduli was plotted on the RheoPlus[®] software package (Anton Paar).

Assessment of Storage and Loss Moduli

To determine the storage (G') and loss (G'') moduli of HA-t hydrogels, solutions were prepared by blending PEG-DA and HA-t at macromer concentrations ranging from 0.25-1.00% w/v diluted with degassed H₂O. The stiffness of the hydrogel was also modified by changing the crosslinker concentration from the working concentration of 20%v/v (equivalent to a 1% w/v hydrogel) to 10%v/v. This was accomplished simply by reducing the volume of PEG-DA in solution. Approximately 0.5ml of the various hydrogel concentrations were allowed to crosslink fully on 25mm Thermanox[®] coverslips (Nunc) for 8hrs at 37°C.

After 8hrs, hydrogels were soaked in DPBS for 2hrs to allow for swelling. Coverslips

4.2 Materials and Methods

were fixed to the rheometer base plate by double-sided tape to prevent slip. A 25mm parallel plate (Anton Paar) was lowered onto the hydrogel, and any remaining surface water was removed by means of blotting tissue. To determine storage and loss moduli, a stress sweep was performed at a constant strain of 5% and oscillations of 1Hz, between 0.05 and 5000Pa at 37°C. Plots of G' and G'' against shear stress were plotted on RheoPlus®. If within the linear viscoelastic region, storage modulus should remain relatively constant, although slip may be present at high shear stress.

4.2.7 Mass Transfer Modelling

To model mass transfer mechanisms within HA hydrogels, an application specific geometry (for example a 24/48/96 well plate) was defined in 3D in the COMSOL Multiphysics® platform. The time dependent transport of diluted species framework was adopted, where the transport reaction is R_i (how quickly the solute is being transported through the system), c_i is the initial concentration, t is time, ∇ the nabla operator, u is the domain velocity and D_i is the diffusion coefficient given by Equation 4.1. A more useful derivation can be given by Equation 4.2, where the flux (amount of salute per area per time) is N_i .

$$R_i = \frac{\partial c_i}{\partial t} + \nabla \cdot (-D_i \nabla c_i) + u \cdot \nabla c_i \quad (4.1)$$

$$N_i = D_i \nabla c_i + u c_i \quad (4.2)$$

4.2 Materials and Methods

The bulk material diffusion coefficient was defined (depending on the hydrogel), and a concentration difference was introduced on either side of the geometry. To better resolve the diffusion length scale, the Einstein relation was adopted, where L is the length scale of diffusion and D is the diffusion coefficient

$$L \sim 2\sqrt{(Dt)} \quad (4.3)$$

The diffusion coefficient of glucose was adapted from Geankoplis as $0.697 \times 10^{-9} \text{m}^2 \text{s}^{-1}$ (Geankoplis, 1993) that is not dissimilar to a HAMC blend hydrogel (Baumann et al., 2009). HAMC was used because it was the softest hydrogel, thereby serving as a useful control for stiffer hydrogels. A finite element mesh was selected and the computation was run between 0-86400secs (0-24hrs) with a 60sec time step. Representative images are presented as a colour spectrum of concentration.

4.2.8 Neural Cell Culture

SY5Y Cell Encapsulation and Differentiation

The SY5Y cell line was gifted generously by Dr. Julian George at the Institute of Biomedical Engineering, Oxford. Cells were initially expanded in normal medium consisting of RPMI 1640 (Sigma), supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 1% MEM (Sigma). Cells were passaged by scraping after reaching approximately 80% confluence. Culture media were changed every 2-3days. Once the appropriate cell number was reached, cells were scraped and counted with a haemocytometer in the presence of Trypan blue.

4.2 Materials and Methods

Cells were encapsulated in HA-t hydrogels at a PEG-DA concentration of 0.12% v/v by diluting the macromer with dH₂O. Hydrogels were transferred to Thermanox coverslips and crosslinked at 37°C for 40mins, and covered in normal medium or differentiation medium supplemented with 100 μ M retinoic acid. Cells were maintained for 1, 7 or 14 days with medium changes every 2-3 days. Hydrogels were characterised mechanically on an Anton Paar Physica rheometer by performing a stress sweep between 1-100Pa with a constant 5% strain rate at 1Hz.

4.3 Results and Discussion

4.3.1 Control of Hyaluronan–Thiol Crosslinking

It has been suggested that the crossover point of the storage and loss moduli (G' and G'' respectively) of viscoelastic materials is analogous to the point where the material crosslinks (Ghosh et al., 2005). To evaluate the time taken for hyaluronan–thiol (HA-t) hydrogels to crosslink, hydrogels of 1, 0.75, 0.50 and 0.25% w/v HA-t, diluted in dH₂O, were mixed with the thiol reactive crosslinker poly (ethylene glycol) Diacrylate (PEG-DA). A time sweep was performed at a constant strain of 5% and 1Hz, at 37°C.

Estimating the time taken for a hydrogel to form is essential when working with cells or tissue explants. There is a limit to how long a cell will remain viable without culture medium, and keeping this duration to a minimum is important. We can clearly see from Figure 4.5 that HA-t concentrations as low as 0.25% w/v crosslink after approximately 21mins. At this point, the storage modulus of the hydrogel exceeds the loss modulus. Viscoelastic theory infers that a hydrogel responds to stress as a function of both how much energy the hydrogel stores and loses, where G' is the storage modulus, or Hookean component, where energy is stored and G'' is the loss modulus, or Newtonian component, where energy is lost as viscous heat. These moduli are in turn functions of angular momentum ω , shear strain γ_0 and time t

$$\sigma = G'(\omega)\gamma_0 \sin(\omega t) + G''(\omega)\gamma_0 \cos(\omega t) \quad (4.4)$$

4.3 Results and Discussion

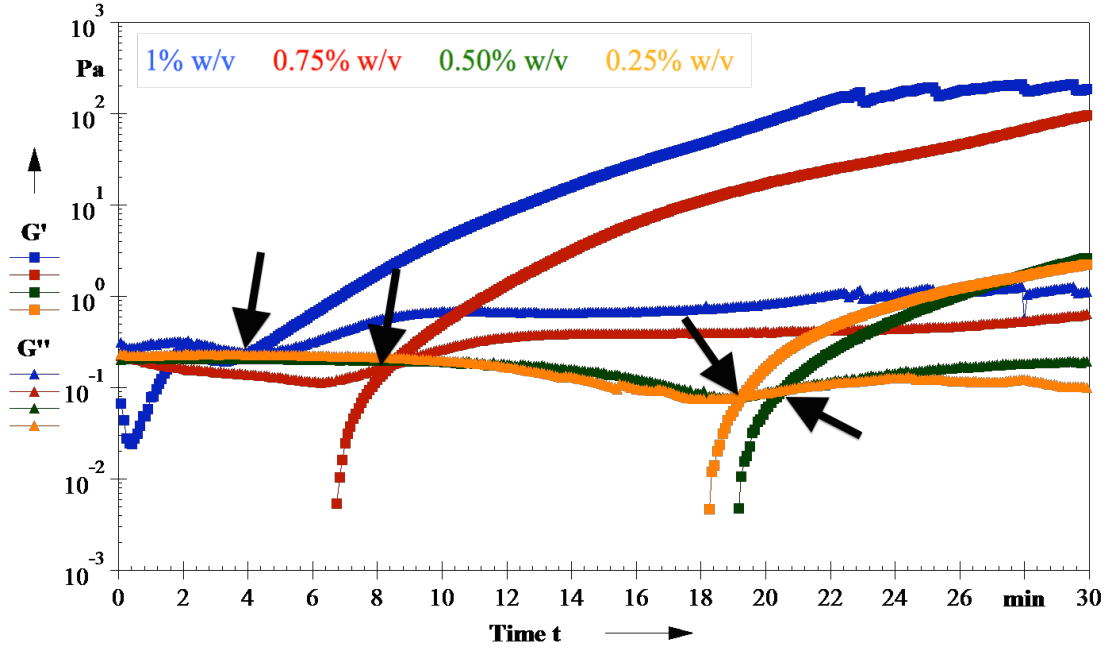


Figure 4.5: Many theorists consider the point at which the storage modulus exceeds the loss modulus to be the point of gelation (black arrows). Reducing the concentration from 1% w/v to 0.25% w/v HA clearly increases the time taken for the hydrogel to form, as expected.

At the point where the storage modulus exceeds the loss modulus, the hydrogel is more solid than it is liquid. As suggested by Ghosh and colleagues, this overlap can be thought of as the crosslinking point (Ghosh et al., 2005). Although this method can be a useful indicator of crosslinking, it should be noted that once the rheometer plate was lifted, the hydrogel retained some viscous properties. An inverted test tube test (where the crosslinking solution is inverted every 5 minutes in a transparent tube until flow stops) would confirm that in practice, it takes longer for a hydrogel to crosslink than these data suggest. The storage/loss modulus crossover could be thought of as the point at which crosslinking begins to affect the mechanics of the gel, rather than the point at which crosslinking is complete.

4.3 Results and Discussion

This is an important finding: although rheology suggests that the material is solid, in practice it will not support a cell, and requires more time for covalent bonds to form between PEG-DA and HA-t. The manufacturer suggests a crosslinking time of 25-30mins at 1% w/v HA, significantly higher than the above time of approximately 4mins. Clearly this calls into question the viability of G' and G'' as an indicator of gelation, often used in other papers.

4.3.2 Controlling the Stiffness of Hyaluronan–Thiol Hydrogels

A simple and effective way to control the stiffness of a hydrogel, is to change the concentration of the crosslinker or the uncrosslinked polymer (or macromer). Depending on the average length of the polymer chains, this can be done simply by adding more water to the macromer (in this case hyaluronan), or by increasing or decreasing the crosslinker concentration. In effect, this is changing the molar ratio between the polymer and the crosslinker. To assess the differences between both methods, a stress sweep was performed at a constant strain rate of 5% and 1Hz with an Anton Paar Physica MCR 301 rheometer at 37°C.

Figures 4.6 and 4.7 demonstrate that the stiffness of a HA hydrogel can be modified predictably by changing the water content of the hydrogel, thereby diluting the macromer. The latter was analysed by ANOVA, where the internal $n=3$ (i.e. each sample was repeated 3 times). This method will not influence the molar ratio of thiol:acrylate groups (Figure 4.1), as both constituents are diluted by the same factor. This decreases the probability of a thiol group reacting with an acrylate

4.3 Results and Discussion

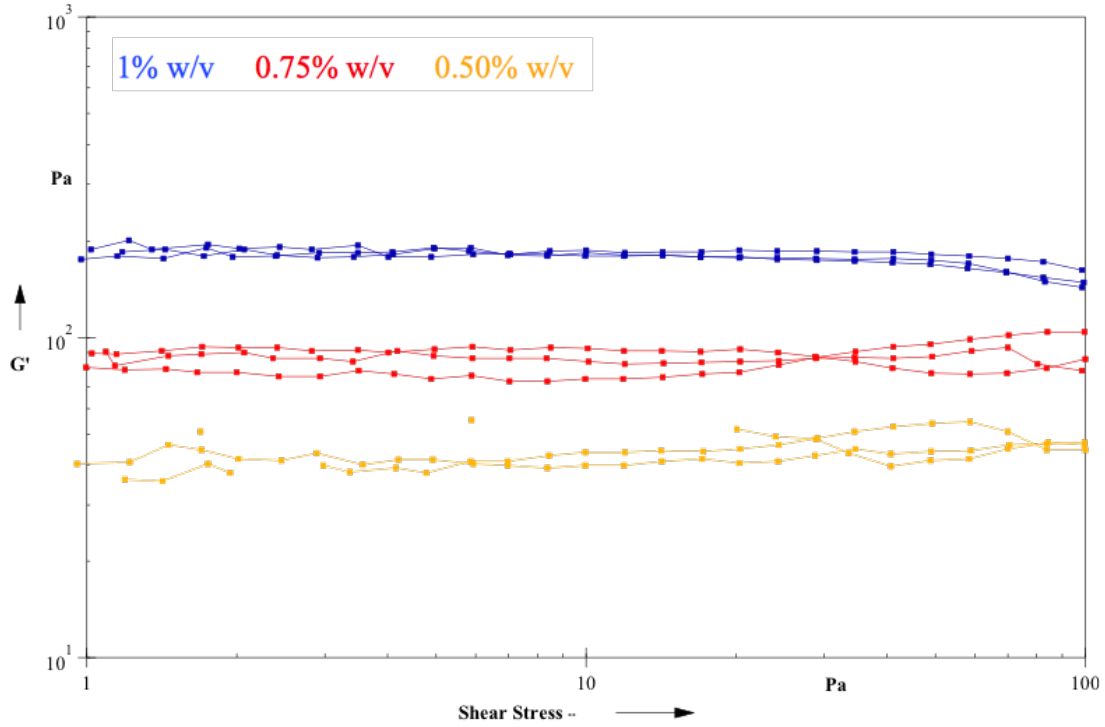


Figure 4.6: Macromer concentration was modulated by increasing the volume of water in the hydrogel prior to crosslinking. A stress sweep was performed for different concentrations (1, 0.75, 0.50%w/v) at a constant strain rate of 5% between, 1-100Pa, to indicate the average storage modulus or stiffness. A clear trend is observed between each concentration although some slip was evident in the 0.50%w/v group (note missing data points).

group. As the volume ratio of macromer/PEG-DA:water increases, the stiffness or G' decreases. Although this method does provide good control over stiffness, it does significantly reduce the number of free carboxylic acid groups important for peptide biofunctionalisation, which will be discussed in detail in the following chapters. This reduction of $-OOH$ concentration would also require a higher volume of the biofunctional group (i.e. peptide) concentration, which may influence the biocompatibility of the material.

Figures 4.8 and 4.9 (where the internal $n=3$) similarly demonstrate that decreasing

4.3 Results and Discussion

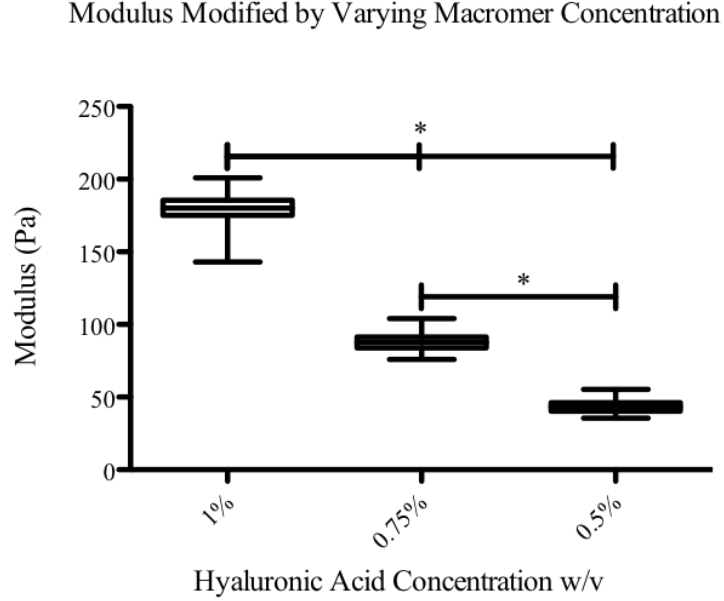


Figure 4.7: Varying the concentration of the macromer by mixing the hydrogel with water prior to crosslinking predictably changes the storage modulus G' of the hydrogel. Internal $n=3$.

the crosslinker concentration can predictably decrease the modulus of the hydrogel. In this case, the number of free thiols (on HA) remains the same, but the probability of an acrylate group reacting with a thiol group decreases significantly. Theoretically this leads to a mesh-like structure of HA-t in relatively close proximity, crosslinked sparsely by PEG-DA.

It is important to note, however, is that owing to the small volumes of PEG-DA, diluting the solutions accurately is experimentally challenging. This is the most likely reason for the modulus overlap of different concentrations noted in Figures 4.8 and 4.9. Overlap could also be a result of the graph axes; the x - and y -axis here are logarithmic, so any overlap must be interpreted carefully. Statistically, with sample variation taken into account, there is a very significant difference in

4.3 Results and Discussion

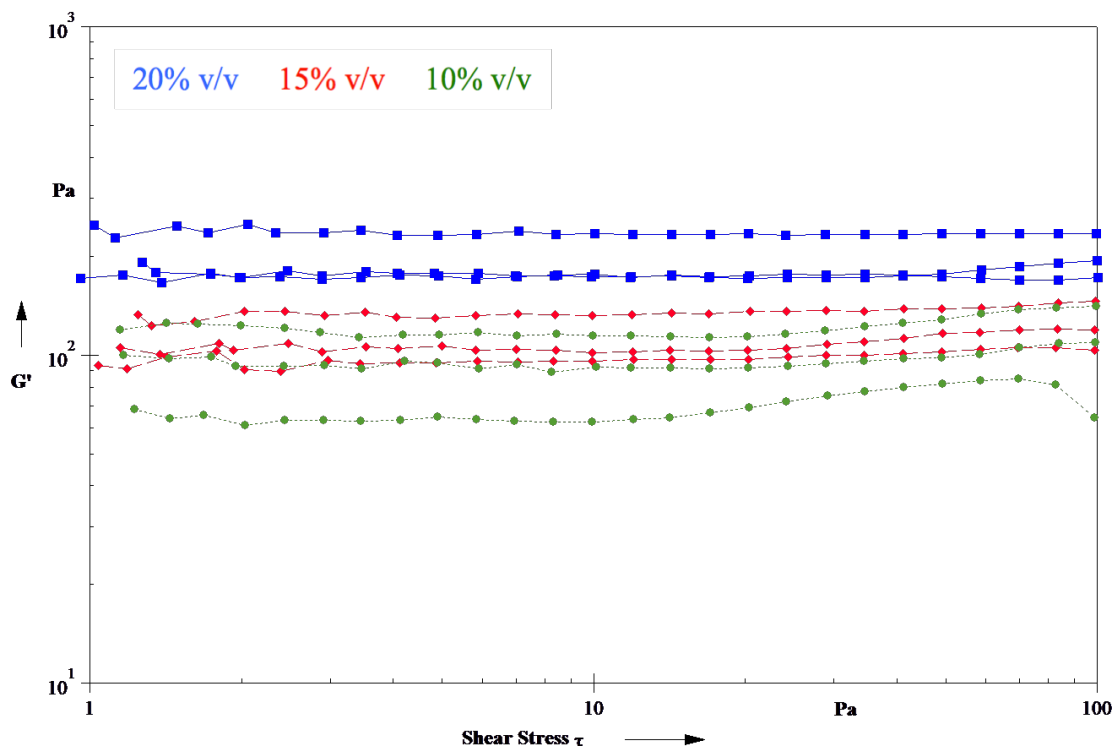


Figure 4.8: Increasing or decreasing the crosslinker concentration from the initial 20% v/v working concentration, rather than decreasing the macromer concentration, can modify crosslinking density. The stiffness of three different concentrations of crosslinker (20, 15, 10% v/v) were tested by performing a stress sweep at a constant strain rate of 5% between 1-100Pa.

all groups, confirmed by ANOVA and a post-hoc Bonferroni test. Statistically, the internal n (in this case each sample was repeated 3 times) was chosen purely for practical reasons; doubling or even tripling each sample repeat would take significantly longer. Similarly, block analysis (to reduce known variability) could have been employed, although the very significant variation between groups noted here would render this test somewhat redundant. The control offered by these two methods exceeds that found in many other hydrogel characterisation papers, which claim control in the order of several thousand Pascals rather than a few hundred Pascals, as found by these experiments (Freudenberg et al., 2009; Segura

4.3 Results and Discussion

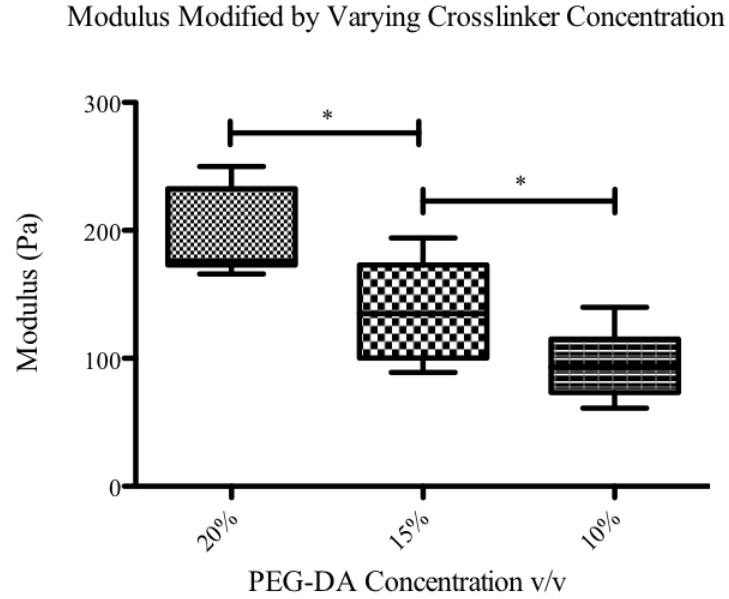


Figure 4.9: Varying the concentration of the crosslinker predictably changes the storage modulus (G') of the HA-t hydrogel. Groups are significantly different (internal $n=3$), $P < 0.05$.

et al., 2005). Neurons *in vivo* respond to extremely small changes in stiffness, and the current method demonstrates that controlling stiffness within very small magnitudes may be better suited to neuronal cell encapsulation.

4.3.3 Effect of Cultured Neural Cells on Hyaluronan–Thiol Hydrogel Properties

The importance of matrix stiffness has been discussed at length in both of the introductory chapters. Few studies to date have characterised the consequences of 3D neuronal culture and differentiation on hydrogel stiffness. To test the hypothesis that differentiation decreases matrix stiffness, the neuroblastoma derived SY5Y cell

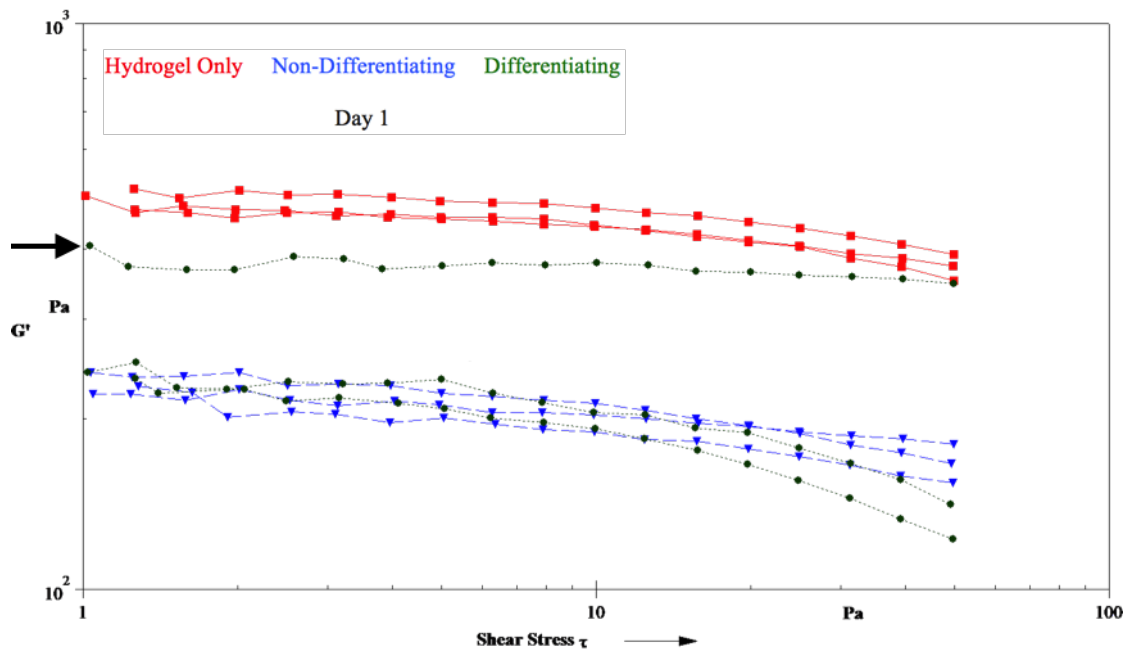
4.3 Results and Discussion

line was encapsulated in HA-t hydrogels and differentiated for 14days by retinoic acid supplementation.

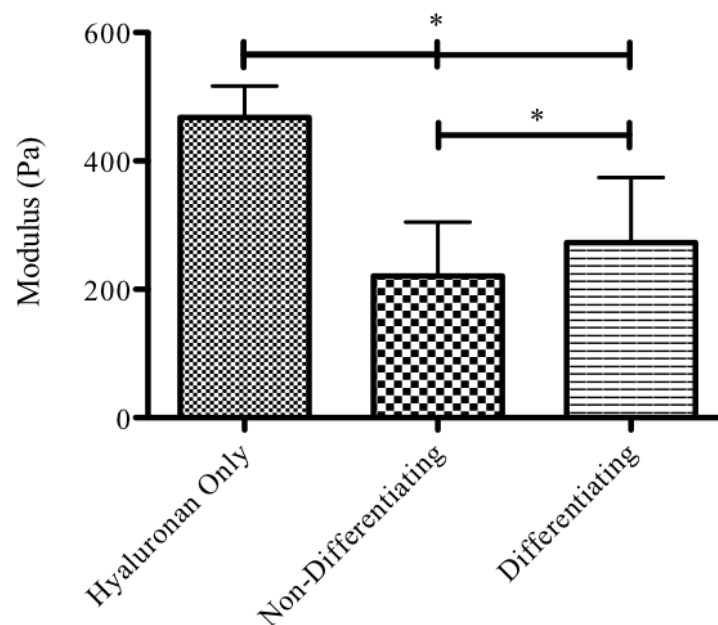
Hypothetically, encapsulated cells should decrease the stiffness of the matrix they are encapsulated within by physically hindering chemical interactions, and thereby increasing the distance between adjacent crosslinking points. The cells may also produce degradation enzymes such as the MMPs that could decrease the stiffness of the matrix further. Figure 4.10 demonstrates that HA-t alone is stiffer than substrates combined with encapsulated cells. This could be explained by the increasing distance between adjacent crosslinking points; if the PEG-DA concentration remains the same the probability that a thiol will react with a acrylate group decreases, thus reducing the stiffness.

Additionally, because the hydrogel is added to a solid cell pellet, there is no increase in water concentration, therefore a decrease in stiffness cannot be attributed to a fluctuation in viscosity alone. The anomalous group (see arrow, figure 4.10a) is most likely due to experimental error during HA-t and PEG-DA mixing and should be considered carefully when designing statistical tests.

Several studies report that matrix modulus influences stem cell differentiation, and this is now widely accepted (Leipzig and Shoichet, 2009; Seidlits et al., 2010). The author knows of no study, however, quantifying the effect of differentiating stem or precursor cells such as the SY5Y cell line on substrate stiffness. The experimental data presented here, therefore, may shed some light on the macroscopic mechanical properties of hydrogels during cellular differentiation.

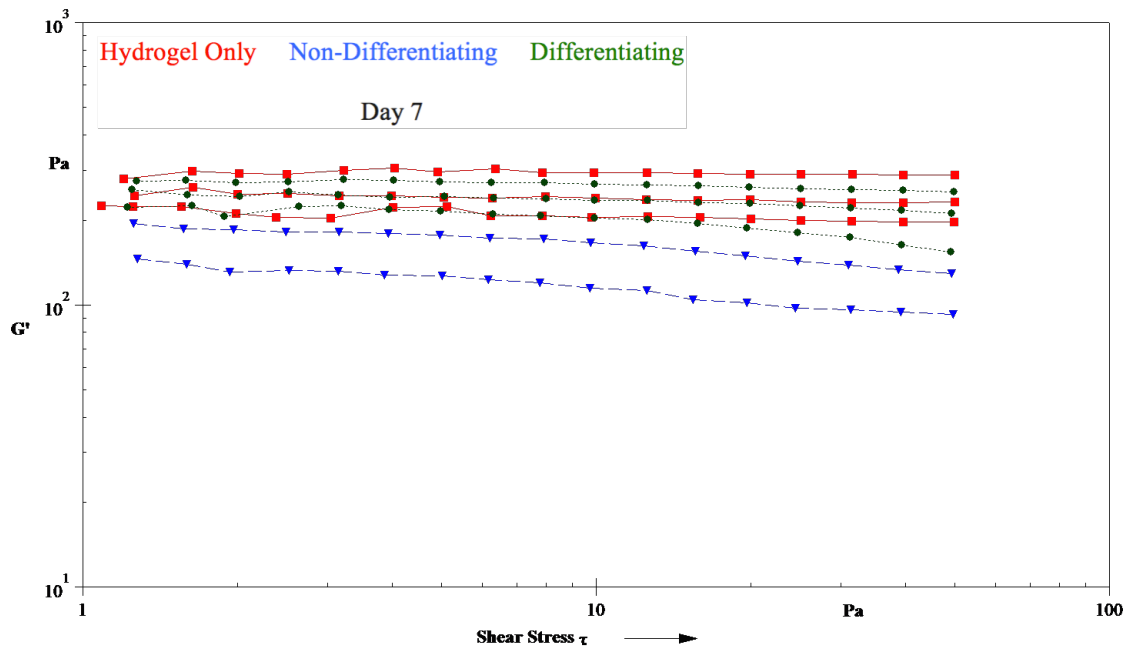


(a) Raw Rheological Data

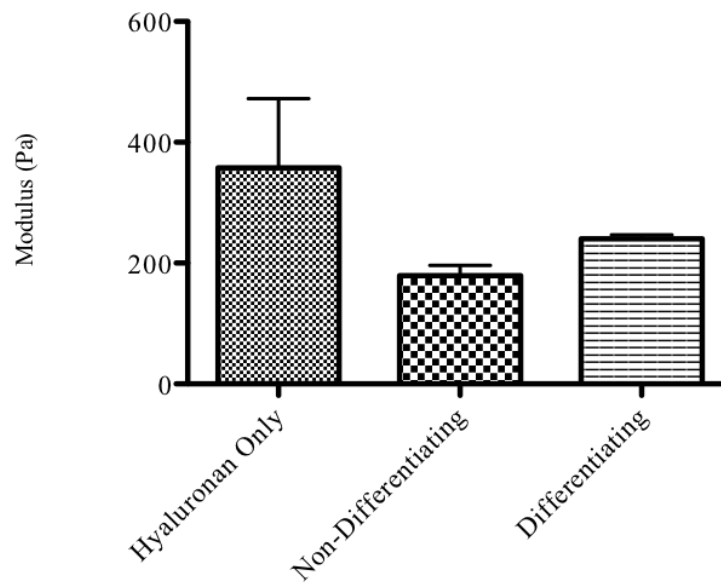


(b) Statistical analysis

Figure 4.10: (a) Rheological characterisation of differentiating SY5Y cells encapsulated in hyaluronan (HA-t) after day 1. Hydrogels were characterised mechanically by performing a stress sweep between 1 and 100Pa; arrow denotes anomalous outlier. (b) Graphical representation of (a) where ANOVA was used to compare groups. Note that the starting modulus of HA alone is approximately 480Pa. Error bars represent a standard error of the mean. $p < 0.05$.



(a) Raw Rheological Data



(b) Statistical analysis

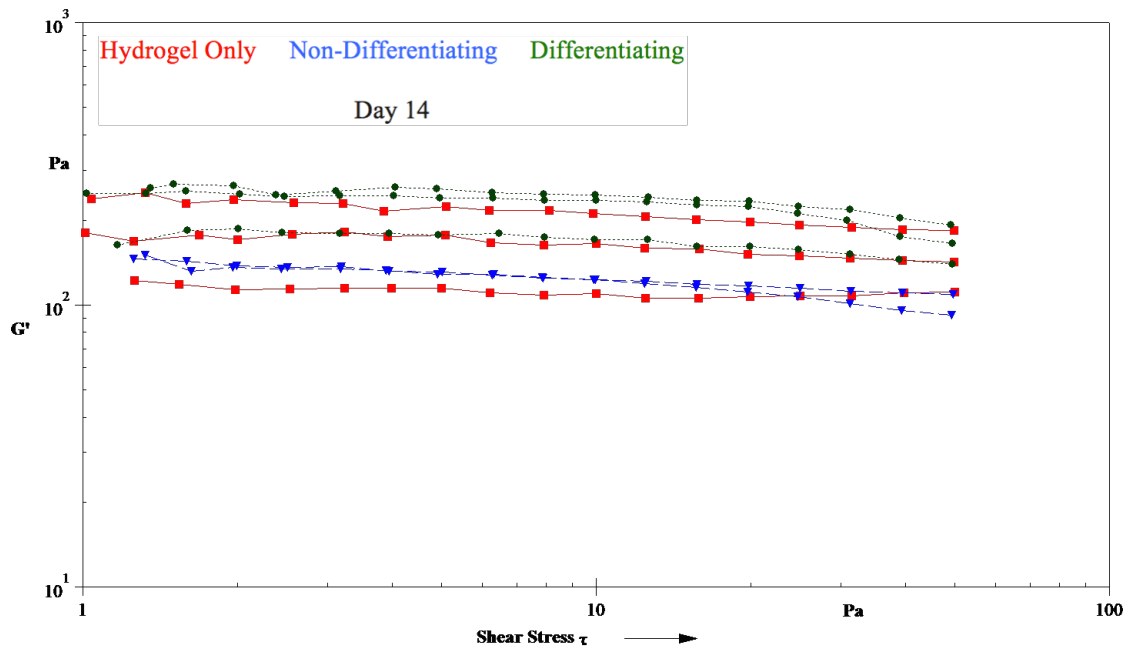
Figure 4.11: (a) Rheological characterisation of differentiating SY5Y cells encapsulated in hyaluronan (HA-t) after day 7. (b) Graphical representation of (a) demonstrates that after 7 days the bulk HA modulus has decreased from approximately 490 Pa to approximately 380 Pa, while encapsulated hydrogel moduli remain roughly the same. Error bars represent a standard error of the mean, although no statistical difference was found by ANOVA.

4.3 Results and Discussion

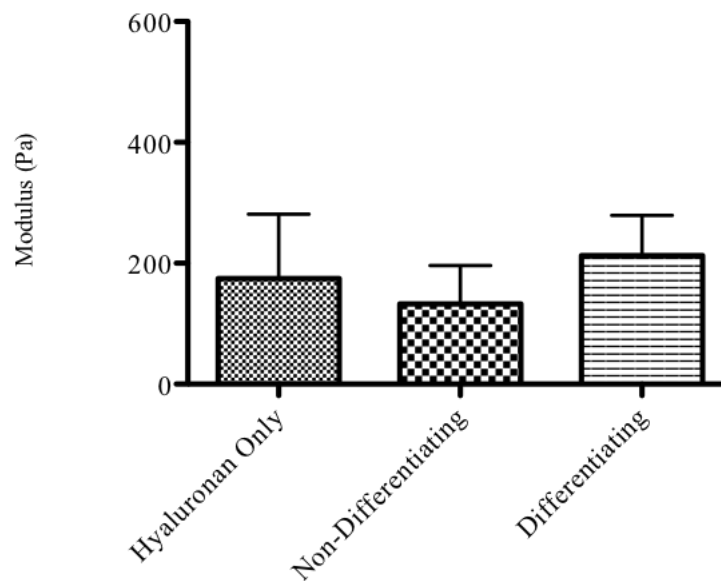
Matrix degradation is well documented, and it has been shown that certain neuronal cells can manipulate the substrate within which they grow. DRGs, for example, degrade their substrate by matrix metalloproteinases (MMPs), that decrease local substrate stiffness and promote neurite outgrowth. Inhibiting the MMPs leads, predictably, to a reduction in neurite outgrowth (Sarig-Nadir and Seliktar, 2010).

Figures 4.10b and 4.12b demonstrate somewhat surprisingly that differentiating neurons do not decrease stiffness as previously hypothesised. In fact, after 7days, no significant difference is noted between groups although the non-differentiating cells appear to have reduced the local stiffness. The loss of data (note missing curves, Figure 4.11a and 4.12a) was attributed to file corruption during analysis, and certainly reduces the statistical significance of this investigation. Similarly, the anomalous outlier noted in Figure 4.10a was attributed to user error, and should be considered carefully in future statistical test design. After 14days (see Figure 4.12, differentiating cells appear to stabilise local substrate stiffness, although some overlap between groups is noted.

This result could be attributed to a few factors. Firstly, overall hydrogel stiffness decreases until neurite-like structure formation supports the hydrogel by forming a network of its own. This network interacts with the hydrogel, stabilising the local stiffness. No visual confirmation was completed to support these data, although previous experiments (data not shown) suggest that cells do extend neurites after this time. It is clear, however, that HA-t hydrogels alone decrease in stiffness after 14days, and hydrogels with encapsulated cells remain relatively stable after the same time.



(a) Raw Rheological Data



(b) Statistical analysis

Figure 4.12: (a) Rheological characterisation of differentiating SY5Y cells encapsulated in HA-t after day 14. The stiffness of the hydrogels with encapsulated cells appears to remain stable. (b) Graphical representation of (a) shows that the stiffness of HA alone has significantly decreased, although encapsulated cells do not have the same effect on the matrix. Error bars represent the standard error of the mean, although no statistical difference was found by ANOVA.

4.3 Results and Discussion

4.3.4 Mass Transfer in Hydrogels

In Chapter 3, the importance of diffusion through hydrogels was discussed briefly. Understanding how macromolecules such as proteins move through a hydrogel will allow us to engineer the material to suit a particular application more accurately. It is also essential to model the movement of chemoattractants and repellants within a hydrogel, as these cues or growth factors are essential for the accurate manipulation of cellular behaviour *in vivo*.

To model the movement of a small molecule such as glucose, the COMSOL Multiphysics platform was employed. Physical characteristics of the hydrogel and the macromolecule were confirmed from the literature (cited elsewhere). It is important to note that the hydrogel modelled herein is a blend of HA and methylcellulose (HAMC), previously described by Baumann *et al*, who suggest that there is no significant difference between the diffusivity of large molecules in water or in HAMC (Baumann et al., 2009). HAMC was chosen as it is the softest hydrogel used in cell encapsulation experiments, and serves as a useful control for the stiffer HA-t and HA-f hydrogels. Simply put, diffusion through HA-t and HA-f hydrogels will be slower. The diffusivity of glucose through water at 37°C is described by Geankoplis as $0.697 \times 10^{-9} m^2 s^{-1}$ and was used as the diffusion coefficient or effective diffusivity in the present model (Geankoplis, 1993).

Understanding the interactions between a solute and a hydrogel are essential, as most important molecules will reach the encapsulated cell by these mass transfer mechanisms. Glucose, sera and other proteins – such as differentiation and growth factors – all interact first with the matrix before reaching the neuron. The aim of

4.3 Results and Discussion

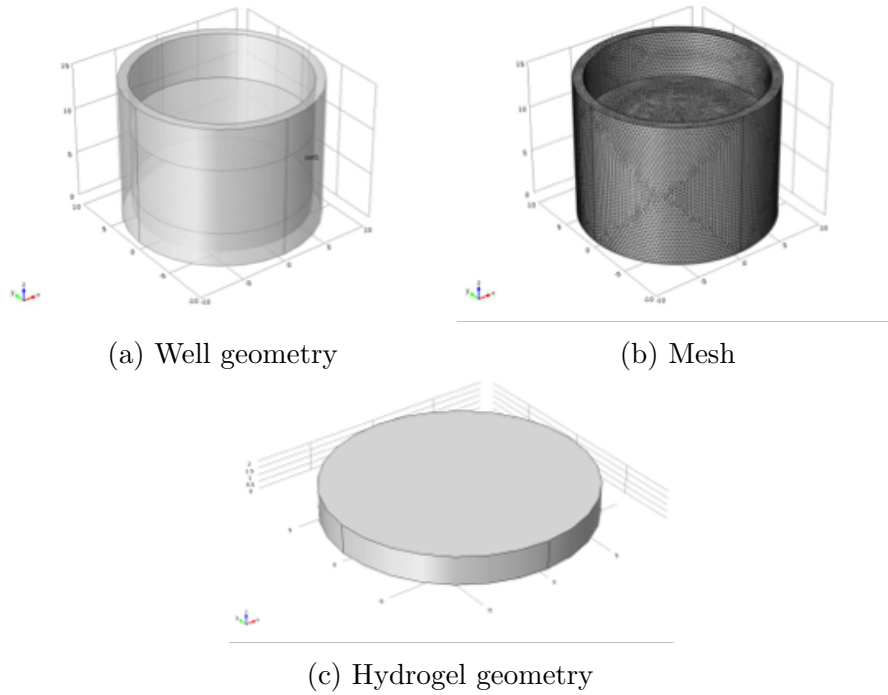


Figure 4.13: (a) Well plates are regularly used in cell culture. The well was replicated in COMSOL, and (b), a fine mesh size was selected. (c) To simplify the model, diffusion was computed in the hydrogel geometry only.

this short section was to construct simple mass transfer models of glucose interacting with a HA hydrogel.

Realistic mass transfer models can be problematic with regards to hydrated polymer networks. In Chapter 3 these difficulties were highlighted, including hydrogel homogeneity, gradient shapes, and polymer-solute interactions. Diffusion specifically has been exhaustively reviewed by Amsden, where the author discusses at length the various mathematical models of diffusion through hydrogels, including free volume obstruction models for heterogeneous hydrogels (Amsden, 1998).

The COMSOL platform provides a model of Fickian diffusion, which was used in this experiment. Glucose was chosen for its relevance to most cell culture; the

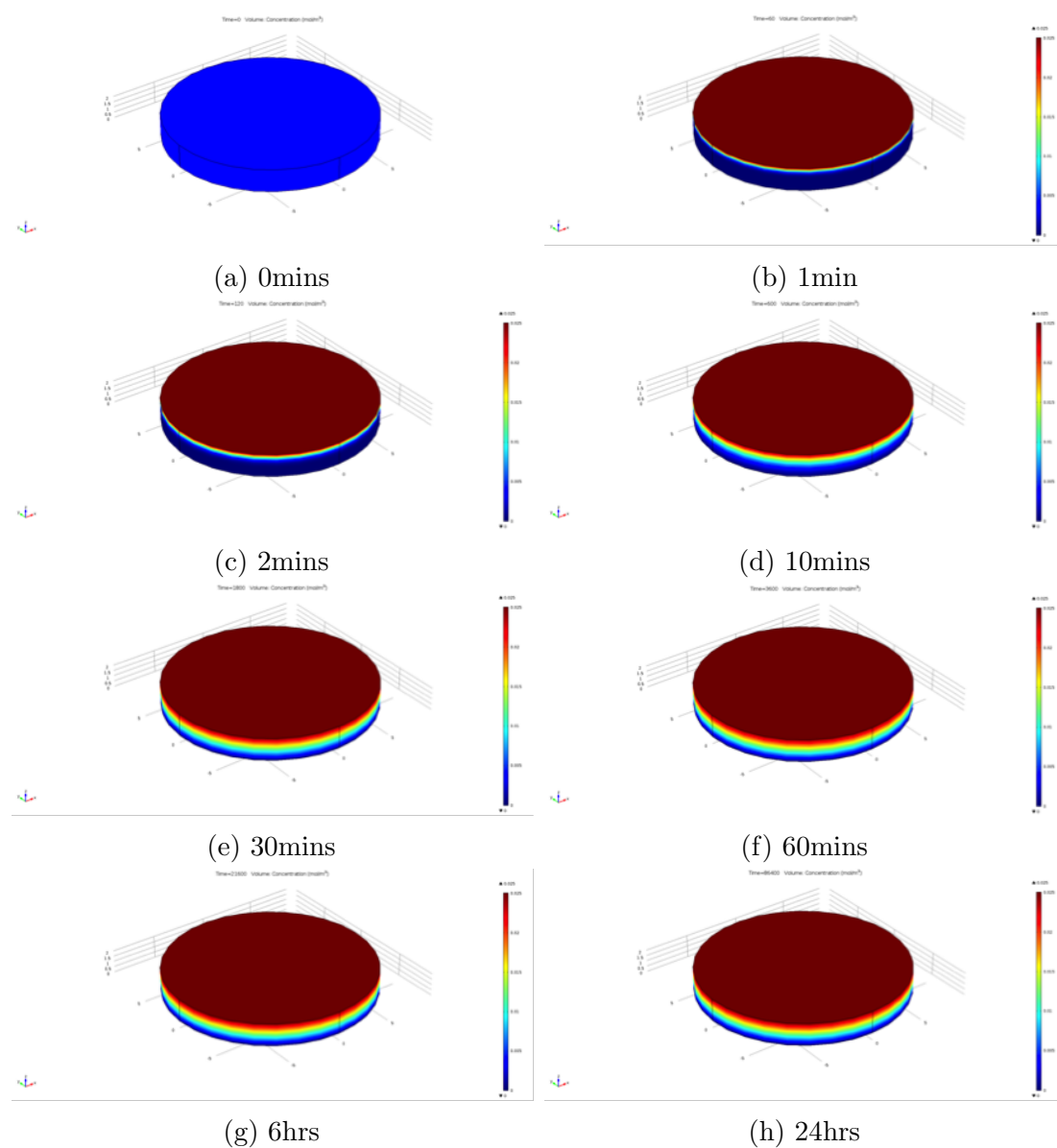


Figure 4.14: Steady state diffusion model of glucose diffusing through a hyaluronan hydrogel, commonly used in previous experiments. Concentration was ramped over 1sec, and remained constant at 25mMol m⁻³ for 24hrs. It is clear that a stable concentration gradient is established relatively rapidly, and remains constant for at least 24hrs. Concentration represented in mol m⁻³.

4.3 Results and Discussion

model could subsequently be modified to accommodate other molecules simply by changing the diffusion coefficient of the new solute. Another very important molecule often chosen in such experiments is oxygen, essential for healthy cell culture. The choice of solute in this work was arbitrarily chosen, as the model could be quickly modified to investigate other molecules. Figure 4.14 demonstrates a time dependent solution to Equations (4.1), (4.2) and (4.3). It is clear that a stable concentration gradient is established within the hydrogel within 30mins.

During the following 24hrs, no steady state is reached due to the boundary conditions, stating that the concentration at the hydrogel-medium boundary will remain constant, a fact that is mirrored in reality. The volume ratio of medium to hydrogel is high enough that the concentration at the boundary will in effect remain constant. This is the most probable explanation for the gradient remaining stable after 24hrs; the large source is constantly introducing more of the solute to the hydrogel. It is also important to note that cells will metabolise glucose and certainly, at high cell densities, the solute gradient would remain constant as suggested by the model above.

Few studies have concentrated solely on the computational modelling of diffusion within a HA hydrogel. Leach and coworkers, in a drug delivery study, cast a crosslinking hydrogel within a perfusion chamber to calculate the mass of a solute released through a composite HA and glycidyl methacrylate (GM) hydrogel (Leach and Schmidt, 2005). The study goes on to use Fick's second law to relate the mass released from the hydrogel to the effective diffusivity of the protein. Although experimentally useful, this study does not incorporate computational data.

4.3 Results and Discussion

Other interesting studies consider two-phase systems where a solute may interact with hydrogel-free inclusions (Hickey et al., 2006; Kingsburry and Slater, 2009) and matrix shape effects (Barba et al., 2009). No paper has been found in recent literature that uses computational models to predict glucose diffusion through HA hydrogels or composites thereof.

In summary, HAMC hydrogels are a good substrate within which to introduce a solute gradient. These gradients are essential for the control of cellular behaviours such as chemotaxis and neurite outgrowth (Keenan and Folch, 2008). The present model is somewhat oversimplified, and does not consider hydrogel-free inclusions or other inhomogeneities. It does, however, provide a robust and predictable platform from which to predict how rapidly a solute will interact with encapsulated cells, and how long the gradient will remain.

4.4 Summary

Hyaluronan is an extremely versatile polymer that can be functionalised with molecules such as 3,3'-dithiobis(propionic hydrazide) (forming HA-t) and furfurylamine (forming HA-f), that allow them to crosslink predictably. The reaction conditions for the synthesis of these hydrogels are extremely favourable with respect to biocompatibility. HA-f was generally disregarded for 3D cell culture due to the crosslinking time and pH. HAMC is an excellent candidate for 3D neuronal cell culture, although the lack of covalent crosslinks may render these hydrogels unsuitable for long term neural culture studies.

Rheology provides an excellent way of characterising important mechanical properties such as stiffness and viscosity. Hydrogel stiffness can be controlled to within very small tolerances by either changing the macromer or crosslinker concentration. Differentiating neuronal precursor cells stabilise the stiffness of HA hydrogels by reducing the degradation rate of the hydrogel, although the exact mechanism by which the cells achieve this was not investigated, and was outside the scope of this study.

The mechanisms of mass transfer were briefly explored, although this study did not attempt to outline the movement of small molecules through a hydrogel. Rather, it aimed to highlight the importance of solute movement within complex 3D environments. Mass transfer events are fundamental to healthy cell culture. Familiarity with these mechanisms can allow tissue engineers to develop materials that are specifically suited to neural cell culture. Here we show that the rate of diffusion within HAMC hydrogels can be predicted, within ± 5 mins.

Chapter 5

Small Angle Neutron Scattering as a Characterisation Tool

5.1 Introduction

Neutron scattering is an extremely versatile technique, which can be employed to characterise various molecules, from peptides and proteins, to large macromolecules such as the polymers. Access to SANS instrumentation is extremely limited, mainly due to the cost of establishing a suitable facility. In this chapter, the use of SANS in hydrogel characterisation will be discussed in detail. An introduction to instrument configuration and sample preparation in the Methods section will be followed by the mathematical background of neutron scattering, and how these scattering events can be used to accurately characterise HA hydrogels.

The complexity of neutron-polymer interaction events are outside the scope of this thesis, although an overview of the theory of small angle neutron scattering is essential for data analysis. An important source for the mathematical theory of SANS (equations 3.9–3.15) was provided by Andrew Jackson at the National Institute of Standards and Technology, Centre for Neutron Research (Jackson, 2008). The reader is referred to this paper for a good introduction. SANS, in the context of this chapter, was employed to better understand the microscopic nature of HA hydrogels, including but not limited to, polymer chain length, diameter, and crosslinking density. We show here for the first time that the relationship between stiffness and crosslinking density is not directly proportional, as first thought by literature (Bian et al., 2013).

5.2 Materials and Methods

5.2.1 Instrument Configuration

Housed at the Rutherford Appleton Laboratory, Harwell Campus, Oxford, The SANS2d instrument is one of the most sophisticated instruments of its type in the world. The apparatus is sheltered within Target Station 2, which contains six additional neutron scattering instruments for a large variety of applications. An extremely useful source of information regarding the SANS2d instrument was found in a paper by Heenan *et al*, and is cited often within this section.



Figure 5.1: The SANS2d instrument was completed in 2009 and is one of the most sophisticated in the world. It is housed at ISIS, within the Rutherford Appleton Laboratories in Oxfordshire. Adapted from (Various, 2009).

5.2 Materials and Methods

An important consideration when designing a neutron scattering apparatus is the intended use. Clearly, it is more cost effective to design an instrument that could be used by the scientific majority. Suitably, therefore, the design engineers settled on an instrument configuration that would allow wide wavelength operations of up to $10\text{\AA}$, with a 40m beam line (Heenan et al., 2005). In order to allow experiments at high and low q ranges, two 1m^2 detectors were chosen, where both the source-sample and sample-detector lengths could be changed, from 2m to 12m (Heenan et al., 2005). An important advantage of choosing two movable detectors is that neutron count rate can be significantly improved when a high q is not required. The reader is referred to figure 5.3 for an accurate representation of the main movable detector of SANS2d.

An important feature of this setup is the vacuum tank housing the two detectors. It is essential to evacuate any volume that the neutron beam will pass through, thereby preventing inaccurate neutron detection. To negate this, the detectors are mounted within a 3.2m wide, 13m long vacuum tank with a 300mm borated wax covering. The latter reduces very significantly the probability of a neutron ‘bouncing’ from the steel tank onto one of the detectors (Heenan et al., 2005). Large dry mechanical pumps can evacuate the tank to 0.1mbar in approximately 40mins, an extremely favourable rate.

5.2.2 Sample Preparation and Data Collection

SANS was used to assess the microscopic characteristics of HA-t hydrogels. Hydrogel solutions were prepared by blending PEG-DA and HA-t at macromer concentrations ranging from 0.5-1.0% w/v diluted with deuterium oxide (D_2O ; Sigma) or dH_2O .

5.2 Materials and Methods

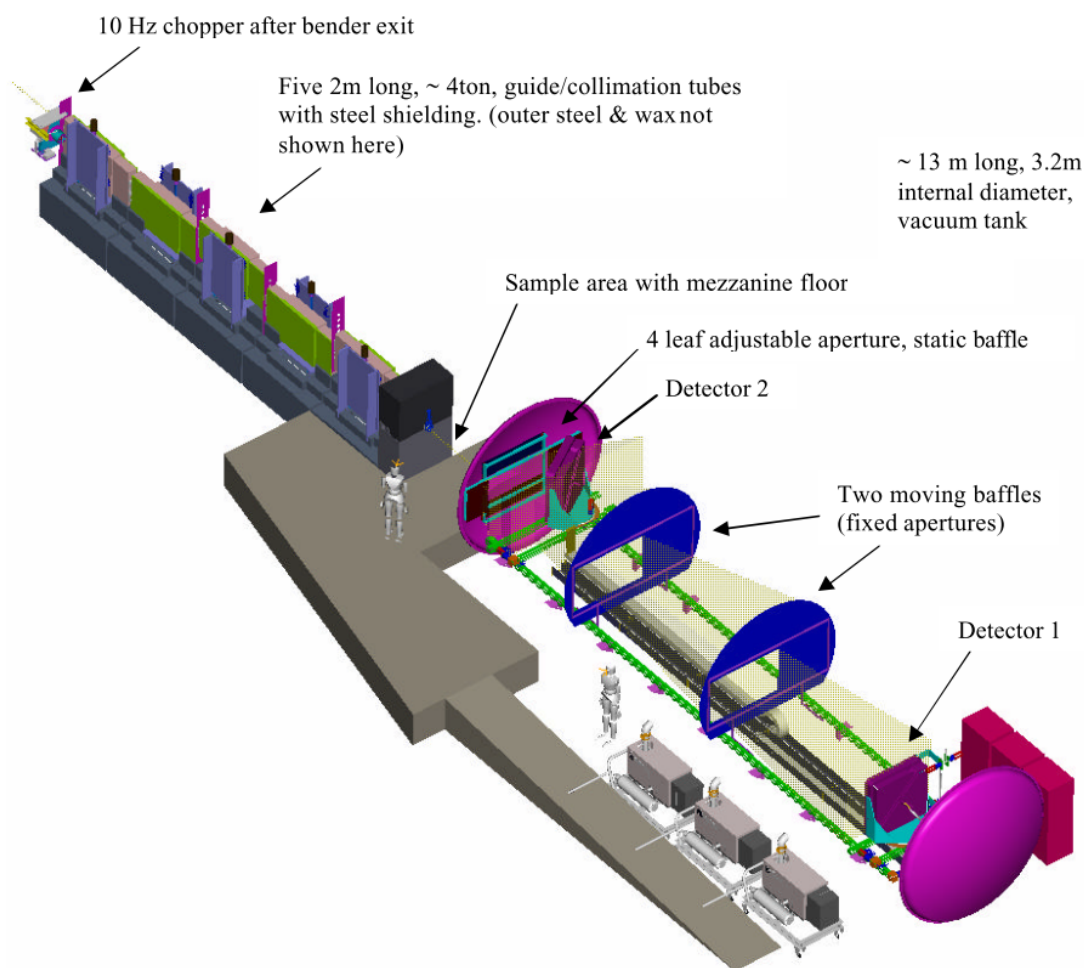


Figure 5.2: An engineering impression of the SANS2d instrument at the Rutherford Appleton Laboratory, from target station to beam exit. Flexible collimation and detection can produce a high q range to detect macroscopic and microscopic scattering events. It should be noted that the external steel and borated wax covering is not shown. Adapted from (Heenan et al., 2005).

Hydrogels were allowed to crosslink overnight at room temperature surrounded by wetted paper to reduce evaporation. The samples were then loaded into quartz Hellma cells of 2mm pathlength, and placed in a thermostatically controlled sample changer to allow automated selection of the sample. Once the chamber was evacuated, small angle neutron scattering (SANS) data were collected on SANS2d,

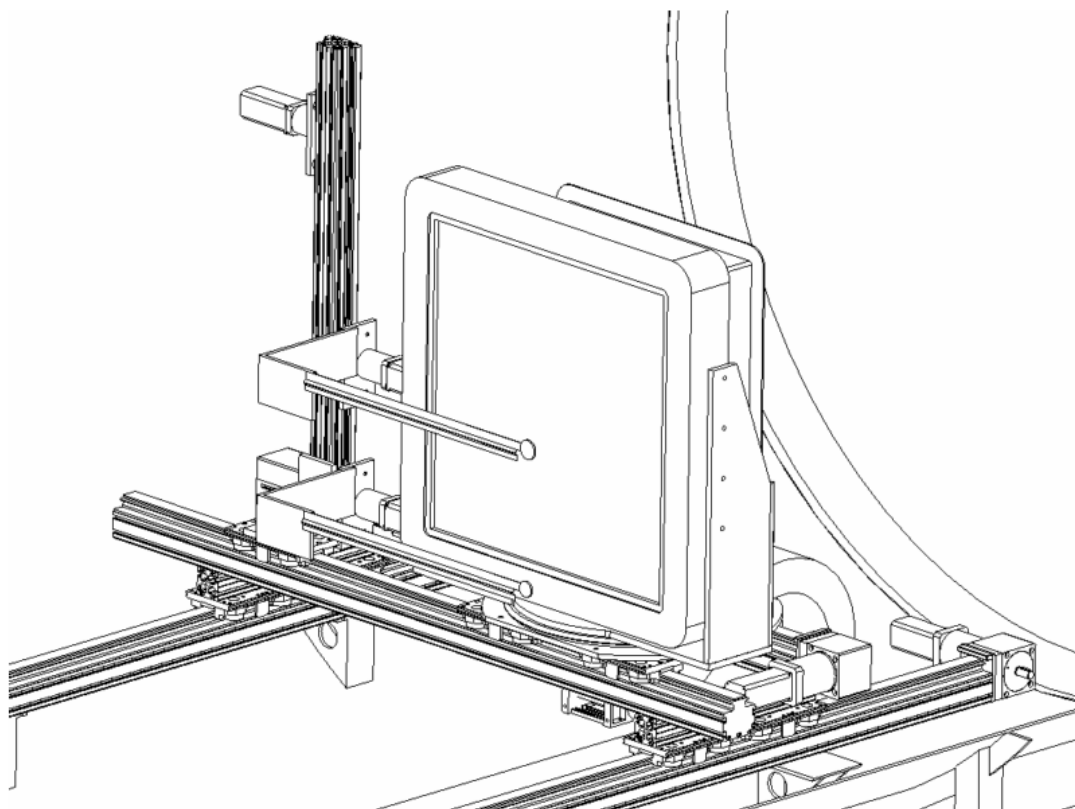


Figure 5.3: The configuration of the main SANS2d detector. Note the trolley, which will allow the detector to move for a large q range. Adapted from (Heenan et al., 2005).

a state of the art SANS instrument on the second target station at ISIS pulsed neutron source, Harwell, Oxfordshire (Heenan et al., 2011).

The incident wavelength neutrons of 1.75 to $16.5\text{\AA}$ coupled with an incident collimation length and sample detector distance of 4m , meant that a q range of $0.005\text{--}0.6\text{\AA}^{-1}$ was accessible. The beam diameter was 8mm . Scattered neutrons were detected on a 1m^2 detector with 77×103 pixels. Raw data were corrected for detector efficiencies and sample transmission, and the scattering of the D_2O solvent subtracted as background.

5.2 Materials and Methods

For analysis, data were converted to scattering cross-section $\frac{\partial\sigma}{\partial\Omega}$ against q using the Mantid software package (Mantid, 2007) and placed on an absolute scale (cm^{-1}) using the scattering from a standard sample (a solid blend of hydrogenous and perdeuterated polystyrene) (Wignall and Bates, 1987). Quantitative data, as a correlation between intensity $I(q)$ and scattering vector q , were modelled mathematically in SASview, a software package developed by the DANSE project under NSF award DMR-0520547. The modified Debye-Bueche and Guinier-Porod models were selected for their versatility in assessing polymeric structure. Data is presented at a scatter plot before and after model fit.

5.3 Results and Discussion

The aim of a SANS experiment, taking account of homogeneity and volume normalisation, is to detect the density of a scattered neutron at a wave vector q from the sample, as a function of scattered neutron intensity $I(q)$. Raw data is represented initially as scatter plot, although as we will see from Figure 5.4, using H_2O as a solvent introduced additional anomalies.

Due to the hydrogen protons of the water molecules in the sample, the scattering length density cannot be accurately measured, hence leading to a loss of phase information. This is known as Babinet's Principle (Jackson, 2008). To negate these difficulties, water was substituted with deuterium oxide, producing a plot with significantly higher contrast (Figure 5.4). In this form, the data do not yield much information about the microstructure of the material, and further computational models are required to evaluate specific microscopic features such as the polymer chain length and crosslinking density. These will be discussed at length in the following sections. For additional background information regarding the principles of neutron scattering, the reader is referred to Chapter 3.

5.3.1 The Modified Debye-Bueche Model

Mathematical models discussed in Chapter 3 can be adapted to yield information on the structural features of HA hydrogels. Especially useful for this section, was the SasView software package, which was used throughout analysis.

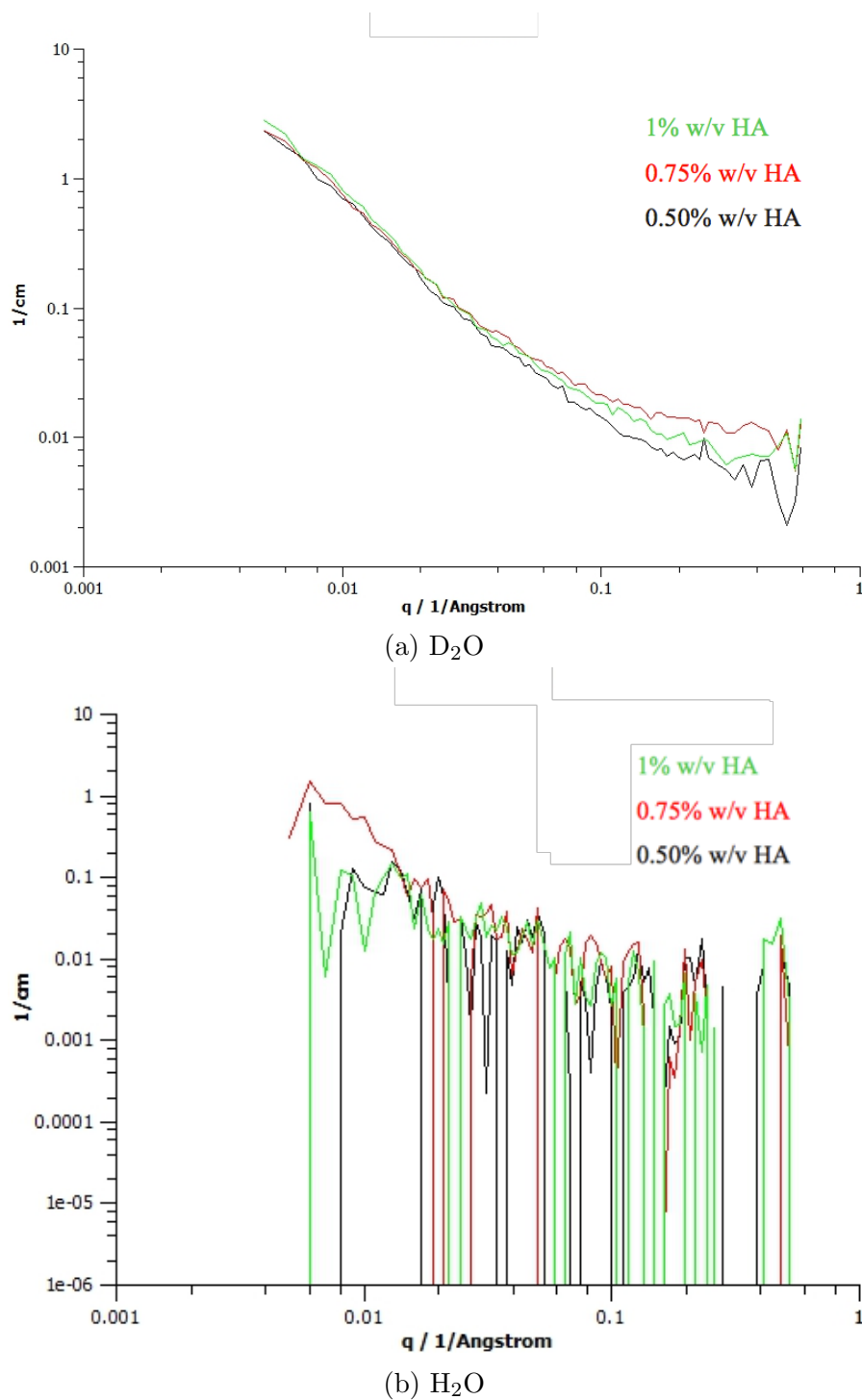


Figure 5.4: HA-t hydrogels at different macromer concentrations of 0.50%, 0.75% and 1.00% w/v (black, red and green respectively) were analysed by neutron scattering in a q range between 0.005-0.6 $\text{\AA}^{-1}$. HA was dissolved in deuterium oxide, showing good contrast, as opposed to (b) HA dissolved in water, where hydrogen protons reduce contrast. Axes represent the neutron intensity $I(q)$ (y axis) and the scattering vector q (x axis).

5.3 Results and Discussion

In a method adapted from (Hyland et al., 2012), the scattering vector q is related to the wavelength λ , and scattering angle θ , by

$$q = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (5.1)$$

Once the scattering data were uploaded to SasView, a fractal model (also known as the modified Debye-Bueche model) was run to assess a material with a repetitive elementary structure such as the repeating monomeric units of HA. In high q regions this data can hint at the specific properties of these repeating units. In mass fractal analysis, the fractal dimension d ranges from 1 to 3; $d = 1$ for stiff rod-like structures, $d = 2$ for Gaussian coils, and $d = 5/3$ for swollen Gaussian coils in a solvent and $d > 2$ indicates a degree of branching.

Scattering intensity from mass fractals is given by

$$I(q) \propto Bq^{-d} \quad (5.2)$$

where q is the scattering vector given by equation 5.1, B is the prefactor indicating structural characteristics, and d is the fractal dimension.

This method has been adopted by (Hyland et al., 2012) to evaluate the structure of cylinder-like polymers. To calculate the correlation length L_c , which can be thought of as the average mesh size ξ , the Debye-Bueche model was used through the fractal model function in SasView.

From Table 5.1 we can see that different concentrations of HA lead to structurally different hydrogels at high q . The fractal dimension d hints at a Gaussian coil-like

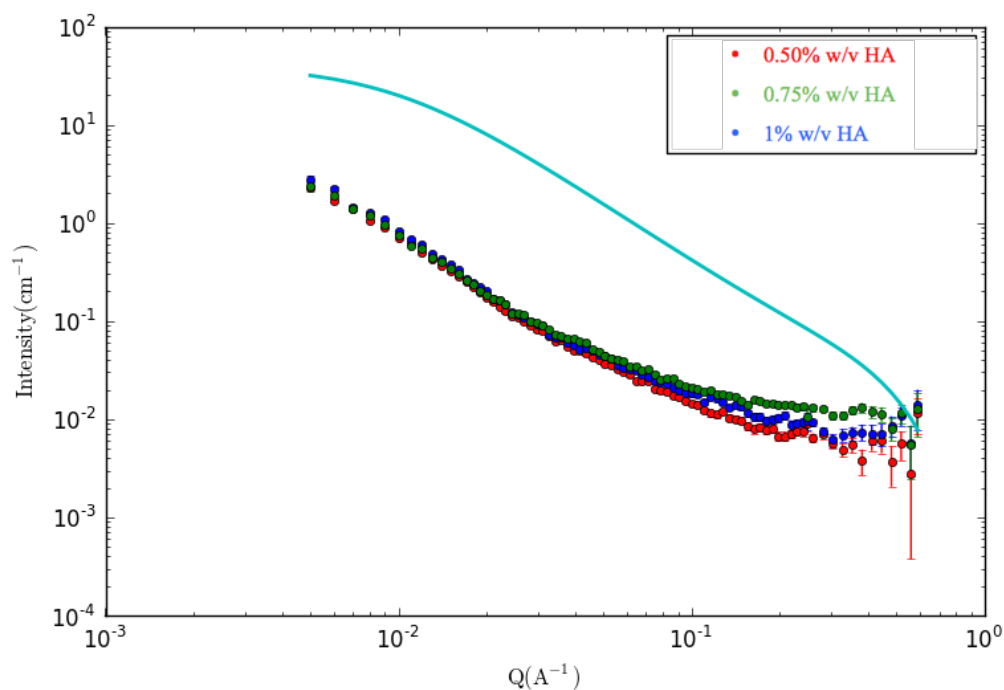
5.3 Results and Discussion

Table 5.1: A modified Debye-Bueche model was used to calculate the correlation length, fractal dimension and polymer chain radius of HA-t Hydrogels at three different macromer concentrations.

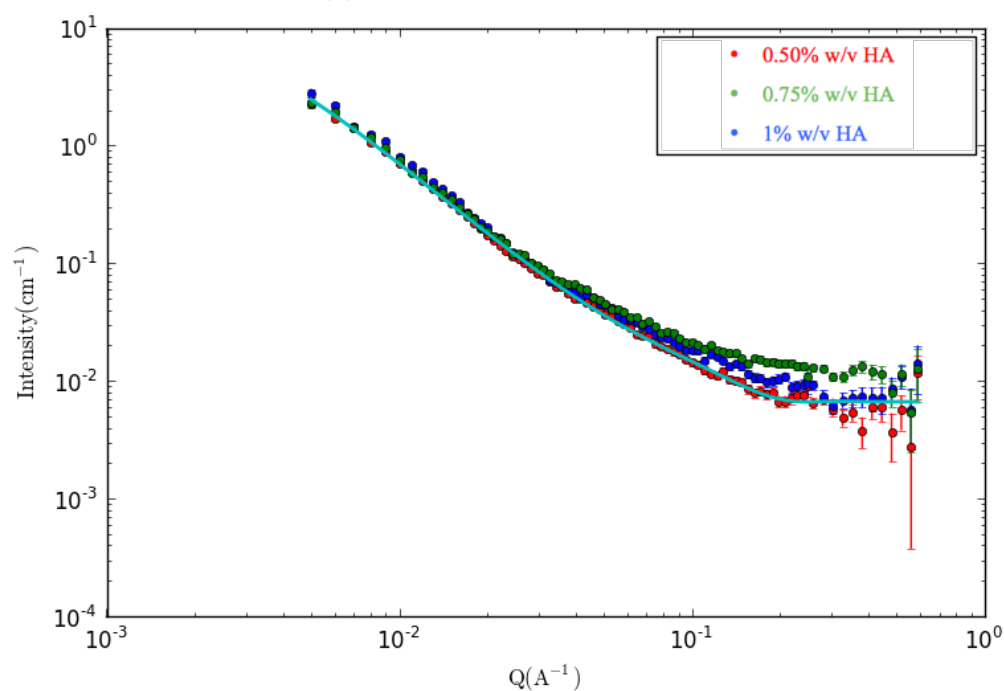
	Hyaluronan Concentration (w/v)		
	1.00%	0.75%	0.50%
Q Range($\text{\AA}^{-1}$)	0.005-0.591	0.005-0.591	0.005-0.591
Background Intensity, I (cm^{-1})	0.009	0.012	0.007
Correlation Length, L ($\text{\AA}$)	554.470	-1294.400	362.560
Fractal Dimension, d	2.077	1.9128	2.092
Radius of Polymer $\text{\AA}$	15	0.80964	18.04
Volume Fraction of Polymer (ratio)	0.086	-0.027	0.001
Scattering Length Density of Polymer $\text{\AA}^{-2}$	$4.246e^{-6}$	$-6.3196e^{-8}$	$2.744e^{-6}$
Scattering Length Density of Solvent $\text{\AA}^{-2}$	$4.495 e^{-6}$	$-2.4762e^{-6}$	$4.9606e^{-6}$

structure within the hydrogels, where in each hydrogel $d \approx 2$. The apparent discrepancies noted in the 0.75% hydrogels (for example correlation length, polymer radius, volume fraction and both scattering length densities) are suggestive of anomalies in the data. This is especially apparent with respect to the correlation length, L which should be disregarded, as both the sign and number are anomalous. This clearly brings into question the validity of the whole sample, which should be very conservatively analysed. Anomalous data points were not removed for the sake of completeness, but in further analysis this could be considered.

Notwithstanding, the Debye-Bueche model is useful for calculating the radius of the chains to be between 15 and 18 $\text{\AA}$. We would not expect this radius to change for different concentrations. In contrast, however, we see an increase in correlation length with increasing concentration. This result was unexpected; theoretically the mesh size (which is a function of correlation length), is inversely proportional to concentration, because we would expect the solvent to increase the space between



(a) The Debye-Bueche Model



(b) Data Fit

Figure 5.5: SANS data revealed some similarities to the modified Debye-Bueche model (a). Scattering events from 1.00%, 0.75%, and 0.50% w/v (blue, green and red respectively) HA-t hydrogels were detected and fitted to the mathematical model (b). The curve of the 0.50% w/v HA-t hydrogel appears to follow the model closely, although error was high at $Q > 0.6 \text{ \AA}^{-1}$.

5.3 Results and Discussion

the polymer chains. An estimation of the polymer volume fraction, however, does confirm that for lower macromer concentrations, the relative volume occupied by the polymer does in fact decrease.

One likely reason for such a discrepancy could be that the selected q range was not large enough (Figure 5.5). Data such as the mesh size are detected at low q and in this case it is possible that without data at low q , the equations distort the true mesh size. Decreasing this value to approximately $10^{-3}\text{\AA}^{-1}$ may yield such data. Similarly, however, the discrepancies between experimental data and computational model could be due to hydrogel inhomogeneities.

Koberstein and coworkers found that the effect of a small q range can drastically influence structural information such as the mesh size, caused by large scale concentration fluctuations (Koberstein et al., 1985). This finding was also confirmed more recently by (D’Erricot et al., 2008) who demonstrate model fitting discrepancies probably due to hydrogel inhomogeneities. Horkay *et al* used a power law in the form of Equation 5.2 to evaluate the organisation and hierarchy of HA, and serves as an important comparison to the data presented herein. Using three different HA concentrations (1,2 and 3% w/w HA), the study demonstrated significant differences in scattering events at high q , a finding that also resonates with the data presented here (Horkay et al., 2012).

Importantly, the author discusses three distinct scattering regions: at $q < 0.006\text{\AA}^{-1}$, the slope is > -3 and represents surface scattering due to loose domains (Horkay et al., 2012); a shoulder-like event at $q \approx 0.008\text{\AA}^{-1}$; and most significantly, the high q region with slope -1, a classic feature of rod-like complexes. The paper also discusses the importance of the downturn at $q > 0.2\text{\AA}^{-1}$ and uses the Guinier law

5.3 Results and Discussion

(see subsection 5.3.2) to calculate the HA chain radius, in this case approximately 0.25nm (Horkay et al., 2012).

5.3.2 The Guinier-Porod Model

The Guinier-Porod model can be used to fit SANS data from scattering polymer chains. For the sake of simplicity, these chains will be regarded as cylinders of measurable length and radius. This model was selected because of the similarities in the relationship between q and $I(q)$. A cursory observation of the curve (figure 5.6) shows a good correlation between the raw data and model.

The general Guinier law, with intensity $I(q)$, Guinier scale factor G , radius of gyration Rg , scattering vector q , dimensionality parameter dim (defined as $3 - s$), and dimensional variable s , is given by

$$I(q) = \frac{G}{Q^s} \exp \left[\frac{-Q^2 R_g^2}{3 - s} \right] \quad \text{for} \quad q \leq q_1 \quad (5.3)$$

$$I(q) = \frac{D}{Q^m} \quad \text{for} \quad q \geq q_1 \quad (5.4)$$

For the cross section of a randomly oriented cylinder of radius R , $Rg = \frac{R}{\sqrt{2}}$, which we would expect of HA-t. Similarly, the radius of a sphere is given by $Rg = R\sqrt{\frac{3}{5}}$, and the cross section of a sheet or lamellae of thickness T , is given by $Rg = \frac{T}{\sqrt{(12)}}$.

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The Porod function yields

$$q_1 = \frac{1}{R_s} \sqrt{\frac{(m-s)(3-s)}{2}} \quad (5.5)$$

Combining both the general Guinier law and Porod function

$$D = G \exp \left[\frac{-Q_1^2 R_g^2}{3-s} \right] q_1^{(m-s)} = \frac{G}{R_g^{(m-s)}} \exp \left[-\frac{(m-s)}{2} \right] \left(\frac{(m-s)(3-s)}{2} \right)^{\frac{(m-s)}{2}} \quad (5.6)$$

This is known as the Guinier-Porod function, and is the mathematical expression that was used to fit raw SANS data in the following figures.

The generalised Guinier law states that 3D spherical objects return $s = 0$, 2D symmetrical objects such as rods return $s = 1$, and 1D symmetrical objects (for example sheets or lamellae return $s = 2$. The dimensionality parameter ($dim; 3-s$) evident in Equations 5.5 and 5.6 therefore, naturally returns values of 3 for spherical objects, 2 for rods and 1 for sheets.

The Porod exponent is related to the radius of gyration, itself a function of crosslinking density, as seen in equations 5.5 and 5.6. It has been demonstrated that the radius of gyration can be used to estimate crosslinking density. It can be theorised, therefore, that hydrogels with a similar Porod exponent m , are crosslinked to the same degree (Elmer, 2007). The radius of gyration estimate should be approached with caution, however, as this value is calculated in the q range before the plateau of the curve begins to decrease (see arrow, figure 5.6).

5.3 Results and Discussion

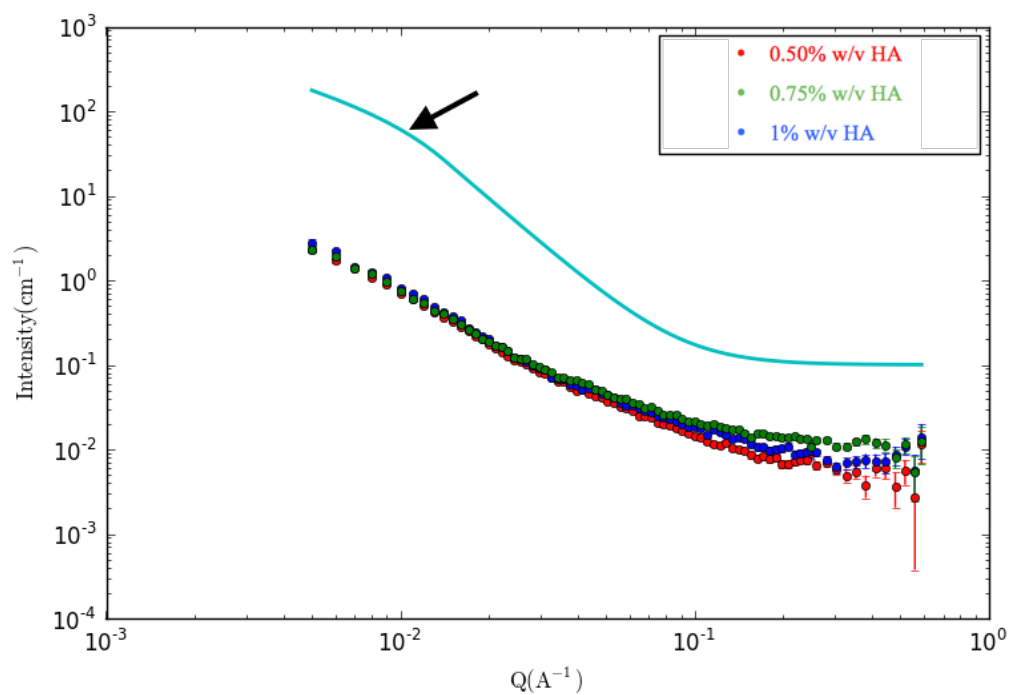
Table 5.2: The Guinier-Porod model was used to calculate the radius of gyration (and therefore the scale of scattering objects), Guinier scale G , and dimensional variable, s , of HA-t hydrogels at 1.00%, 0.75% and 0.50% w/v.

	Hyaluronan Concentration (w/v)		
	1.00%	0.75%	0.50%
Background Intensity (cm^{-1})	0.008	0.012	0.006
Porod Exponent, m	1.965	1.905	1.934
Radius of Gyration, R_g ($\text{\AA}$)	130.140	157.200	134.310
Guinier Scale, G (cm^{-1})	0.007	0.036	0.020
Dimensional Variable, s	1.188	0.849	0.937

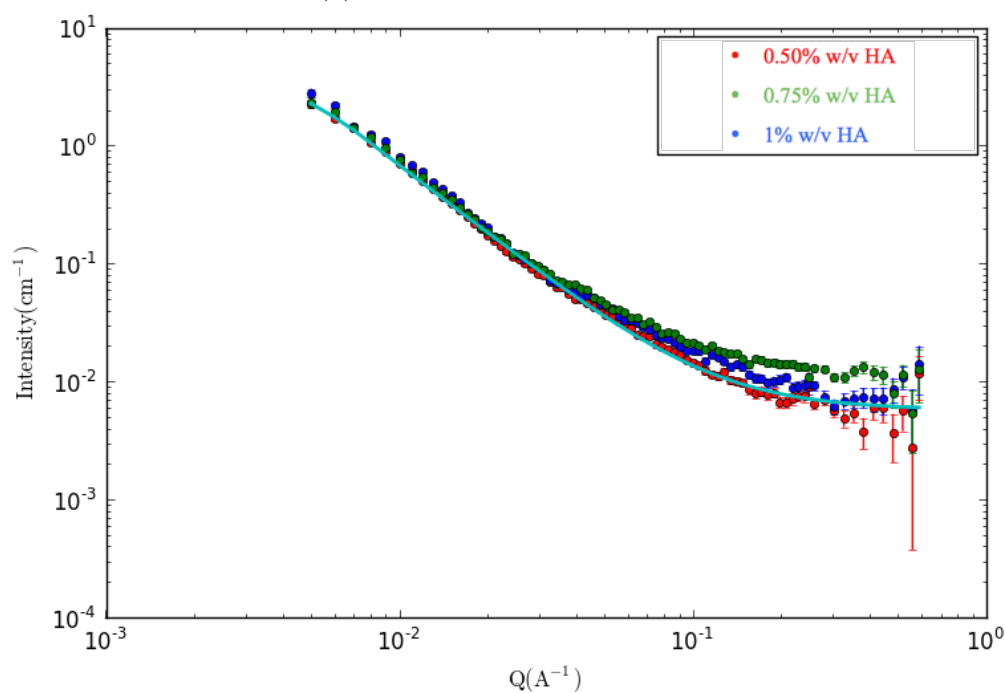
One of the most significant findings of this experiment is that the crosslinking density between HA concentrations do not change significantly. This can be seen in Table 5.2 where the Porod exponent $m \approx 2$ for all concentrations. The Porod exponent has been linked to crosslinking density via the radius of gyration, as discussed by Elmer (Elmer, 2007). The reason for a decrease in stiffness in hydrogels with different macromer concentration is therefore unlikely to be the result of the crosslinking density as suggested by the Guinier-Porod function, although repeats of the results are essential.

Hyaluronan hydrogel structure and polymer chain conformation was first characterised by SANS in 2003, by Buhler and Bou  . The authors used the Sharp-Bloomfield and de Cloizeaux models to fit their data, and calculate the total persistence length LT , which represents the effective rigidity of the polymer chain (Buhler and Bou  , 2003). Although this method suggests a value for persistence length, the model is somewhat primitive, and does not take into account any other structural or conformational parameters offered by the computational models used here.

The importance of electrostatic interactions were also evaluated by Horkay and



(a) The Guinier-Porod Model



(b) Data Fit

Figure 5.6: The Guinier-Porod model (a) provides a useful estimation about the structure of a scattering object in a solvent. Scattering events from 1.00%, 0.75%, and 0.50% w/v (blue, green and red respectively) HA-t hydrogels were analysed and fitted to the mathematical model (b). In a manner similar to the Debye-Bueche, the 0.50% w/v HA-t hydrogel appears to fit the model closely.

5.3 Results and Discussion

coworkers, although importantly, the crosslinking of HA was performed at pH 9, therefore any comparison must be approached cautiously (Horkay et al., 2006). The data do hint at scattering similarities, noted in Figure 5.7. In another study, the same group use a power law in the form of Equation 5.2 to calculate the polymer chain radius, and it is the first example of a directly comparable data set (Horkay et al., 2012).

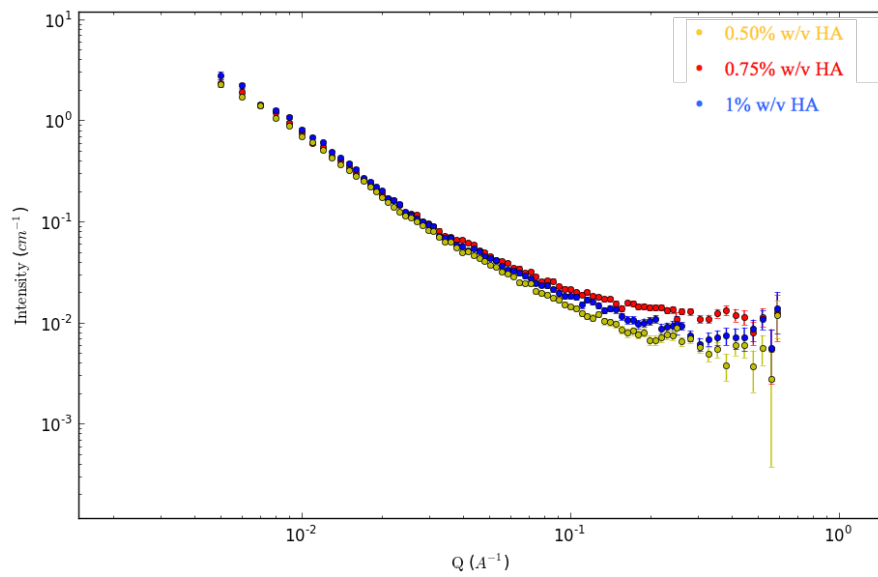
1. At low q , large HA chain clusters are distinct, and their structure is independent of concentration
2. At high q , the scattering intensity is governed by osmotic concentration fluctuations and thermal fluctuations

In this region, $I(q)$ varies with concentration as $c^{0.5}$. Mathematically this can be described by

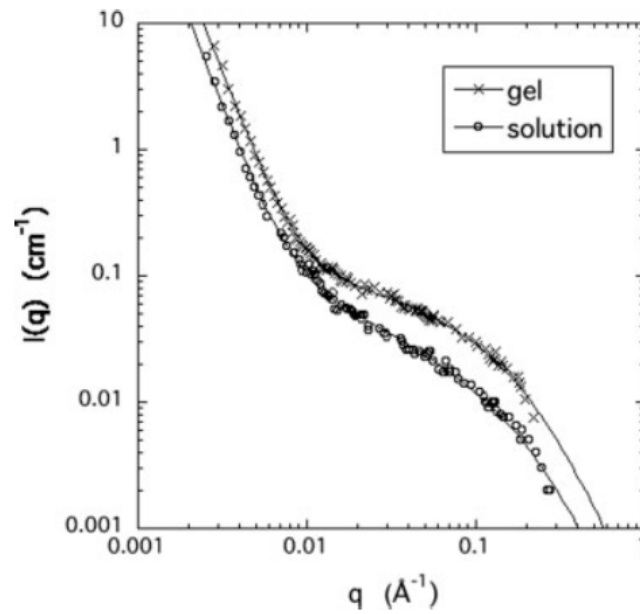
$$I_{os}(q) \propto c^{\frac{3-2D_f}{3-D_f}} \quad (5.7)$$

where the fractal dimension $D_f = 1$ for rigid rods. Hence $I(q)$ should vary by $c^{0.5}$ (Horkay et al., 2012). Although the scattering events in our data vary somewhat, the scattering variation at a higher q is possibly also due to osmotic concentration fluctuations.

Characterising the structure of HA hydrogels is a non-trivial exercise. The present study was limited by the q range, which could distort the mathematical fit of the data. However, the data presented herein provide useful approximations of several important microscopic properties. For example, we know that our HA hydrogels



(a) HA at pH7



(b) HA at pH9

Figure 5.7: Scattering effects of HA hydrogels at pH7 (a) and pH9 (b) show some curve similarities, especially in the $0.01 < q < 0.1$ range. Hydrogel and solution refer to crosslinked and uncrosslinked HA hydrogels respectively. (b) Adapted from (Horkay et al., 2006).

5.3 Results and Discussion

are stiff rod-like structures with clusters of chains (probably due to entanglement and non-crosslinked segments). The clusters are distinct and do not interact (where $I(q)$ is proportional to concentration). Most significantly, crosslinking density does not change with concentration. We also show that we can calculate several other parameters such as polymer chain length and radius, although as discussed previously these data should be approached with caution due to the narrow q range selected.

The microscopic characterisation of HA is important for neural tissue engineering, especially to our understanding of the way neural cells interact with their environment. Cells have surface receptors for HA (known as the hyaladherins) including CD44 and RHAMM (receptor for hyaluronan-mediated motility; also known as CD168) (Turley et al., 2002). CD44 and RHAMM are expressed extensively throughout the brain, including neural precursor cells and glia (Casini et al., 2010; Liu et al., 2004; Lynn et al., 2001; Preston and Sherman, 2012). Significantly, RHAMM is expressed in the adult mouse SVZ and by migrating neuroblasts in the rostral migratory stream (RMS) (Lindwall et al., 2013).

The importance of microscopic characterisation of HA, therefore, should not be understated. Influencing cellular behaviour *in vitro* requires an in-depth understanding of the synthetic scaffold to accurately mimic the *in vivo* ECM. These properties include the polymer chain length and radius, and the degree of crosslinking etc.

5.4 Summary

The present study presents – to the best knowledge of the author – the first SANS characterisation of a fully PEG-DA crosslinked HA hydrogel, an important addition to our understanding of these materials. Several papers have used SANS studies to characterise the microstructure of HA, albeit uncrosslinked (Bonnet et al., 2008; Buhler and Boué, 2003; Horkay et al., 2012; Morfin et al., 2011; Nyström et al., 2010).

From the current experimental data, we know that HA-t presents both chain-like segments, clusters of chains, and uncrosslinked monomers. Each one of these microscopic features will lead to differences in the way the cell responds to the matrix. This effect is unlikely to produce macroscopic effects on the encapsulated cells, but may lead to differences on a much smaller length scale, such as cell surface receptor clustering in the chain-rich regions of the hydrogel. It is widely accepted that receptor clustering directly influences important mechanisms such as cell adhesion and somal migration.

Small angle neutron scattering (SANS) is a highly sensitive technique that can be used to characterise the microstructure of hydrogels. We show here for the first time that HA-t macromer concentration is not proportional to the crosslinking density within the hydrogel. Decreasing the concentration reduces the stiffness by a different mechanism, possibly by increasing the mesh size, thereby allowing the influx of free D₂O. In addition, this study presents for the first time that HA-t hydrogels are rod-like chains that cluster together, producing HA-rich inhomogeneities within the hydrogel. We also know that these clusters do not interact.

Chapter 6

Surface Modification of Polystyrene With Mimetic Peptides that Modulate Differentiation and Neurite Outgrowth

6.1 Introduction

The field of tissue engineering and regenerative medicine is rapidly moving away from simply observing a cell on a material. Current opinion in the field is that in order to produce a tissue that closely mimics the *in vivo* environment, we must also provide the types of signals (whether molecular, mechanical or otherwise) that the cells use to develop properly. A vast number of studies have shown that very specific signalling pathways can be targeted, including those important for adhesion, differentiation, and migration.

Novel peptides have been designed to mimic the neural cell adhesion molecule (NCAM), polysialic acid (PSA) and sialylated NCAM (a post-translational modification of NCAM discussed in Section 2.6); the latter two are completely novel to the field. *In vivo*, these proteins bind to the fibroblast growth factor receptor (FGFr), and mediate several downstream signalling cascades that lead to complex genotypic and phenotypic changes. We show in this chapter that the structure of these peptides can be modelled computationally. Their bioactivity is explored extensively on modified 2D surfaces, namely the adhesion, differentiation and neurite outgrowth of neuronal cells such as the dorsal root ganglia (DRGs), and the neural stem precursor cells (NSPCs) found in the subventricular zone (SVZ). *In vivo*, these cells migrate towards the olfactory bulb via the rostral migratory stream (RMS), where they differentiate terminally into several cell types. The molecular mechanisms by which these molecules bind to the surface are postulated, although are not explored in detail. Chapter 6 should be considered an important prerequisite to Chapter 7, where the effect of mimetic peptides are briefly explored in 3D.

6.2 Materials and Methods

6.2.1 Peptide Synthesis

NCAM mimetic and control peptides were synthesised by Fmoc-mediated solid phase peptide synthesis (SPPS). The protocol was kindly provided by Dr. Roger Tam at the Donnely Centre for Cellular and Biomolecular Research at the University of Toronto. Firstly, a fluorenylmethyloxycarbonyl (Fmoc) protected Wang-glycine (Wang-Gly) resin was added to a filtered peptide synthesis tube and swollen in dimethylformamide (DMF) for 30mins. The resin was deprotected with 20% piperidine in DMF for 2x20mins.

After 40mins the solvent was drained and the resin was thoroughly washed 3x in dichloromethane (DCM). 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluoro-phosphate (HCTU), was prepared in a 3 molar equivalent to resin (e.g. for 2mmol resin prepare 6mmol HCTU). Similarly, 3 molar equivalents of Fmoc-Lys(Alloc) (e.g. for 2mmol resin, prepare 6mmol Fmoc-Lys(Alloc)) was prepared and set aside. To a falcon tube, 10ml DMF was added followed by the prepared Fmoc-Lys(Alloc) and HCTU and mixed thoroughly for 20mins on a rocker.

After 20mins, a small quantity was added to a 200 μ l tube and a drop of picrylsulfonic acid was combined and agitated briefly. 100 μ l of DMF and 100 μ l N,N-Diisopropylethylamine (DIPEA) was used to dilute the picrylsulfonic acid and to catalyse the reaction respectively. A red colour indicates that the resin is completely deprotected. Once deprotected, the dissolved Fmoc-Lys(Alloc) and HCTU was

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combined with the resin. DIPEA was added in a 6 molar equivalent to resin (e.g. for 2mmol resin add 12mmol DIPEA). The reaction vessel was sealed and any excess pressure released. Once sealed, the vessel was agitated overnight.

To ensure that the reaction is complete, a free amine test was repeated (see above). The resin – now coupled to Fmoc-Lys(Alloc) – was loaded into a microwave peptide synthesiser (CEM; Matthews NC, USA). Amino acids were dissolved as per manufacturer's recommendations. The following process of adding subsequent amino acids was automated, and waste/reagent vessels checked every day.

Once complete, the resin was removed completely from the vessel and washed with DCM. The C-terminus of the peptide was initially protected by a solution consisting of 9 molar ratio of DIPEA and 11 molar ratio of acetic anhydride to resin, dissolved in DMF. The reaction vessel was sealed and agitated for 3hrs. The N-alloc group was deprotected using tetrakis (triphenylphosphine) palladium(0) ($\text{Pd}(\text{PPh}_3)_4$) and an excess of dimethylamino trihydroborate (Me_2NBH_3) in DCM in a nitrogen atmosphere. The solution was agitated at room temperature for 24hrs and the free amine test repeated.

A maleimide linker, which would ultimately be used to conjugate the peptide to the material, was prepared by dissolving N,N'-Diisopropylcarbodiimide (DIC) in 6 molar ratio and maleimidopropionic acid pre-mixed in DCM in a 4 molar ratio for 30mins. Once no free amines were detected, the peptide was cleaved from the resin. To cleave the peptide, 12ml trifluoroacetic acid 1.5ml distilled water and 1.5ml triisopropylsilyl (TIPS) were combined and added to the resin and agitated at room temperature for 6hrs. Once precipitated (diethyl ether on ice for 15mins), the product was centrifuged and dried in air. The remaining solid was purified initially

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with a C18 column and different acetonitrile (ACN) concentrations between 0-75% in water. Solutions from each ACN pass were then purified by high performance liquid chromatography (HPLC; Shimadzu, Japan). Different fractions were initially characterised by mass spectroscopy and the most promising fractions were further analysed by ^1H NMR at 500MHz (Varian Mercury). It should be noted that some of the peptides used in the following studies were also synthesised externally, notably by China Peptides, Shanghai, China.

6.2.2 Peptide Structural Prediction

Structural prediction algorithms were used to evaluate tertiary peptide structure of several mimetic peptides. Amino acid sequences were initially uploaded to the PEP-FOLD structural prediction algorithm developed by Maupetit *et al* (Maupetit *et al.*, 2009, 2010) via the Mobyli bioinformatics portal (Alland *et al.*, 2005; Néron *et al.*, 2009). This framework is based primarily on a Hidden Markov Model derived Structural Alphabet that describes overlapping canonical conformations of peptides, generating different models based on conformational probability.

The NCAM mimic FGL (GGEVYVVAENQQGKSKAGG), the polysialic acid mimic PSAm (SSVTAWTTGG) and the novel sialylated NCAM mimic PSAm-SG5-FGL (GSSVTAWTTG-SG5-GEVYVVAENQQGSKA) were subject to the same computational boundary conditions. The presence of extra glycine residues in FGL and the SG5 linker in the PSAm-SG5-FGL are due to cell binding optimisation for cell culture, and were included for completeness. PEP-FOLD generates five ‘most probable’ conformations, the first of which was then interpreted by Visual Molecular Dynamics (VDM), developed by Humphrey *et al* (Humphrey *et al.*, 1996).

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Figures are presented as a tertiary structure model with amino acid structure, and a surface rendering model. Conformational probability was represented by a conformational probability and Ramachandran plot, both available as a VMD extension.

6.2.3 Functionalisation of Two Dimensional Polystyrene Surfaces

Prior to functionalisation, the peptide was conjugated to a maleimide group, which will enable the peptide to adhere to the polystyrene surface. Once purified, the peptide solutions were dissolved in several concentrations depending on application. For general bioactivity testing, a range of concentration between 20-100 μ M were used. Each lyophilised peptide was weighed out on an analytical balance and dissolved in dH₂O. The solutions were sterile filtered through a 0.22 μ m syringe filter and stored at 4°C, or used immediately.

Polystyrene culture plates (Nunc) were coated with a solution of poly-D-lysine (PDL; Sigma) dissolved in dH₂O, and allowed to adhere overnight at 4°C. Prior to peptide adhesion, the solutions of PDL were aspirated followed by the addition of the peptide solutions in various concentrations (see above). Peptides were allowed to adhere to the PDL coated plates overnight at 4°C. Solutions were aspirated immediately prior to cell culture. No washing was performed between steps to prevent the loss of any adhered peptides.

6.2 Materials and Methods

6.2.4 Cell Culture

Rat Dorsal Root Ganglion Explant Culture

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (University of Toronto, Toronto, Canada). Embryonic day 15 (E15) Sprague-Dawley rats were perfused. Dorsal root ganglia were dissected, removed from the spinal column, and pooled in serum-free Neurobasal culture medium (Gibco). All dissection was performed within a HEPA filtered air column. Once removed, individual explants were transferred to functionalised polystyrene surfaces or hydrogels depending on the experiment. Three explants were transferred to each well of a 24 well plate. Control surfaces were generally non-functionalised polystyrene.

Mouse Neural Stem Precursor Cell Isolation

Wild type CD1 mouse pups (Charles River Laboratories, Margate, UK) were sacrificed at post-natal day 4 (p4). Animals were decapitated at the brain stem, and the skin was incised dorsorostrally along the midline. The cranium was similarly incised dorsorostrally from the foramen magnum, and resected with two small incisions dorsolaterally along the temporal area to aid removal. The brain was removed after separation from the olfactory bulb and kept in serum-free normal medium consisting of DMEM F-12 Ham (Sigma) supplemented with 1% pen/strep. 500 μ m coronal sections were produced by a tissue chopper and examined under a microscope to identify the lateral ventricles (LV). Approximately 2-3 sections (see Figure 6.1) were transferred to and maintained in normal medium at 0°C. The

6.2 Materials and Methods

lateral ventricles were identified and the subventricular zone (SVZ) dissected with a sterile large gauge needle, and transferred to a 15ml tube in normal medium at 0°C.

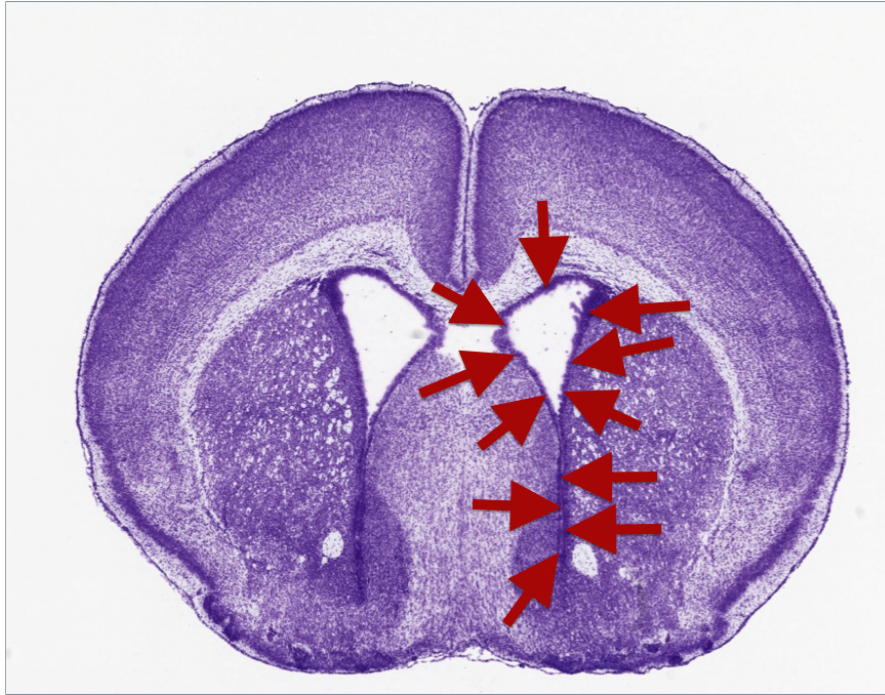


Figure 6.1: Coronal section of the mouse brain showing the location of the subventricular zone (arrows). Adapted from (AllenInst, 2012).

Once dissected, NSPCs were dissociated by 100% accutase (Sigma) for 10mins at 37°C. This step was repeated if dissociation was not complete. Dissociated NSPCs were counted with Trypan Blue and cultured on poly-HEME coated flasks for 4-5days in normal medium, supplemented with $20\mu\text{g ml}^{-1}$ basic fibroblast growth factor (bFGF) and $20\mu\text{g ml}^{-1}$ epidermal growth factor (EGF).

6.2 Materials and Methods

Mouse Neural Stem Precursor Cell Culture

Mouse NSPCs isolated from the SVZ of 6week old mice were maintained as neurospheres for 7days after isolation, and cultured in serum-free Neurobasal medium supplemented with pen/strep, L-Glutamine, EGF, FGF and heparan (Hep). Cells were passaged again, and maintained for a further 7days in the same conditions (P1). These P2 neurospheres were then dissociated and plated at a density of $1.5 \times 10^4 \text{cm}^{-2}$ within a standard TCP culture plate (96 or 24well). Surfaces were functionalised by adsorption of FGL, cyclic FGL (cFGL), the polysialic acid mimic PSAm, a novel peptide consisting of PSAm joined to FGL by a nonreactive linker SG₅, and RGD peptides onto a $50 \mu\text{g ml}^{-1}$ poly-D-lysine (PDL) coating. The negative control was PDL coating only (no peptide).

Mouse Neural Stem Precursor Cell Differentiation

Cells were maintained in two culture medium conditions after dissociation: untreated (condition 1; EGF, FGF and Hep only) or treated, where the medium was supplemented with a 10x concentration of dibutyryl cyclic adenosine monophosphate (dbcAMP) and interferon gamma ($\text{IFN}\gamma$) – 1mM and 100ng ml^{-1} final concentration respectively – after 1day in condition 1. Culture medium was not changed completely at this stage.

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6.2.5 Immunocytochemistry

Rat DRG Neurite Outgrowth

Following the DRG culture and neurite outgrowth assay, explants were fixed for a minimum of 1hr in 4% paraformaldehyde at 4°C. Plates were washed 2x carefully in DPBS. It should be noted that significant fluid shear stresses at this, or any other wash steps, could lead to detachment of the explants from the plate. Ganglia were permeabilised in 0.1% Triton X-100 in DPBS for 30mins at room temperature. Plates were washed 2x in DPBS at room temperature, and blocked with 10% FBS diluted in DPBS overnight at 4°C.

Primary antibodies were mouse anti-NF-200 (Neurofilament 200), diluted 1:2000 with 3% goat serum and 1% BSA in DPBS, and phalloidin anti-actin which was added to the explants at 4°C overnight. Ganglia were washed carefully for 3x5mins in DPBS and incubated with goat anti-mouse secondary Alexa Fluor®488, diluted 1:500 in DPBS for 2hrs at room temperature. After 2hrs, explants were washed finally 3x5mins in DPBS and counterstained with DAPI Fluoroshield®(Sigma) for 20mins prior to imaging on a confocal microscope (Zeiss).

Mouse NSPC Differentiation and Neurite Outgrowth

After a 9day culture period, mouse NSPCs were fixed for a minimum of 1hr in 4% paraformaldehyde at 4°C. Cells were washed for 2x5mins at room temperature in DPBS prior to permeabilisation, and blocked with 0.1% Triton X-100 and 5% BSA diluted in DPBS for 1hr at room temperature. A rocker was used to improve permeabilisation. After 1hr, plates were washed 2x5mins in DPBS and incubated

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with primary chicken anti- β III-Tubulin (Abcam) diluted 1:2000 in DPBS, with 1% BSA and phalloidin anti-actin at 4°C overnight. After approximately 24hrs, cells were washed 3x5mins in DPBS at room temperature and incubated with goat anti-chicken Alexa Fluor®633 (Invitrogen), diluted 1:500 in DPBS and 1% BSA for 2hrs at room temperature. Plates were finally washed 3x5mins in DPBS and counterstained with DAPI Fluoroshield (Sigma) for 20mins prior to imaging on a confocal microscope (Zeiss).

6.3 Results and Discussion

6.3.1 Structural Prediction of the NCAM-Mimetic Peptide, FGL

To gain an understanding of the possible structure of a synthetic NCAM mimicking peptide (FGL; GGEVYVVAENQQGKSKA), a polysialic acid mimicking peptide (PSAm; SSVTAWTTGG), and a novel sialylated NCAM mimicking peptide (PSAm-SG₅-FGL; GSSVTAWTTG-SG₅-GEVYVVAENQQGKSKA), their amino acid sequences were uploaded to the PEP-FOLD structural prediction algorithm developed by Maupetit *et al* (Maupetit et al., 2009, 2010). This was accomplished via the Mobyli bioinformatics portal (Alland et al., 2005; Néron et al., 2009), as discussed in the Methods section above. The data generated by the PEP-FOLD computation were then interpreted by Visual Molecular Dynamics (VMD), a freeware software package developed by Humphrey *et al* (Humphrey et al., 1996).

Predicting peptide structure is notoriously troublesome, due to the complexities of peptide-peptide interactions. PEP-FOLD produces several conformational models based on the computation of complex algorithms. These conformational algorithms compute the angles between adjacent peptides, in addition to modelling secondary and tertiary folding interactions. The probability of a specific peptide conformation can also be calculated to statistically verify the data; the Ramachandran plot calculated the allowable bond angles between N and C terminal carbon atoms, represented respectively by Phi (Φ) and Psi (Ψ) (figure 6.2).

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At the time of writing, no existing literature was found confirming the structural characteristics of FGL, PSAm, or PSAm-SG₅-FGL. These data, therefore, provide the first insight into the tertiary structure of these sequences *in vitro*. Very few papers found coupled bioactivity with structural data, although these techniques consider the entire fibronectin domain rather than the specific FGL sequence (Neiiendam et al., 2004). Indeed, modelling the entire FN-III domain may not represent the structure of FGL entirely accurately.

Although these studies – that used NMR to predict protein structure – do confirm that FGL exists as a loop, it was not confirmed with any computational models. Some slight structural differences exist as expected, although the helix loop remains the dominant structural feature, most likely flanked on either side by a β -sheet.

Similarly, no data exist establishing the theoretical structure of the PSAm peptide and the novel sialylated NCAM mimetic peptide PSAm-SG₅-FGL. Although limitations exist with these mathematical predictions, they do shed some light on the amino acid residues available for cell binding. Significant attention has been awarded to the structure and function of NCAM in its entirety: homophilic NCAM interactions were investigated by (Kasper et al., 2000) and heterophilic interactions – with receptors such as FGFr – were examined by (Kochoyan et al., 2008). Once again, these studies do not examine specific sequences, but rather consider the entire NCAM molecule.

Consider PSAm-SG₅-FGL. Larger sequences such as these often fold into complex structures that may hinder cell-peptide interactions. Without predicting their tertiary structure (albeit with some error), we do not know whether a neuron will have access to the peptide. Referring to Figure 6.4, we note that the PSAm portion

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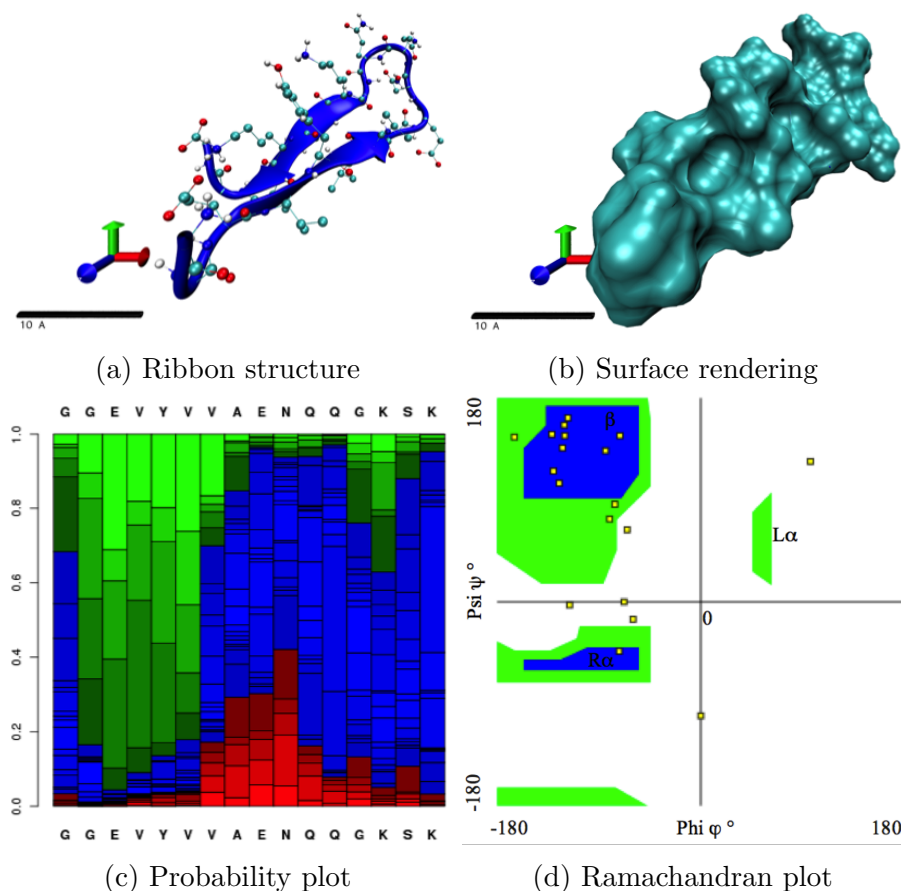


Figure 6.2: The structure of the NCAM mimic FGL was interpreted by PEP-FOLD, and visualised in VMD. Secondary structure ribbons with associated side groups, and a surface-rendering model are presented. Probability plot displays the probability (*y-axis*) of each structural alphabet at each residue position (*x-axis*), and correspond to helical (red), extended (green), and coil (blue) conformations. Ramachandran plot shows allowable (blue) and generously allowable (green) bond angles (Φ , Ψ) in degrees. Areas corresponding to left and right-handed alpha helices ($L\alpha$ and $R\alpha$ respectively), and β sheets, are also shown.

of the sequence does not hinder the tertiary folding of FGL. This is likely due to the non-reactive crosslinker SG₅ that was specifically incorporated to reduce the probability of binding inhibition by PSAm. This figure should be approached conservatively, as the Ramachandran plot suggests that several residues lie outside the allowable bond angle. This infers that the predicted structure of this sequence

6.3 Results and Discussion

may not represent the true *in vitro* or *in vivo* conformation of this sequence.

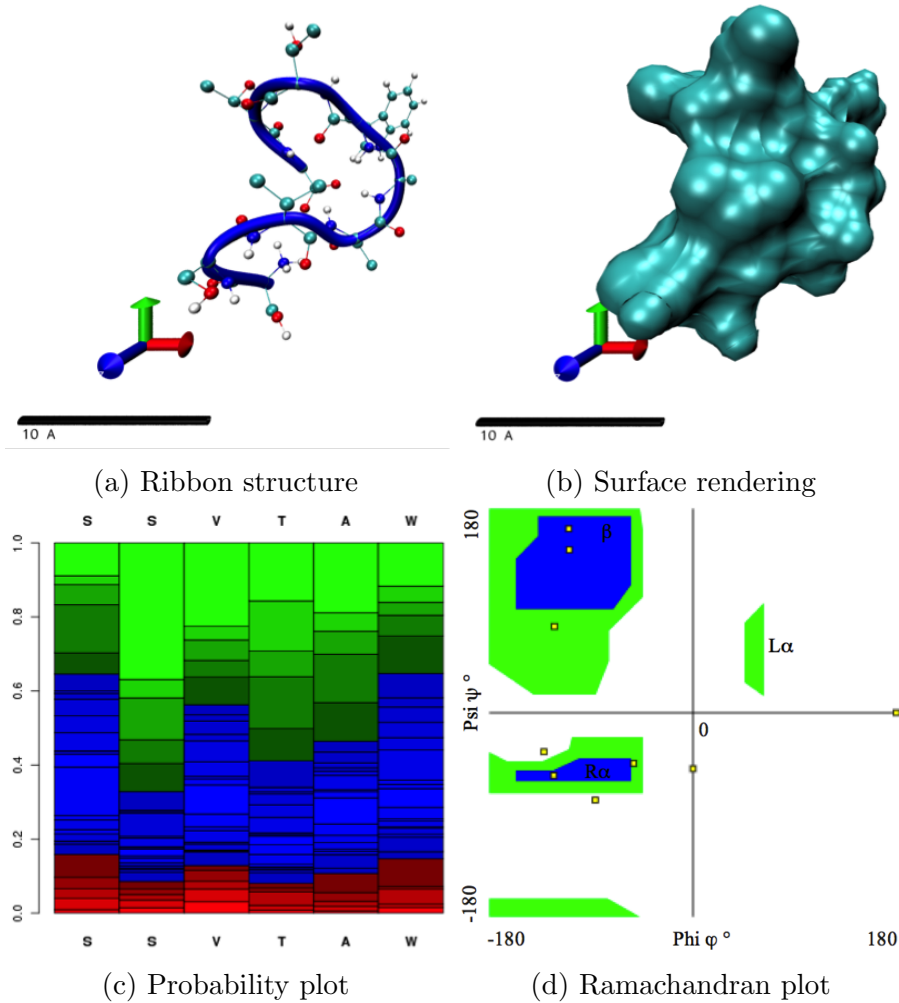


Figure 6.3: The structure of the polysialic acid mimic PSAm was interpreted by PEP-FOLD, and visualised in VMD. Secondary structure ribbons with associated side groups, and a surface-rendering model are presented. Probability plot displays the probability (*y-axis*) of each structural alphabet at each residue position (*x-axis*), and correspond to helical (red), extended (green), and coil (blue) conformations. Ramachandran plot shows allowable (blue) and generously allowable (green) bond angles (Φ , Ψ) in degrees. Areas corresponding to left and right-handed alpha helices ($L\alpha$ and $R\alpha$ respectively), and β sheets, are also shown.

Another important consideration, is the theoretical interaction of PSA and NCAM *in vivo*. PSA is an important regulator of NCAM binding, inhibiting neurite

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and somal migration most likely via steric inhibition, or modulation of the ECM space (Bonfanti, 2006). The mimetic peptide presented herein (Figure 6.3) does not resemble naturally occurring polysialic acid, which exists natively as a homopolymer of acetylneuraminic acid (Bonfanti, 2006). As we will see later in this chapter, PSA mimics do elicit bioactivity by neural progenitors and neurons, although the exact mechanism remains unclear, especially in light of the structural data presented here. The effect of these mimetic peptides on neural progenitors will be discussed later in this chapter, but it is important to note that any bioactivity, especially if evoked by the PSA mimic, is unlikely to be comparable to native PSA.

6.3.2 Peptide Synthesis and Purification

Here a brief examination of the data confirming peptide purity will be presented. At the time of writing, this data represent an FGL peptide that was synthesised externally by China Peptide, a synthesis company based in Shanghai, China. After extensive bioactivity testing with this batch, a sequencing problem was identified, where the entire peptide was coupled in reverse (e.g. [from C to N terminus] RGD, rather than [from C to N terminus] DGR). Additionally, the maleimide linker was attached to the wrong terminus. The conformational change was confirmed by modelling (not shown). Thus, the bioactivity of this FGL batch is non-existent (due to the scrambled conformation), although the purity and mass spectroscopy data has been included for completeness. Figure 6.5 shows that a pure peptide was produced. Subsequent mass spectroscopy (Figure 6.6) confirmed that the mass of the peptide was identical to the predicted mass.

To confirm the mass of the synthesised peptide, mass spectroscopy was used to

6.3 Results and Discussion

Table 6.1: High-performance liquid chromatography data, demonstrating the purity of the synthesised peptide to be >95% pure. Data provided by China Peptide.

Rank	Time (mins)	Concentration (%)	Area
1	8.363	0.3306	56317
2	8.446	0.6709	114265
3	8.599	97.7	16641704
4	9.116	0.4304	73307
5	9.214	0.6355	108237
6	9.321	0.2307	39297

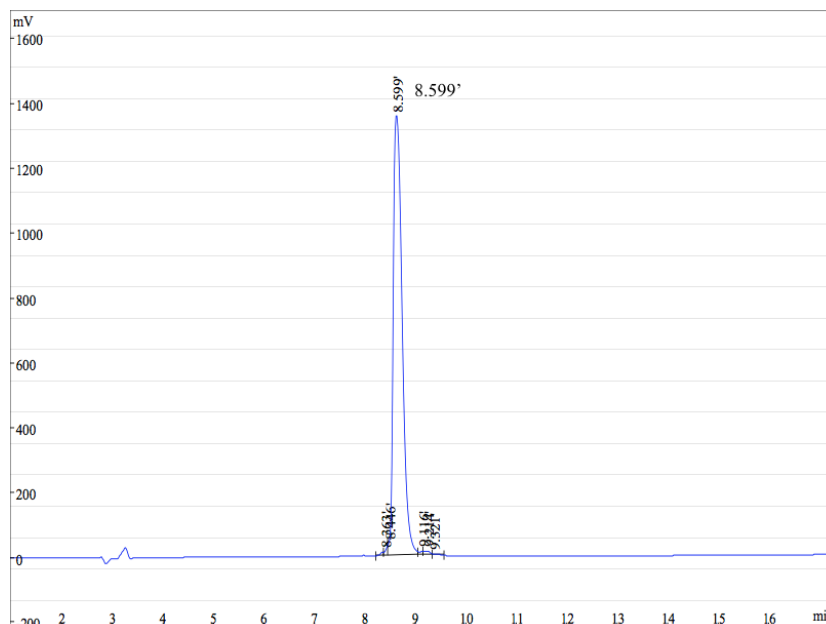


Figure 6.5: High-performance liquid chromatography (HPLC) was used to confirm the purity of the synthesised peptide under the peak at 8.599mins. It should be noted that this data was provided by China Peptide, and was not produced by the author.

correlate with the predicted mass of 1956.15Da. The reader is referred to Figure 6.6, where the measured mass was confirmed as 1957.4 Da.

6.3 Results and Discussion

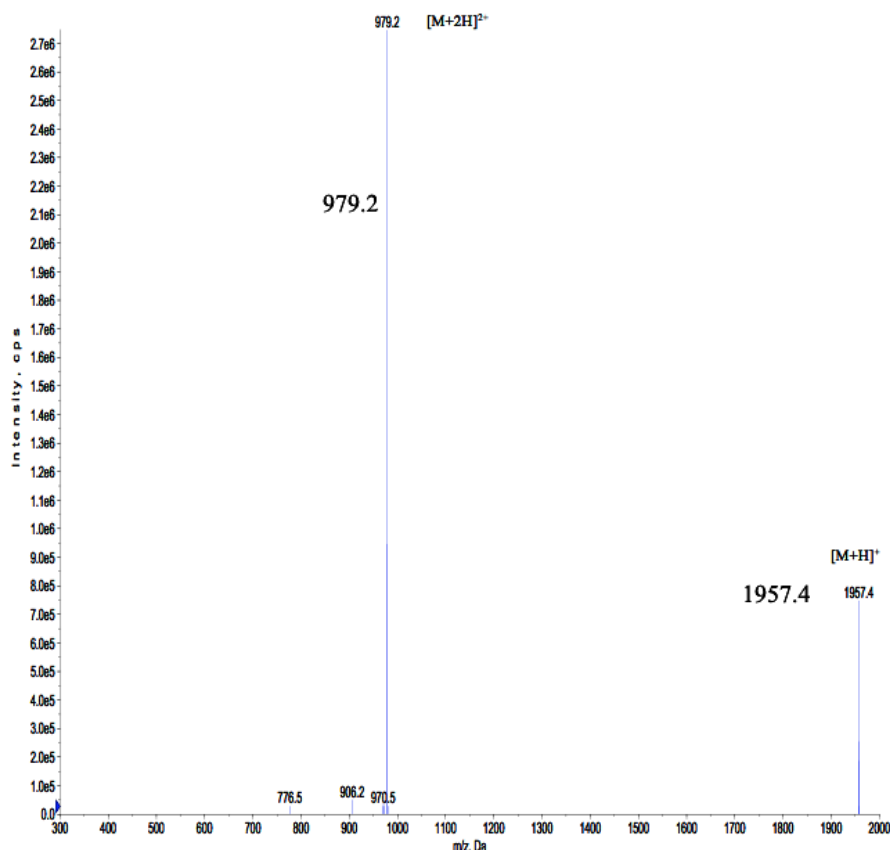


Figure 6.6: Mass spectroscopy was used to confirm the mass of the synthesised peptide, in this case 1957.4Da. It should be noted that this data was provided by China Peptide, and was not produced by the author.

This data was provided by China Peptide and is not the work of the author. This data is essential when confirming hydrogel functionalisation in later chapters. For example, when confirming the covalent binding of FGL to a HA hydrogel by methods such as FTIR, a knowledge of the mass of the synthesised peptide is essential. More simply, however, this data confirms that a compound with the correct mass has been produced, even though it does not confirm whether each amino acid is bound to those adjacent to it. Additional protein characterisation can be accomplished by NMR analysis, although interpretation is complex.

6.3.3 Rat Dorsal Root Ganglion Adhesion and Neurite Outgrowth

To assess the effect of FGL on adhesion, rat dorsal root ganglia (DRGs) were considered a good model, as they respond rapidly to changes in their physical and chemical environment, are simple to culture, and easily imaged at low magnifications. In addition, quantifying neurite length is to some extent simpler when compared with single cell culture. Briefly, DRGs from embryonic day 15 (E15) rats were dissected and removed from the spinal column and maintained in serum-free Neurobasal medium. Tissue culture polystyrene (TCP) plates were coated with PDL and subsequently with peptide solutions including FGL, RGD and a combination of both (RGD+FGL). Peptide solutions were allowed to adhere overnight at 4°C. The control surface was PDL only, without subsequent peptide adsorption. Explants were maintained in serum-free Neurobasal medium supplemented with pen/strep, L-Glutamine and B27 for 24hrs. After culture period, explants were fixed for a minimum of 1hr in 4% paraformaldehyde. Explants were immunostained by a standard protocol for NF-200 and actin with a DAPI counterstain, before imaging on a confocal microscope (Zeiss).

Initial NF-200 staining suggested little to no significant difference in overall neurite length between the control (PDL only) and the surfaces treated with FGL and/or RGD. Poly-D-lysine is known to positively influence adhesion. It would therefore not be unreasonable to expect some neurite outgrowth from these explants. All cultures were also maintained in the medium supplement B27, which has been shown to positively affect adhesion and neurite outgrowth. RGD with FGL significantly

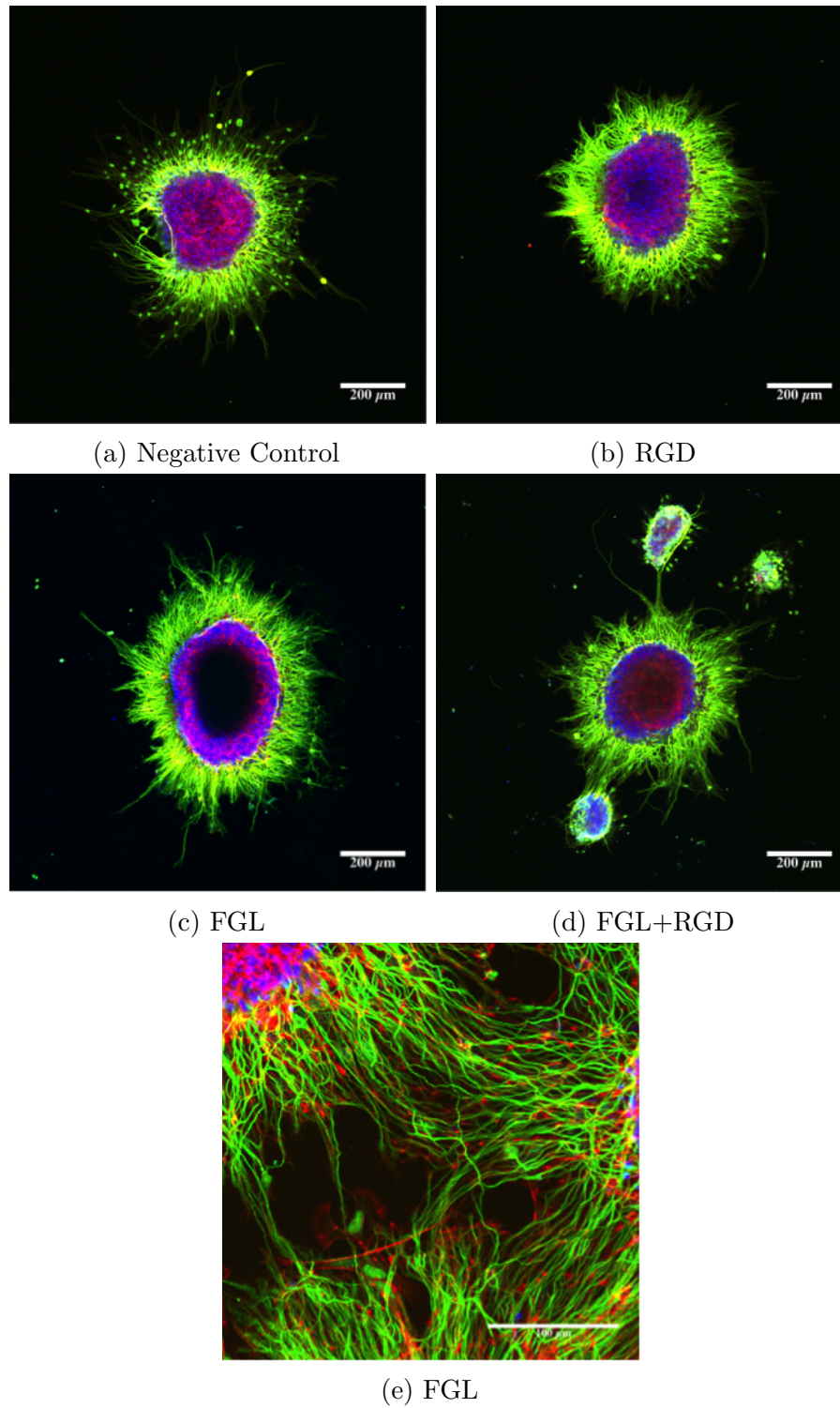


Figure 6.7: E15 Rat DRGs stained for NF-200 (green), actin (red) and DNA (DAPI; blue) after culture on polystyrene surfaces functionalised with FGL and RGD peptides. Quantification (Figure 6.8) shows that functionalisation significantly influenced neurite outgrowth after FGL adsorption. Scale bar 200 μm .

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decreased neurite outgrowth, an observation that was also noted within 3D HAMC hydrogels. Density of neurite outgrowth does seem to increase with FGL adsorption, although this is difficult to quantify and remained statistically inconclusive. A novel method of assessing the area of DRG neurite outgrowth was developed by the author, and can be found in Appendix A. To assess the area of neurite outgrowth, the inner cell mass area (defined by the DAPI positive inner cell mass boundary) was subtracted from the total area of neurite extension. This data was analysed by one-way ANOVA and a Bonferroni post-hoc test to compare pairs of data sets.

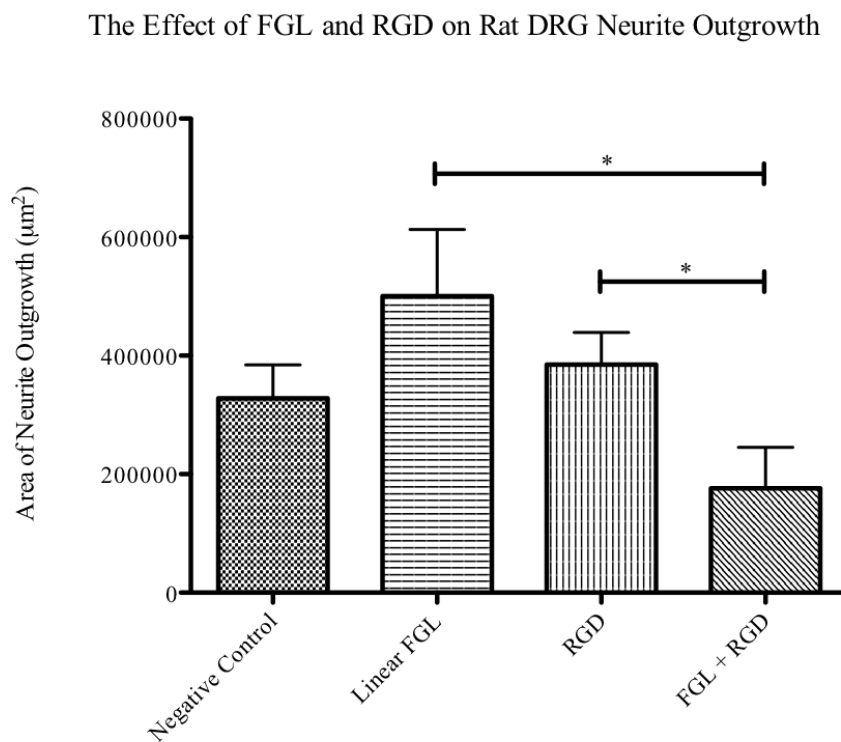


Figure 6.8: The area of neurite outgrowth was calculated by drawing around the inner cell mass, and subtracting this value from the total DRG area. One-way ANOVA analysis (where internal $n = 3$) was followed by a Bonferroni post-hoc test ($P < 0.05$).

6.3 Results and Discussion

It was also hypothesised that NF-200 fluorescence could be used as a quantifiable measure of neurite density. For a complete method, the reader is referred to Appendix B. Two methods were used: firstly by thresholding the entire DRG and measuring the integrated density related to the area of the DRG, and secondly by tracing around the whole DRG (including all processes) and measuring the integrated density in a similar manner. Comparing the two methods, there was no significant difference in accuracy, so the simpler thresholding method was adopted.

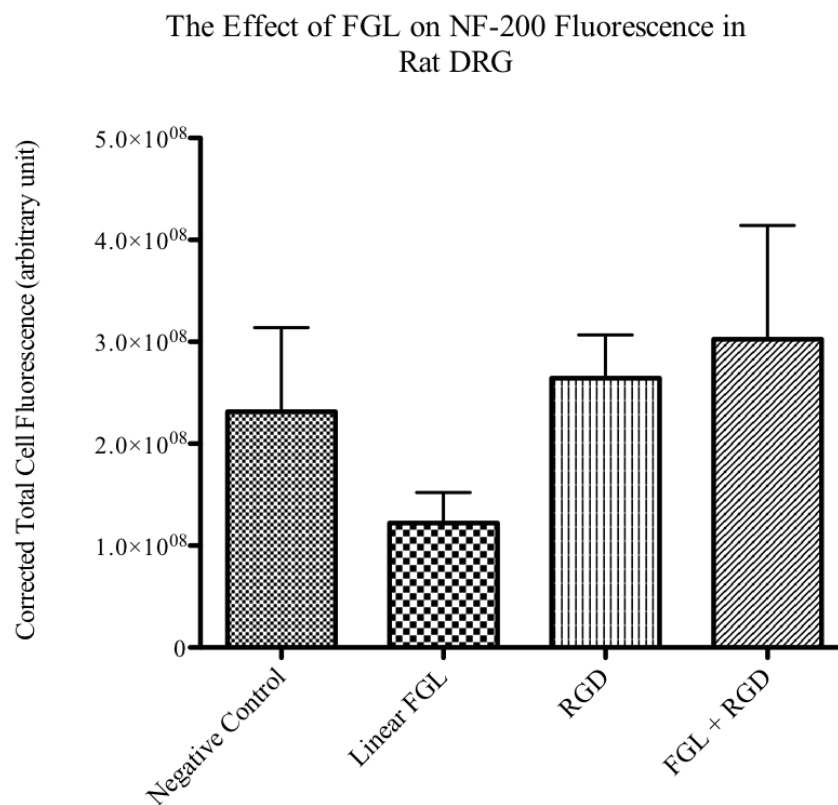


Figure 6.9: The corrected total cell fluorescence (CTCF) was calculated by thresholding individual images and calculating the integrated intensity of fluorescence as a function of DRG area. There was no statistical significance between groups (where internal $n = 3$) in this case.

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An important finding in this study is that FGL appears to decrease neurite outgrowth length, which was also noted in the samples treated with RGD. This result could likely be due to the inhibition of specific signalling cascades essential for neurite outgrowth, probably via integrin-mediated mechanisms. A study by Cox and colleagues in 2001 set out to demonstrate that substrate concentration (in this case fibronectin) could regulate cell polarity and protrusion. The paper suggests that, on intermediate fibronectin concentrations ($3\text{-}10\mu\text{g ml}^{-1}$), integrin-mediated interactions activate Cdc42, Rac1 and RhoA. On higher fibronectin concentrations ($20\text{-}40\mu\text{g ml}^{-1}$), however, only RhoA is activated, inhibiting Cdc43 and Rac1 (Cox et al., 2001).

The inhibition of Cdc42 and Rac1 ultimately inhibits polarisation and migration of the cell body. These mechanisms could be important in DRG neurite outgrowth, as RGD binds to the cell via integrin-mediated interactions possibly inhibiting Cdc42 and Rac1. This finding does not explain the reduced outgrowth on FGL functionalised surfaces as FGL binds directly to FGFr. To confirm, an immunoassay such as a western blot for phosphorylated Cdc42, Rac1 and RhoA could be performed to demonstrate receptor activation or inhibition.

As the first 2D study of FGL functionalisation in this thesis, the aim was to demonstrate a positive effect on neurite outgrowth. Contrary to the hypothesis, FGL did not appear to greatly influence neurite outgrowth. Careful analysis with novel methods developed by the author does suggest that FGL has a role in adhesion of DRGs. The author knows of no study where the influence of FGL was tested in rat DRGs.

6.3.4 FGL, RGD and Related Mimetic Peptides Influence development of Mouse NSPCs

Undoubtedly the most significant aspect of this chapter with respect to novelty and impact, is the role of mimetic peptides of sialylated and unsialylated NCAM in differentiation and neurite outgrowth. The aims of this experiment were to assess the toxicity of these molecules qualitatively, and to ascertain whether they play a role in the development of neural stem cells. Previous studies have probed the interaction of FGL with DRGs, and confirm that the mimic does in fact influence adhesion. At the time of writing, the effect of such treatments have not extended to stem cells, and certainly not NSPCs isolated from the LV of young mice.

A further important distinction to make, is that although FGL has received much attention in the last decade, mimetic peptides of PSA and PSA–NCAM have not. Indeed, the mimetic peptide of PSAm–NCAM was designed for the first time with the help of Dr. Roger Tam at the Donnely Centre for Cellular and Biomolecular Research at the University of Toronto, Canada. This peptide is entirely novel and has not yet been presented to the larger scientific community. Methods of peptide immobilisation and subsequent cell culture were extensively outlined elsewhere. Briefly, following optimisation experiments (data not shown), maleimide-functionalised peptides (FGL, cyclic FGL, PSAm, PSAm–FGL and RGD) were allowed to adhere to poly-D-lysine coated TCP plates. Mouse NSPCs were cultured for 10days in culture medium supplemented either with EGF, FGF and heparan or dbcAMP and IFN γ . Cells were stained with a standard protocol for β III-Tubulin, actin and DNA (DAPI). Fluorescence micrographs are presented

6.3 Results and Discussion

in Figures 6.10 and 6.11. To quantitatively assess the effect of FGL and related peptides on neurite outgrowth, cells that stained positive for DAPI were counted regardless of β III-Tubulin staining (clusters of cells, unless clearly distinguishable, were counted as a single cell). Cells extending neurites were counted only if a clear extension was visible.

These experiments clearly demonstrate that the supplementation of culture medium with dbcAMP and IFN γ significantly increased positive β III-Tubulin staining, suggesting a role in the neuronal differentiation of mouse NSPCs. This result is not novel, and the role of these molecules in differentiation is well established in the field. The lack of cells staining positive only for actin indicates that, by this protocol, cells preferentially differentiate into a neuronal rather than a glial lineage, as expected, although no specific glial markers were stained to confirm this. Additionally, as expected, the proportion of cells staining positive for the neuronal marker β III-Tubulin was significantly reduced in the untreated (no supplemented dbcAMP and IFN γ) medium condition, although a repeat of the experiment is necessary due to the low cell number. Untreated cells displayed no neurite outgrowth on any surface modification condition.

The most significant result in this chapter (and indeed in this thesis) is that surfaces modified with novel NCAM and PSA mimetic peptides significantly increase the proportion of mouse NSPCs staining positive for β III-Tubulin and extending neurites ($P < 0.01$). This study is limited in that the cell number was relatively low, but does provide a good foundation for future studies. Low cell density could affect several important cellular processes, so this would need to be considered carefully in future experiments. It has been established that self-renewal and differentiation

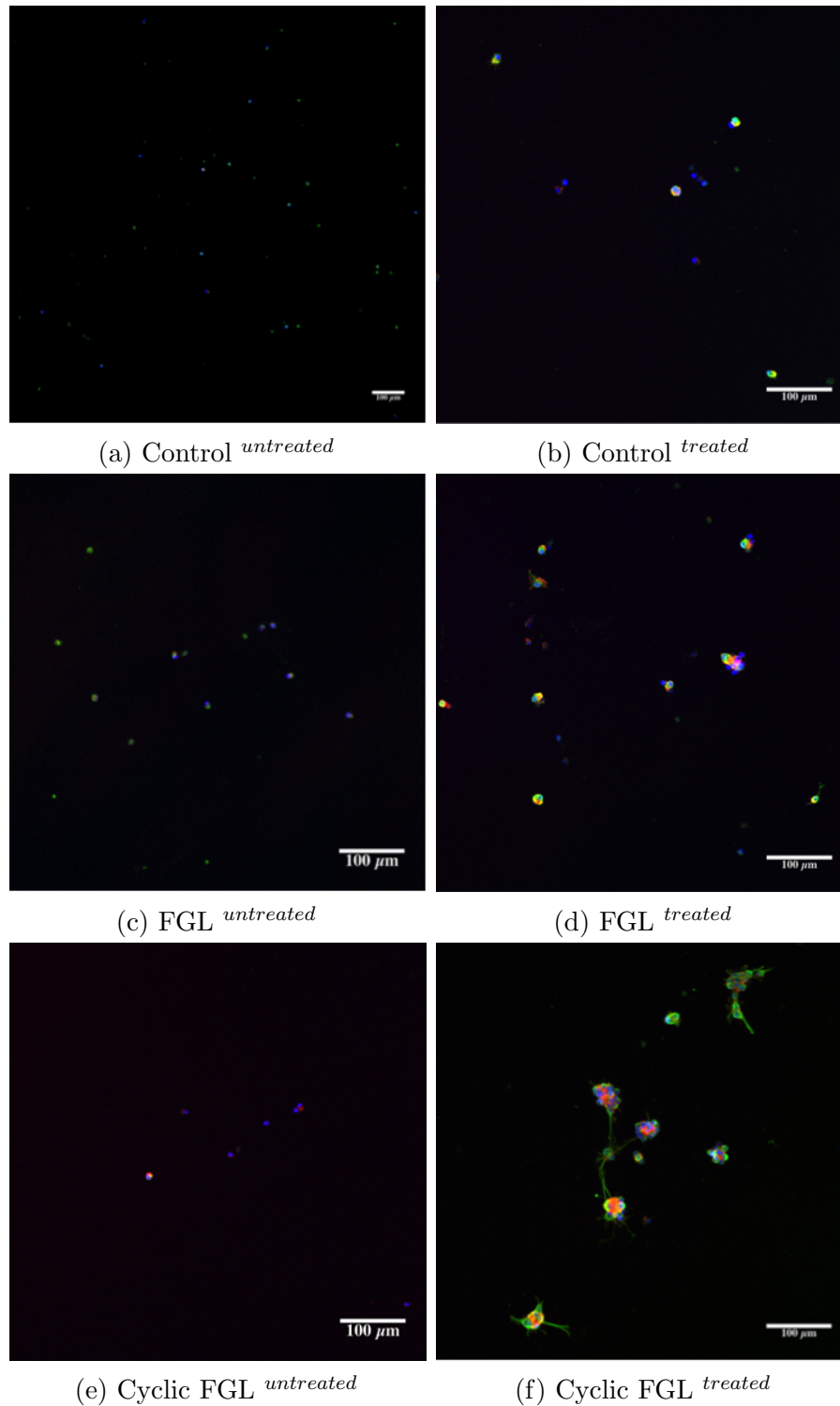


Figure 6.10: TCP surfaces were functionalised with various NCAM and PSA mimicking peptides, including the novel cyclic FGL peptide. Mouse NSPCs were cultured for 10days in medium supplemented with EGF, FGF and Heparan, and (**right column**) subsequently treated for 9days in culture medium supplemented with dbcAMP and IFN γ . Cells were finally stained for β III-Tubulin (green), actin (red) and DNA (DAPI; blue). Scale bar 100 μ m.

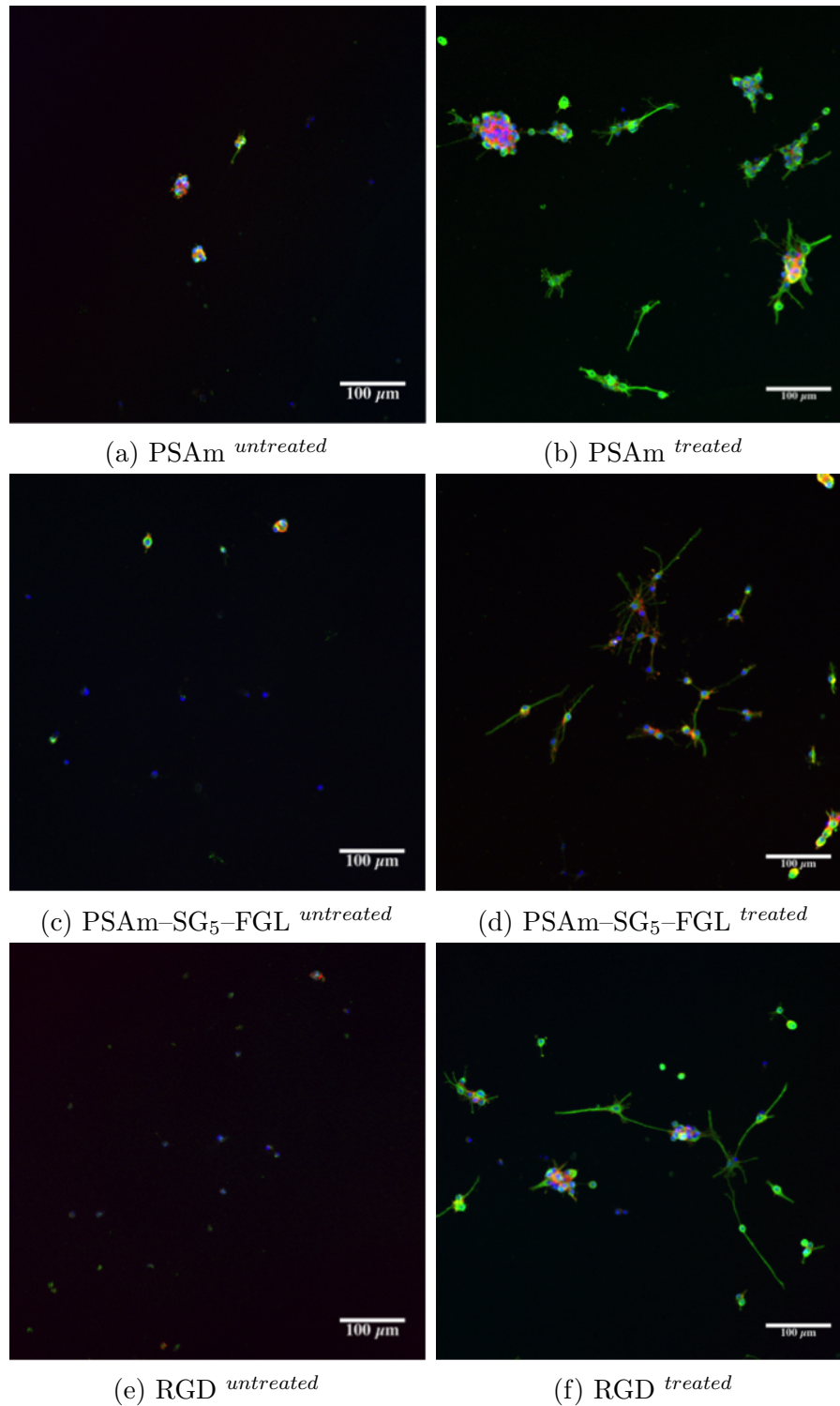


Figure 6.11: TCP surfaces were functionalised with various NCAM and PSA mimicking peptides, including the novel PSAm-FGL mimic. Mouse NSPCs were cultured for 10days in medium supplemented with EGF, FGF and Heparan, and (**right column**) subsequently treated for 9days in culture medium supplemented with dbcAMP and IFN γ . Cells were finally stained for β III-Tubulin (green), actin (red) and DNA (DAPI; blue). Scale bar 100 μ m.

6.3 Results and Discussion

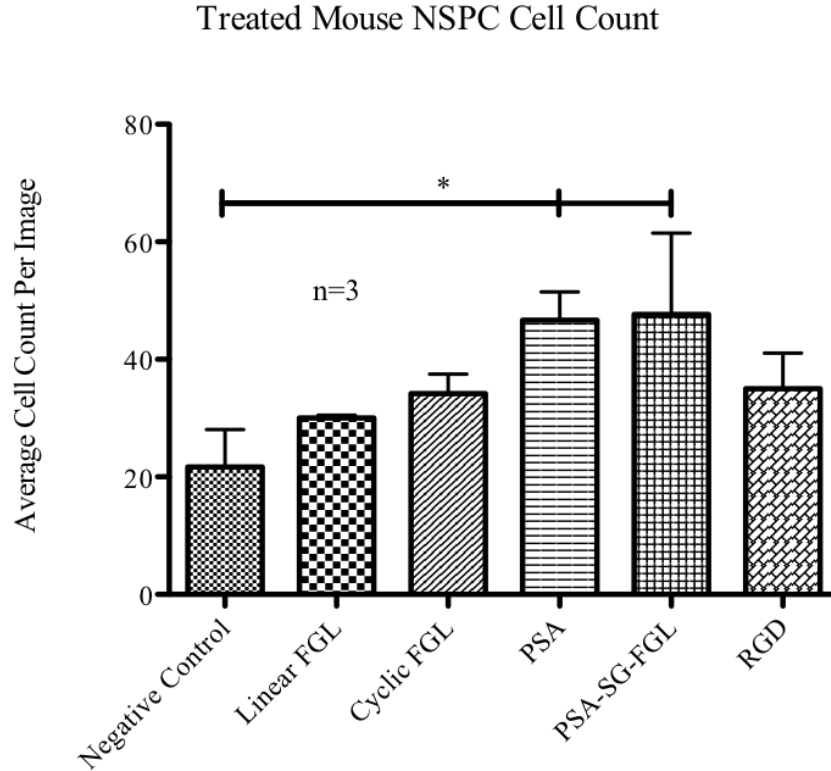


Figure 6.12: Mouse NSPCs treated with dbcAMP and $\text{IFN}\gamma$ were counted prior to neurite outgrowth analysis. The DAPI positive cells from two images per well were counted, and the mean plotted for each group. This was repeated three times ($n = 3$). One-way ANOVA with a post-hoc Bonferroni was performed ($p < 0.05$).

are regulated to some extent by signalling via FGFr (Maric et al., 2007). Although this study did not confirm by western blotting or PCR, it is postulated that the differentiating effects of FGL are mediated through FGFr. Theoretically (and by confirmation with biochemical techniques) FGL has the ability to bind to both FGFr1 and FGFr2 (Kiselyov et al., 2003; Christensen et al., 2006). Excluding PSAm (which has not been tested in any literature to date), all peptides with this functional sequence will be able to bind to any neuron or glial cell with the FGFr1 and FGFr2 receptor.

6.3 Results and Discussion

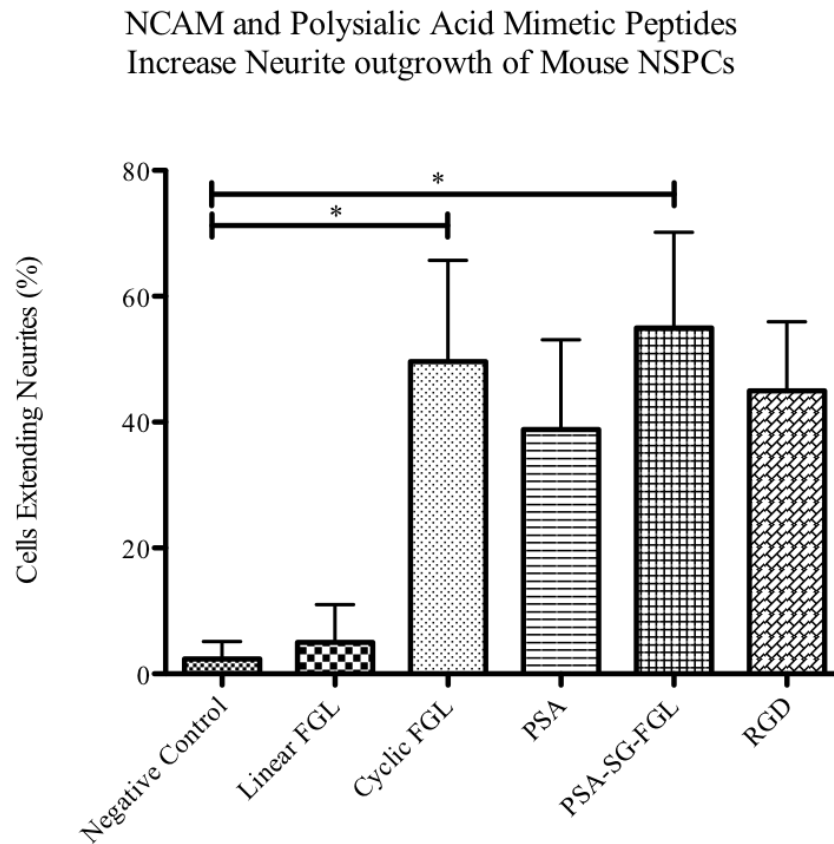


Figure 6.13: Mouse NSPCs were cultured on polystyrene surfaces functionalised with the NCAM mimetic peptide FGL, cyclic FGL, PSAm, and a novel peptide consisting of FGL and PSAm linked by the non-reactive peptide linker SG₅. Cells were treated with dbcAMP and IFN γ for 9days, and the percentage extending neurites were counted ($P < 0.01$).

The downstream signalling cascade is complex, and was reviewed by the author exhaustively in Chapter 3; for the benefit of the reader this has been replicated in Figure 6.14 below. It is clear that FGL has several roles in neural development. Of particular interest here is the Erk1/2, ShcA, FRS2 α , and PLC γ pathways, important in neurite outgrowth, synaptogenesis and cell survival (Neiiendam et al., 2004; Chen et al., 2010).

6.3 Results and Discussion

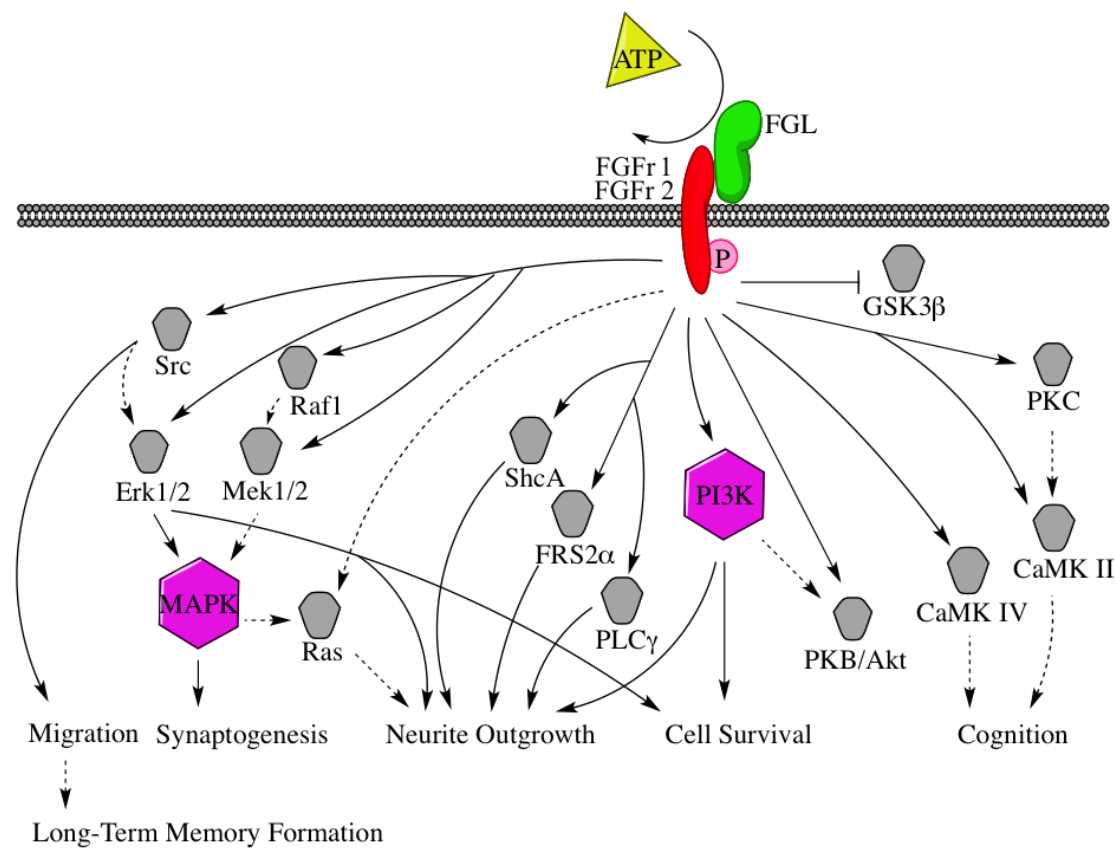


Figure 6.14: A comprehensive delineation of FGL signalling via FGFr. The FGL peptide binds to both FGFr1 and FGFr2, triggering the phosphorylation of several downstream signalling partners. The cellular response to FGL includes migration, synaptogenesis, neurite outgrowth, and cell survival. Solid lines have been confirmed experimentally whereas dotted lines represent hypothesised interactions based on homophilic and heterophilic NCAM data. ©Phillipus J. Putter.

It is important to note that in the present experiments, the phosphorylation of the FGF receptors or any of their downstream mediators was not assessed, and clearly any conclusions regarding FGL–FGFr binding must be accompanied by such data. Notwithstanding, FGL and the related novel mimetic peptides appear to significantly affect the differentiation of mouse NSPCs. Only one study has been identified to date where the influence of FGL on NSPC neurogenesis, proliferation and differentiation were evaluated. In the study by Klein *et al*, FGL significantly

6.3 Results and Discussion

enhanced proliferation and the induction of an oligodendroglial phenotype; crucially, the differential effects of NCAM and FGL were mediated via different receptors (Klein et al., 2014). The data by Klein represent the first study of the role of FGL in the mobilisation of NSPCs from the SVZ. The data presented in this thesis reinforce our understanding of the role of FGFr mediated signalling in differentiation of NSPCs.

6.4 Summary

The proper function of developmental neurobiological mechanisms such as adhesion, differentiation and pathfinding are essential for the accurate formation of the brain. Of specific interest in this chapter was the effect of mimetic peptides of sialylated and unsialylated NCAM, including FGL and the novel PSAm–NCAM mimic developed with Dr Roger Tam at the University of Toronto. In order to study the effect of these molecules in a 3D environment, it is important firstly to quantify their influence in 2D.

We show here for the first time that FGL, cyclic FGL, PSAm and PSAm–SG₅–FGL influence the fate decisions made by mouse NSPCs isolated from the SVZ of young mice. It is important to note that there are significant differences between mouse and human NSPCs, so it would be careless to suggest any clinical relevance at this early stage. These molecules seem to regulate the adhesion of rat DRGs on a 2D surface, possibly by increasing the number of focal adhesions formed. The exact mechanism remains unclear, and the analysis is certainly limited, but is likely mediated by FGFR via several signalling cascades. Improvements could be made by assessing the influence of these molecules on specific genes involved in brain development. A novel method of DRG neurite outgrowth quantification was developed, based on existing protocols; quantifying the area of neurite outgrowth and fluorescence may be a useful technique to quantify cellular bioactivity. In summary, FGL is a good candidate for 3D immobilisation within HA hydrogels. It is important to correctly identify the molecular mechanisms regulating these behaviours if any valuable conclusion is to be made.

Chapter 7

Three Dimensional Neural Cell Culture and Hydrogel Functionalisation

7.1 Introduction

The true power of hydrogel matrices for cell encapsulation and culture lies in the the techniques of 3D cell culture and biofunctionalisation. It is generally accepted that a three dimensional environment can provide a more realistic representation of the native *in vivo* environment. The *in vivo* environment, however, is not simply defined by spatial constraints, but by a vast array of molecules with very specific roles in cellular signalling.

Tissue engineers have exploited hydrogel chemistry to tag on peptides and other active molecules that ultimately interact with an encapsulated neural cell. Consider RGD (Arg-Gly-Asp), a mimetic peptide of fibronectin that is known to provide integrin mediated binding sites to a vast array of cell types. When covalently bound to a hydrogel, these peptides can affect cellular adhesion, spreading and proliferation (Shu et al., 2003). Several groups have also experimented with hydrogel patterning, whereby an active molecule such as a peptide is selectively attached to the material by photopatterning techniques that provide higher spatial control (Luo and Shoichet, 2004b).

Chapter 6 explored the effects of the highly specific mimetic peptides of NCAM, polysialic acid (PSA), and sialylated NCAM, subsequently bound to a 2D polystyrene surface. Chapter 7 aims to build on the 2D data by encapsulating neuronal cells and their precursors in HA hydrogels. Finally, we attempt to demonstrate the functionality of FGL and RGD on rat DRGs when immobilised in a HA hydrogel.

7.2 Materials and Methods

7.2.1 Functionalisation of Hyaluronan–Methyl Cellulose hydrogels

Lyophilised peptides were weighed on an analytical balance and dissolved in distilled water at a range of concentrations between 20 and 100 μ M (the final concentration in the hydrogel). Peptide solutions were filtered through a 0.22 μ m syringe sterile filter and stored at 4°C or used immediately. Peptides such as RGD or FGL were subsequently added to HAMC and blended carefully by pipetting. Peptides were allowed to conjugate covalently to the free carboxylic acid groups on HA overnight at 4°C prior to cell culture, with pure HAMC serving as control. In the case of HAMC, peptides were added during the blending step to ensure sufficient mixing prior to cell culture. For cell culture refer to following sections.

7.2.2 Cell Culture

NTERA2.D1 Cell Line Encapsulation within HA-Thiol Hydrogels

Encapsulated and differentiated NT2 cells were kindly provided by Dr. J George, Oxford University. The NTERA2.D1 cell line (NT2; kindly provided by Dr. E Hill, Aston University) was first cultured in complete medium containing DMEM Glutamax (Gibco), 10% foetal bovine serum (FBS; Gibco) and 1% penicillin / streptomycin (P/S; PAA), which was replaced every 2-3days. Cells were maintained at 37°C and 5% CO₂ until approximately 80% confluent. Once adequate cell

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numbers were obtained, cells were washed in DPBS and cleaved from culture plates using 100% Accutase (Sigma). Cells were counted with a haemocytometer in the presence of Trypan blue. Pellets of equal density (6.6×10^5 cells/pellet) were prepared by centrifugation.

HA-t hydrogels were prepared at 1%w/v, a concentration recommended by the manufacturer. Laminin was added to aid cell adhesion at a final concentration of $20 \mu\text{g ml}^{-1}$. The uncrosslinked hydrogel solution was added to the prepared cell pellet and gently pipetted to homogeneously suspend cells. Solutions were carefully pipetted onto Thermanox cover slips and incubated for 20mins at 37°C to crosslink fully. Finally, complete medium was gently added to the hydrogels and replaced every 2-3days.

NTERA2.D1 Cell Line Differentiation within HA-Thiol Hydrogels

To differentiate the cells, culture medium was aspirated from hydrogels after day 1 and supplemented with $10 \mu\text{M}$ all-trans retinoic acid (RA; Sigma), initially dissolved in DMSO (Sigma). Cells were maintained at 37°C , 5% CO_2 and 100% relative humidity for a minimum of 4 weeks, replacing medium every 2-3days. After 4 weeks, medium was aspirated and replaced with complete medium supplemented with $0.1 \mu\text{M}$ cytosine β -D arabinoside (AraC; Sigma) and maintained for 10days, replacing culture medium every 3-4days.

After 10days, medium was aspirated and replaced with complete medium supplemented with $3 \mu\text{M}$ fluorodeoxyuridine (Sigma) and $5 \mu\text{M}$ uridine 1- β -D-ribofuranosyluracil (Sigma) and maintained for 18days, replacing culture medium every

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4-5days. After this period cells are considered terminally differentiated and may be maintained in complete medium indefinitely, changing culture medium every 4-5days.

Neural Stem Precursor Cell Isolation and Differentiation

For details of NSPC isolation and differentiation the reader is referred to the methods in Chapter 6.

Neural Stem Precursor Cell Encapsulation and Culture

Dissected NSPCs were maintained on non-adhesive poly-HEMA flasks, for 4-5days. After this culture period, neurospheres were collected and dissociated with 100% accutase (Sigma) for 10mins at 37°C and were counted with Trypan Blue (Sigma). Different densities were considered, but generally between $1-2 \times 10^6 \text{ ml}^{-1}$ for encapsulation and $2.8 \times 10^4 \text{ ml}^{-1}$ for 2D controls were optimal. To encapsulate, an uncrosslinked solution of HA-t was prepared to a working macromer concentration of 1% w/v. To this, PEG-DA was combined in three different volumetric ratios of 20, 16 and 12%v/v diluted with degassed water. Hydrogels were transferred into 24 well plates and allowed to crosslink for 35mins at 37°C. After this period normal medium was added to the hydrogels, and either maintained with or without differentiation medium.

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Neural Stem Precursor Cell Differentiation

After encapsulation, the medium was replaced with Neurobasal+ medium, consisting of normal medium supplemented with EGF ($20\mu\text{g ml}^{-1}$) and bFGF ($20\mu\text{g ml}^{-1}$). NSPCs were maintained in standard culture conditions for 6days prior to immunocytochemistry. It should be noted that medium was not replaced during the culture period.

Rat Dorsal Root Ganglia Dissection and Encapsulation

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (University of Toronto, Toronto, Canada). Embryonic day 15 (E15) Sprague-Dawley rats were perfused Dorsal root ganglia from embryonic day 15 rats were dissected, removed from the spinal column and pooled in serum free Neurobasal culture medium (Gibco). All dissection was performed within a HEPA filtered air column. Once removed, individual explants were transferred to culture wells containing pre-blended HAMC hydrogels.

For details of HAMC hydrogel preparation, see Methods within Chapter 4. Briefly, HA and MC were mixed in complete serum-free Neurobasal medium supplemented with pen/strep, L-Glutamine and B27 to form a 6mg/ml solution, which was gently agitated overnight at 4°C. Solution was diluted with complete medium to form a 0.75% w/v hydrogel. DRGs were placed on top of each hydrogel, number depending on well size but generally 3-5 per well. Due to the mechanical stability of HAMC hydrogels, culture medium was not added to the hydrogels after encapsulation, as residual culture medium was sufficient for experiments lasting less than 24hrs.

7.2 Materials and Methods

7.2.3 Immunocytochemistry

NTera2.D1 Differentiation and Neurite Outgrowth

After a 4week differentiation period, hydrogels were washed 1x in DPBS and fixed in 4% paraformaldehyde (Sigma) for 6hrs at RT. Hydrogels were then washed 2x30mins in DPBS on a low frequency rocker. Cells were permeabilised with 5% BSA (Sigma) and 0.2% Triton X-100 (Sigma) dissolved in DPBS for 6hrs before incubation with anti β III-Tubulin and anti-GFAP antibodies at 4°C overnight. Hydrogels were washed 3x30mins with 0.2% BSA and 0.2% Triton X-100 dissolved in DPBS. Hydrogels were then incubated with conjugated secondary antibodies for 5hrs at RT. Hydrogels were washed in the above buffer for 3x30mins and counterstained with DAPI (Fluoroshield; Sigma). Composite images were produced on an epi-fluorescence microscope (Nikon).

Mouse NSPC Viability

After a 6day culture period, cells encapsulated within hydrogels and 2D TCP controls were washed gently with PBS for 20mins at 37°C. Cells were incubated with live/dead stain (Invitrogen) at a concentration of 4 μ M Calcein and 4 μ M ethidium homodimer-1 for at least 40mins at 37°C. The live/dead stain concentration was chosen after optimisation (not shown). Composite fluorescent micrographs were produced on an epi-fluorescence microscope (Nikon) and adjusted on ImageJ.

7.2 Materials and Methods

Rat DRG Viability and Neurite Outgrowth

To assess the viability and neurite outgrowth of rat DRGs after 24hrs in a functionalised HAMC hydrogel, cells were incubated per manufacturer's recommendation with Calcein-AM and DAPI; HAMC does not support extensive washing steps required for more sophisticated immunostaining. After immunostaining, explants were imaged on a confocal microscope (Zeiss).

Calculation of Neurite Outgrowth Area

The method for evaluating the area of DRG neurite outgrowth was analogous to that found in Subsection 6.2.5, and is fully explained in Appendix A.

7.3 Results and Discussion

7.3.1 3D Encapsulation of NTera2.D1 Cells

The ultimate aim of this chapter is to gain an understanding of cell–matrix interactions by using 3D hydrogel culture techniques and peptide biofunctionalisation. As with any material, however, the properties of the material must be carefully optimised to produce a material that is specifically suited to 3D neuronal cell culture. The attentive reader would notice the significant deviation from primary neuronal precursors or stem cells, rather adopting an immortalised teratoma cell type. The NTera2.D1 cell line was chosen for its rapid proliferation and simple differentiation in 2D, although the extent of neurosphere formation in 3D was unknown. These first experiments, therefore, were carried out for two main reasons. Firstly, to investigate the viability of HA-t hydrogels for neuronal cell culture and secondly, to test whether neuronal precursor cells such as the NT2 cell line have the ability to extend neurites in 3D.

HA hydrogels were prepared initially at 1% w/v to which laminin was added at a final concentration of $20\mu\text{g ml}^{-1}$. Once crosslinked, a standard 52day differentiation protocol was followed based primarily on mitotic inhibition. Composite fluorescence images of β III-Tubulin and glial fibrillary acidic protein (GFAP) with a DAPI counterstain were acquired with an epi-fluorescence microscope. From Figure 7.1, it is evident that NT2 cells express β III-Tubulin and GFAP, which are neuronal and glial cell markers respectively. Cells not subject to the differentiation protocol also express these markers. This may be attributed to a cessation of cell division mediated by ECM mechanics.

7.3 Results and Discussion

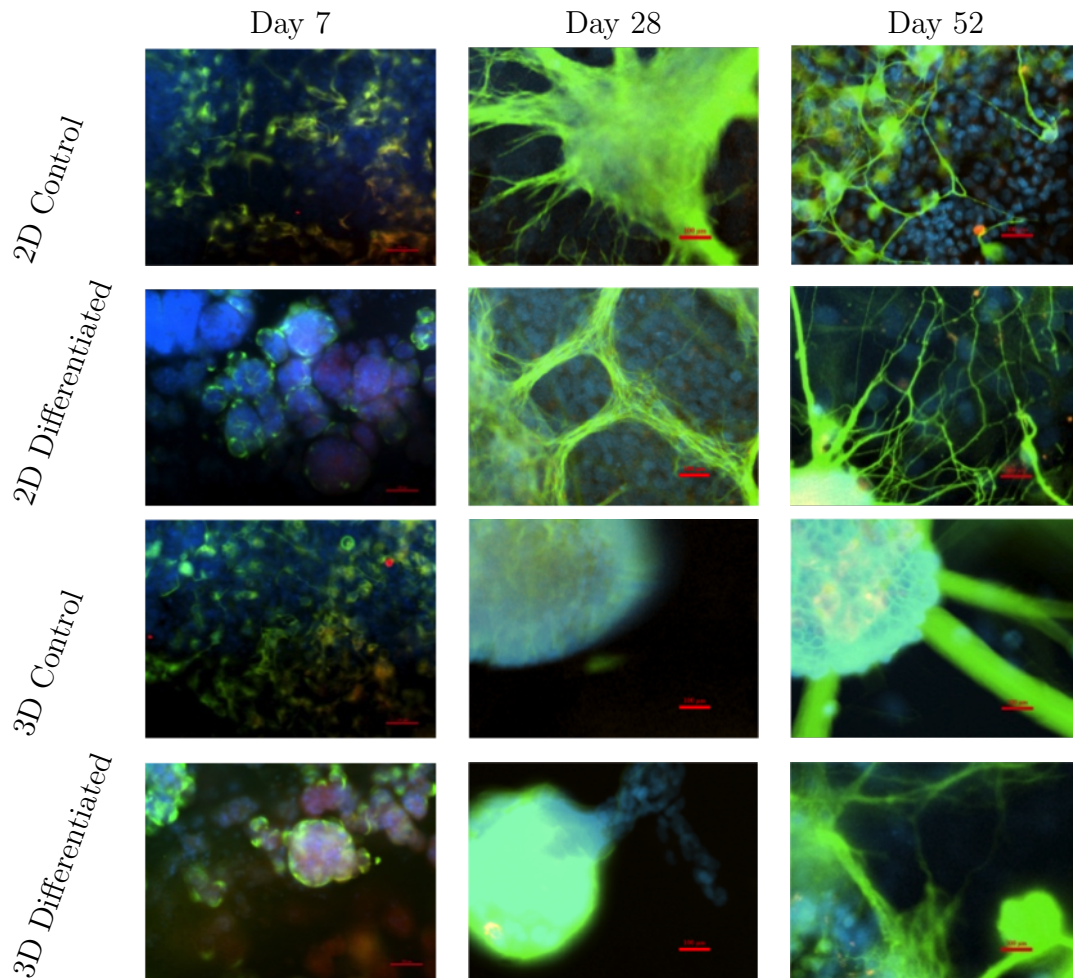


Figure 7.1: Ntera2 cells were encapsulated and differentiated within 1% w/v HA-t hydrogels for 52days. A standard tissue culture polystyrene surface served as a 2D control. Non-differentiation medium consisted of normal medium without mitotic inhibitor supplementation. Hydrogels were stained for β III-Tubulin (green), GFAP (red) and DNA (DAPI; blue). Scale bar 100 μ m.

It has been established through numerous cell culture experiments that the rate of mitosis is reduced when the cells are subject to 3D hydrogel culture. The present study also confirms that this protocol directs NT2s into a neuronal rather than glial lineage, demonstrated by the absence of GFAP between day 7 and 52. Mitotic inhibition is known to direct some precursor cells along a neuronal differentiation

7.3 Results and Discussion

pathway (Surprenant and Shea, 1998).

An increase in neurite extension and β III-Tubulin fluorescence is observed between day 7 and 52, which suggests that this protocol efficiently directs the precursors into a neuronal lineage. It is interesting to note that neurites seem to fasciculate in a manner similar to 2D culture experiments, although this takes much longer in 3D. A repeat in a softer hydrogel (as characterised rheologically in Section 4.3) may increase the overall length of neurite extension. Due to their size, cell bodies tend to cluster together, forming neurosphere-like structures in 3D. At the time of writing, no study has been published on the 3D encapsulation and subsequent differentiation of NT2 cells. This result should be treated cautiously: the presence of β III-Tubulin in all groups suggests that the cells may inherently express neuronal cell markers. Staining for other proteins such as NF-200 may aid the formation of a conclusion regarding NT2 differentiation.

Possible false-positive staining notwithstanding (clearly a limitation with these data), the most significant result from this study is the presence of long neurite bundles between neurosphere-like cell clusters. The aim of this preliminary 3D culture experiment was to elucidate the effects of a 3D environment on well-established 2D differentiation protocols. It is clear that although the differentiation controls also produced these structures, NT2 neurites do have the ability to navigate through a HA-t hydrogel. The exact mechanisms of pathfinding and migration were outside the scope of this study, but it does prove that even the stiffest HA-t hydrogels (approximately 120Pa) can support differentiation and neurite outgrowth in 3D.

7.3.2 Culture of Neural Progenitor Cells in Hyaluronan Hydrogels

We have seen that HA-t hydrogels provide a suitable biocompatible environment within which to culture neuronal cells. The limitations of NT2 cells are significant, and deviate enormously from a healthy stem cell phenotype provided by primary neural stem progenitor cells (NSPCs). The NSPC, also known as the subventricular zone (SVZ) cell, is an excellent candidate for three dimensional cell encapsulation and neural tissue engineering. These cells are highly proliferative and, as we will demonstrate, remain viable when cultured within a synthetic extracellular environment. To assess the viability of NSPCs, they were first cultured in 2D followed by a 3D encapsulation study. This study was important for two main reasons. Firstly, it was essential that NSPCs were evaluated as a potential candidate for future HA-t encapsulation studies; whether encapsulated primary progenitor cells proliferate and extend neurites were important questions in addition to viability. Secondly, as one of the first NSPC encapsulation experiments, it was essential that immunocytochemical and imaging methodologies were optimised.

Culture methods have been extensively summarised elsewhere, but briefly, NSPCs were isolated from the lateral ventricles of postnatal day 4 (p4) mouse pups and cultured on non-adherent poly-HEMA coated flasks for 4-5 days. Neurospheres were dissociated, and single cells were encapsulated at two densities ($1 \times 10^6 \text{ ml}^{-1}$ and $2 \times 10^6 \text{ ml}^{-1}$) within 1% w/v HA-t hydrogels with three concentrations of PEG-DA crosslinker: 20% v/v, 16% v/v and 12% v/v. The mechanics of these hydrogels were investigated in Chapter 4. The control surface was standard TCP. Cell pellets were

7.3 Results and Discussion

suspended in the hydrogels and allowed to crosslink for 35mins. After crosslinking, Neurobasal+ medium was added supplemented with EGF ($20\mu\text{g ml}^{-1}$) and bFGF ($20\mu\text{g ml}^{-1}$) and maintained in normal culture conditions for 6days. After 6days, hydrogels and controls were washed and incubated with Calcein-AM and ethidium homodimer-1 prior to imaging on a Nikon epi-fluorescent microscope. To visualise viability in 3D, Bitplane's Imaris processed z-stacks were deconvoluted and spots corresponding to positive Calcein-AM and ethidium homodimer-1 staining were automatically detected.

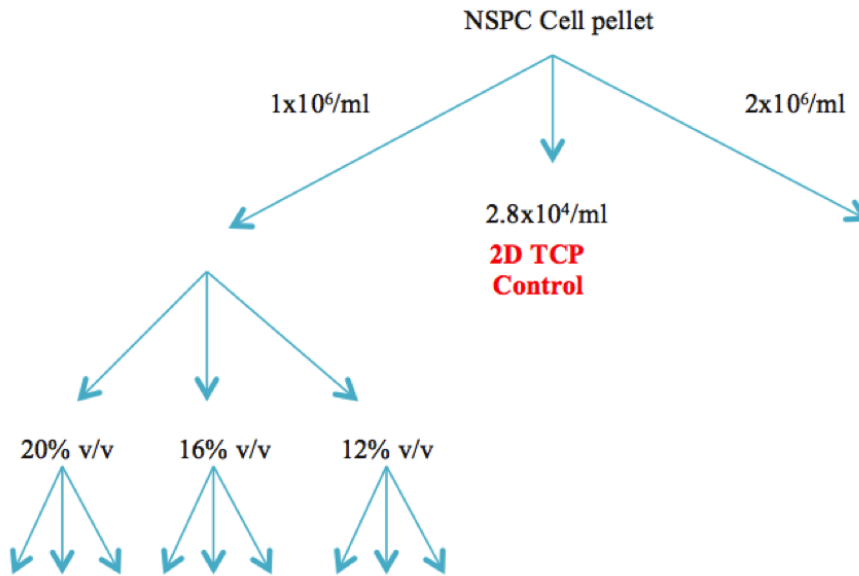


Figure 7.2: A summary of mouse NSPC encapsulation experimental design. Cell suspensions of 2 different densities ($1\text{e}^6\text{ml}^{-1}$ and $2\text{e}^6\text{ml}^{-1}$) were prepared, and encapsulated in 3 different PEG-DA concentrations: 20% v/v, 16%v/v and 12% v/v. Hydrogels were maintained at 37°C for 6days in culture medium supplemented with EGF and bFGF. Each experimental group was repeated in triplicate.

Mouse NSPCs proliferate rapidly on control surfaces, and extended neurite-like processes within HA-t after 2-3days (Figure 7.3). This rapid proliferative state is not unusual in 2D with highly plastic cells such as these. It is important to note

7.3 Results and Discussion

that assessing proliferation in 3D is complex, and without probing for proliferative markers highlighting dividing cells, this data remains inconclusive. In the present study, either increasing or decreasing the concentration of the crosslinker (in this case PEG-DA) produced hydrogels of different elastic moduli which had a significant effect on neurite outgrowth.

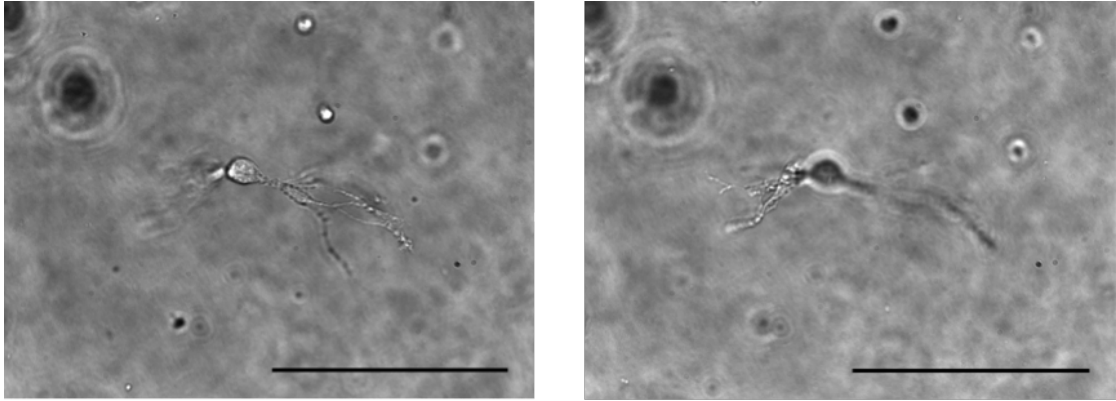


Figure 7.3: Neurite-like projections were observed emanating from NSPC cell bodies after only 3 days. Micrographs represent two z-positions, showing that projections are not extending in a single plane, rather exploring their environment in 3D. Scale bar $100\mu\text{m}$.

Stiffness is known to influence various aspects of cellular behaviour including proliferation and differentiation (Leipzig and Shoichet, 2009). It was therefore hypothesised that decreasing stiffness would influence the degree of neurite outgrowth due to a larger polymer mesh size. This was confirmed after day3, when cells encapsulated in 12% v/v PEG-DA hydrogels (stiffness approximately 100Pa, see chapter 4) extend neurite-like processes (Figure 7.3). These processes cannot be referred to as neurites in the present experiment due to a lack of neurite specific immunostaining or protein blotting; future studies will indeed confirm the presence of neurites by probing for proteins such as NF-200. This result was surprising and never recorded by this laboratory before. Indeed, this result was not found in the

7.3 Results and Discussion

literature and may represent the first data confirming the ability of mouse NSPCs to explore their environment after only 72hrs.

Similarly, encapsulated cells in 16% v/v PEG-DA hydrogels extended neurite-like processes after 6days, possibly due to an increase in protrusion force as a result of differentiation, or by a decrease in local modulus. From earlier differentiation experiments, we can confirm that HA hydrogels do decrease in stiffness after several days in solution. The decrease in local modulus may also be a result of proteinases produced by the cell (Owen and Shoichet, 2010; Daley et al., 2008), or by the intrinsic degradation of individual polymer chains by the solution. For a discussion of proteolytic remodelling of the ECM see section 2.13. Although no neurite-like projections were present after 6days in 20%v/v PEG-DA hydrogels, the author is confident that, given enough time, NSPCs encapsulated in these hydrogels would exhibit similar behaviour after the hydrogels have reached an optimum stiffness.

Figures 7.4 and 7.5 represent phase contrast and fluorescent live/dead micrographs of encapsulated NSPCs respectively. Although difficult to visualise, neurite-like projections were present in all but the stiffest (20% v/v PEG-DA) hydrogels after 6 days. Although statistical analysis was troublesome due to low n , it is clear that cells remain viable in all hydrogels after this time period. No proliferation assays were performed to assess whether the cells are in fact dividing. The biocompatibility of HA has been well documented in other studies, although the author knows of no data confirming the viability of p4 mouse NSPCs within HA-t hydrogels.

To assess the 3D distribution of viable and non-viable cells, Bitplane's Imaris software was used to stitch z-stacks and produced a representative 3D image

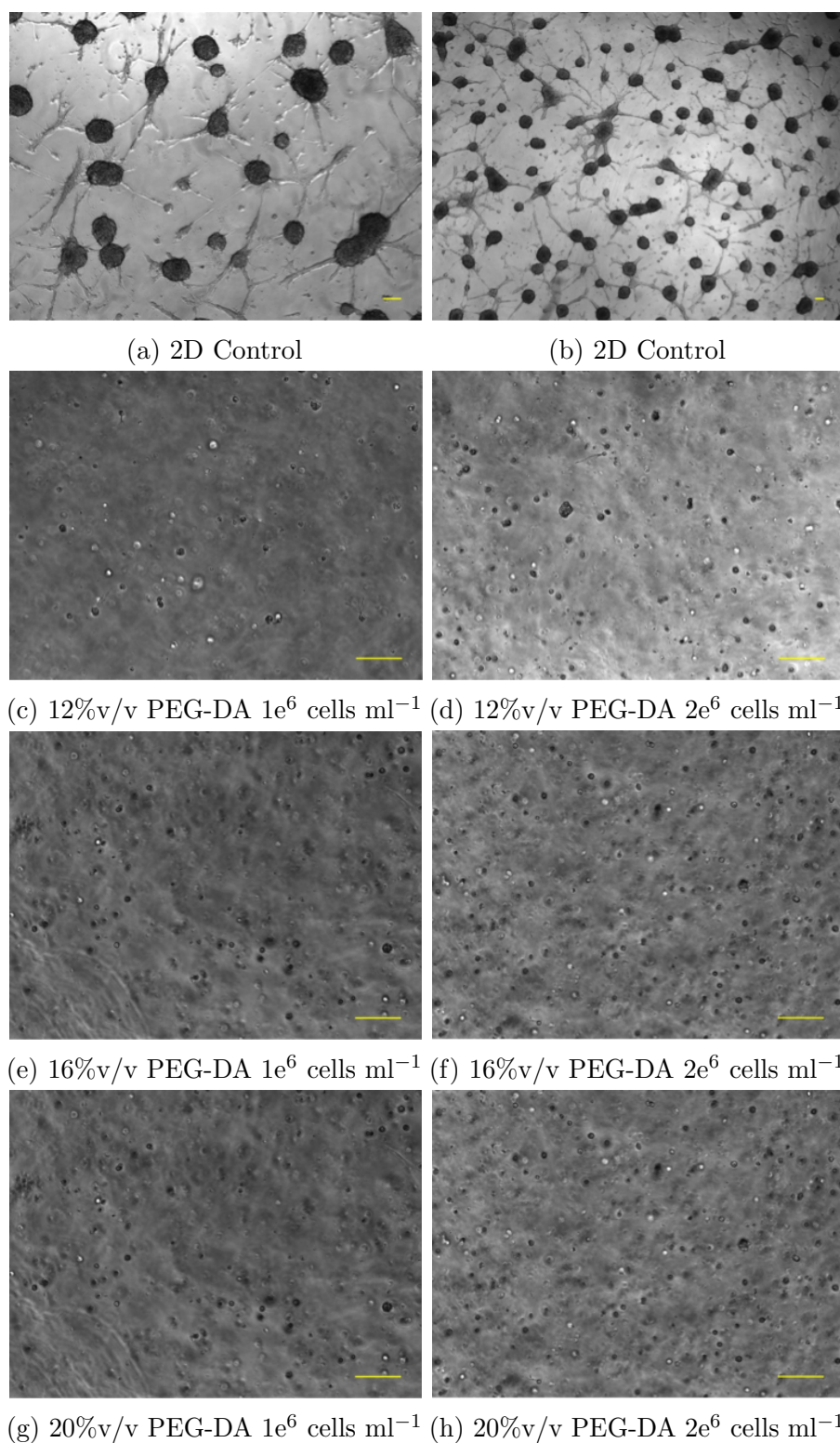


Figure 7.4: Phase contrast micrographs after day 5 show that NSPCs encapsulated within HA hydrogels of different stiffnesses adopt a rounded morphology, in contrast to the neurite-like structures visible on the 2D tissue culture polystyrene (TCP) control surfaces. Scale bar $100\mu m$.

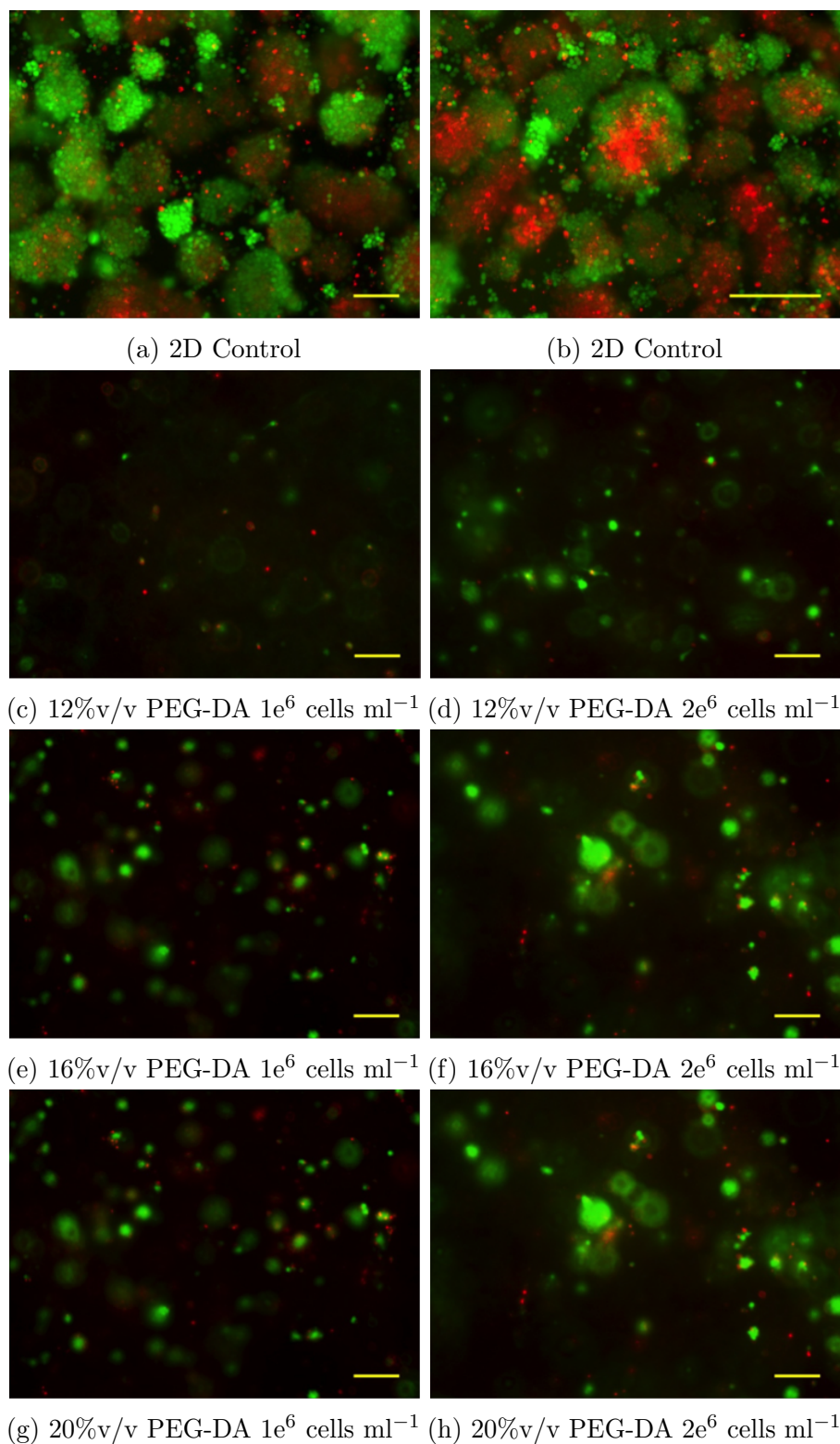


Figure 7.5: Composite fluorescent images of NSPCs encapsulated within HA-Thiol Hydrogels crosslinked with PEG-DA, showing live (green) and dead (red) cells after day 6. Cells remain viable for up to 6days in all concentrations. Scale bar $100\mu m$.

7.3 Results and Discussion

showing a good distribution of viable NSPCs (Figure 7.6). This technique has – to the best knowledge of this author – not been implemented to visualise the 3D arrangement of mouse NSPCs in a HA hydrogel. Figure 7.6 accurately highlights the distribution of live and dead neural cells in a well defined volume. It is important to note however, that this test does not consider apoptotic cells (i.e. cells that are in the process of dying), and genetic investigation with methods such as PCR could be used to better quantify the number of cells and how healthy they are. The hypothesis was that deep cells would be less viable than the more superficial cells. It is evident that this is not the case as first thought. Importantly for neural tissue engineering, this result has lead to two conclusions: firstly, HA hydrogels can support a healthy population of neural cells (although proliferation was not measured) and secondly, 3D spot detection is a viable method for the quantification of 3D viability.

This method of viability analysis does not require the removal of the cells from the hydrogel, which is extremely favourable. For example, advanced analytical techniques such as protein or DNA quantification would require the removal of the cellular component. Furthermore, if a fluorescent tag was incorporated into the DNA of the neural cell, this method would enable the long term 3D imaging of live cells. The presence of live cells throughout the hydrogel is a promising result, suggesting that diffusion is not a limiting factor in 3D HA-t culture. The mechanisms of diffusion were investigated briefly in Chapter 4, and confirm that a solute such as glucose can diffuse rapidly into the hydrogel, forming a predictable gradient. This may in fact be the reason for the high live cell numbers. Thus an important result in this study is that the nutrients and waste (produced by the

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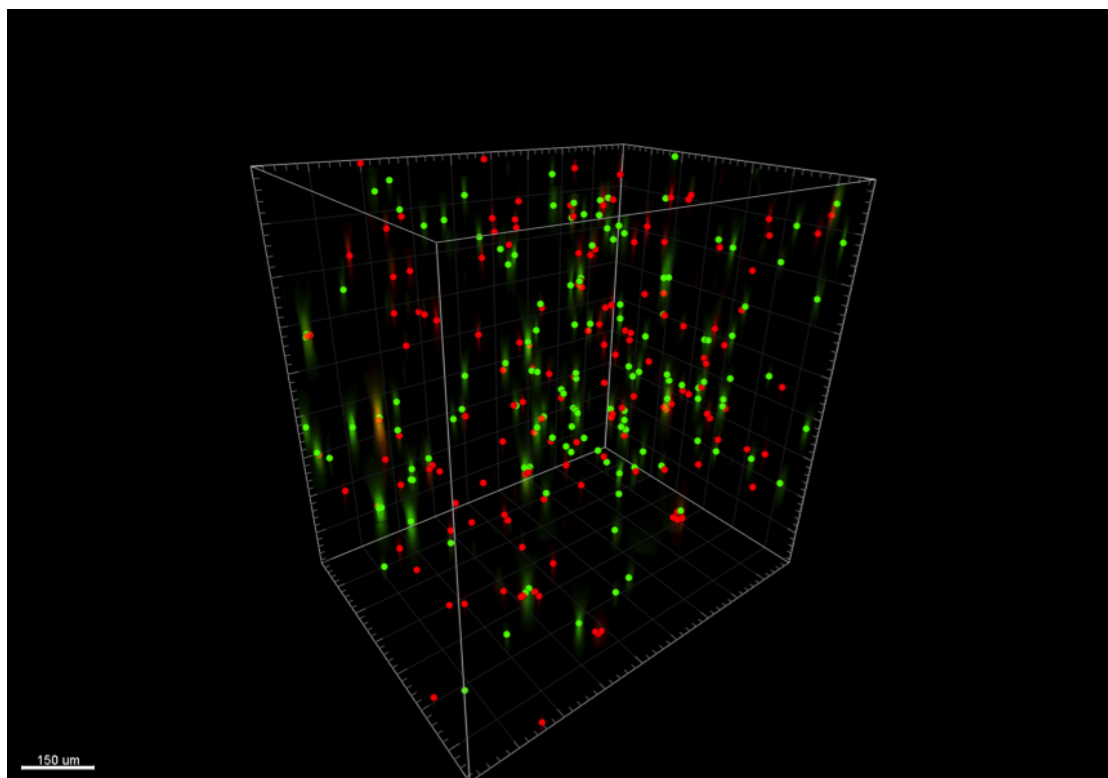


Figure 7.6: Using a stack of fluorescent images, individual cells were detected by Bitplane’s Imaris software. Once deconvoluted, live (green, Calcein AM) and dead (red; ethidium homodimer) cells were overlaid with spots to simplify visualisation. NSPCs encapsulated within HA-Thiol hydrogels remain viable after 6days when homogeneously dispersed. Scale bar 150 μ m.

cell) does not appear to affect viability in a gradient-dependent manner. Neurite outgrowth studies have historically been confined to 2D, mainly due to the infancy of hydrogels for cell encapsulation. Studies evaluating NSPC neurite outgrowth by Imaris have been documented by few, including (Seidlits et al., 2010; Ajioka et al., 2007; Zhang et al., 2006), but these studies do not investigate a large hydrogel volume for, specifically, cell viability.

In summary, mouse NSPCs remain viable in HA-t hydrogels of different stiffnesses, although quantification is difficult in a 3D. Quantification of viability in 3D can be

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simplified by stitching fluorescent z-stacks and counting the number of live and dead cell in-situ. Cells extend neurite-like projections rapidly, as soon as 3days in the 12% v/v PEG-DA hydrogels. This observation is novel in this laboratory, and the author knows of no pre-existing literature reinforcing these data.

7.3.3 Culture of Rat DRGs in Functionalised Hyaluronan Hydrogels

FGL may act as a promoter of cell adhesion in 2D, but what the effect of this peptide may be in 3D has, to date, remained unknown. To test this hypothesis, rat DRGs were encapsulated in a blend of HA and methylcellulose (HAMC). Briefly, DRGs from embryonic day 15 (E15) rats were dissected and placed in a crosslinking 0.75% HAMC hydrogel. Hydrogels were functionalised with either RGD or the NCAM mimic FGL, with pure HAMC serving as control. Cells were maintained in serum free Neurobasal medium supplemented with pen/strep, L-Glutamine and B27 for 24hrs. After culture period, cells were incubated with Calcein-AM and DAPI before imaging on a confocal microscope (Zeiss). The entire experiment was repeated, with an almost identical outcome.

From confocal imaging data it is evident that DRGs remain viable in HAMC hydrogels after 24hrs, demonstrated by positive Calcein-AM staining of cells. The live cell stain Calcein-AM was used due to the fragility of HAMC hydrogels; repeated washing steps required in conventional immunostaining would render the hydrogel too weak. From Figure 7.7, it is clear that explants extend neurite-like projections in HAMC only hydrogels, and hydrogels functionalised with FGL.

7.3 Results and Discussion

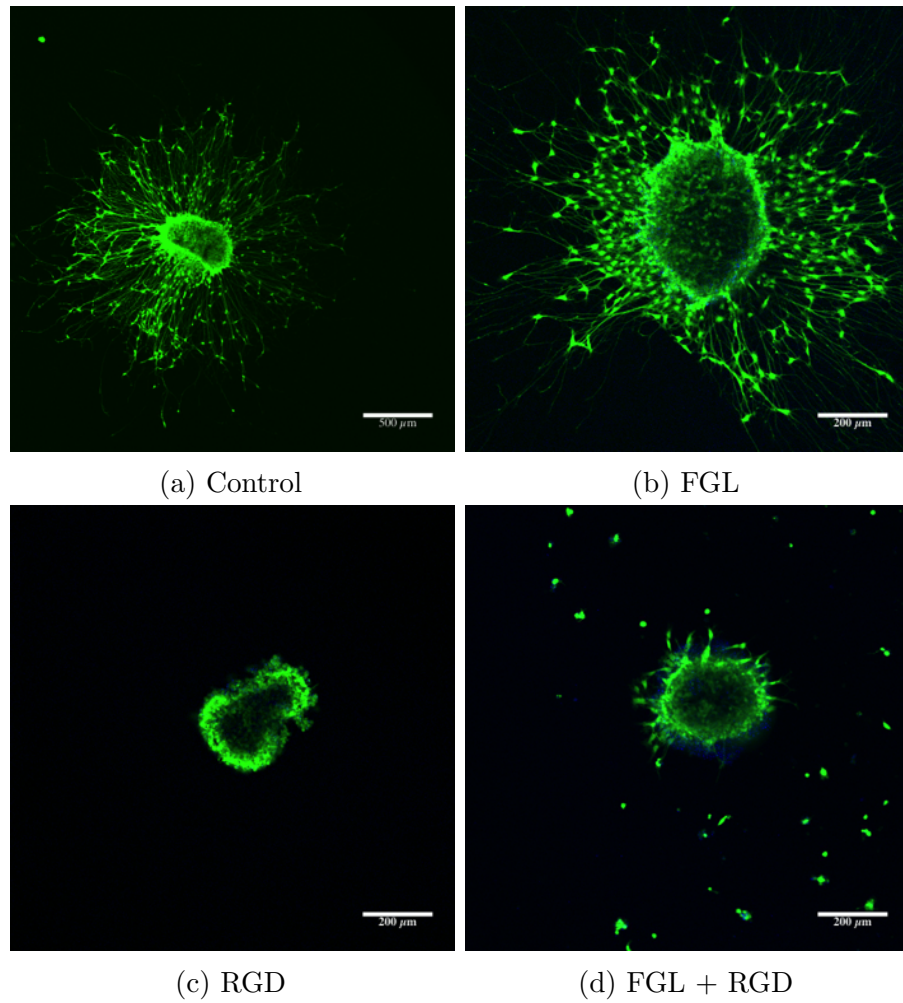


Figure 7.7: Embryonic day 15 rat DRGs were encapsulated within a HA–methylcellulose hydrogel functionalised covalently with FGL, RGD or a combination of both. Control was HAMC only. Cells were stained with Calcein AM (green) and DAPI (blue). Scale bar 200-500 μm .

The FGL peptide did not make a significant difference to the area of neurite extension, as demonstrated by Figure 7.8. No neurite-like extension was visualised in any hydrogel functionalised with RGD, including a hybrid of both RGD and FGL. This result is consistent to some extent with the 2D polystyrene functionalisation test (Chapter 6) where, unexpectedly, RGD did not increase the length of neurite

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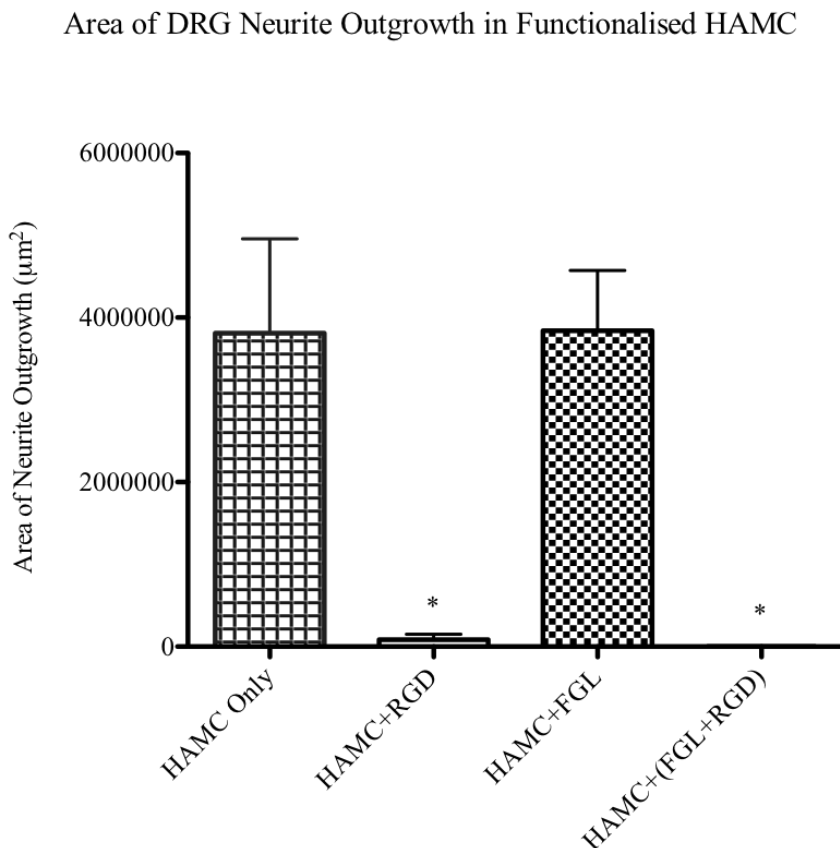


Figure 7.8: HAMC was functionalised with RGD, FGL and (RGD+FGL). The area of neurite outgrowth was calculated by subtracting the inner cell mass area from the outgrowth area. Both RGD and (FGL+RGD) demonstrated significantly lower neurite outgrowth than the control (HAMC only) group ($P < 0.05$). Error bars represent the standard error of the mean.

extension, and may be explained by ligand saturation. For example, increasing the number of ligands (in this case RGD or FGL) means that the cells are in effect ‘stuck’ within the hydrogel. The probability that an individual cell would initiate neurite outgrowth against this integrin–ligand gradient is unlikely. A limitation with respect to Figures 7.8 and 7.9 is that the visual and statistical analysis is restricted to 2D; in reality the explant may be extending neurites in a 3D space.

7.3 Results and Discussion

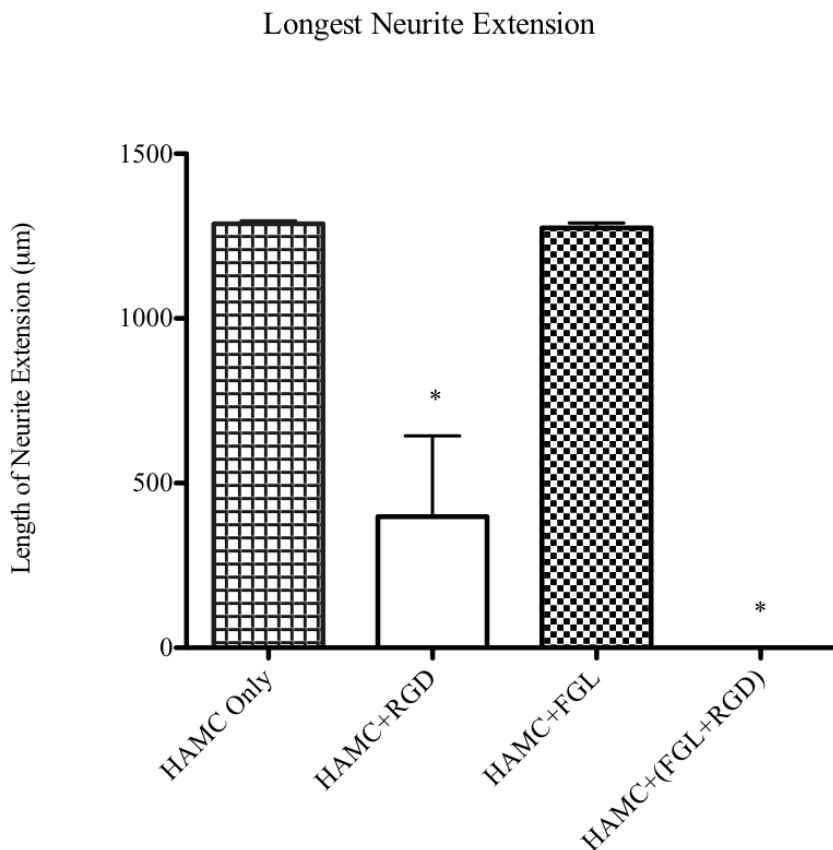


Figure 7.9: HAMC was functionalised with RGD, FGL and (RGD+FGL). The longest neurite length was calculated as an average of the three longest neurites emanating from the inner cell mass. Both RGD and (FGL+RGD) demonstrated significantly lower neurite length than the control (HAMC only) group ($P < 0.001$). Error bars represent the standard error of the mean.

The effect of FGL on cell adhesion is inconclusive with respect to this experiment, possibly due to a low FGL concentration. Although this may initially seem like a negative result, FGL is thought to influence downstream signalling mechanisms through FGFr, and not via the integrins. The present experiment did not assess the state of activation of FGFr by FGL, so conclusions about its effects in 3D should be interpreted with caution.

7.4 Summary

HA hydrogels are excellent candidates for neuronal cell encapsulation. As discussed in previous chapters, it is possible to modify these materials mechanically to maintain an optimum matrix for cell viability and neurite outgrowth. An important addition provided by Chapter 7 is that these materials can very easily be modified with peptides that have been functionalised with a reactive chemical group such as maleimide or propyl maleimide. Although the covalent functionalisation was not directly measured, the encapsulated peptides clearly influence adhesion and neurite outgrowth (Figure 7.7). Neuronal cells such as the NTera2.D1 cell line and the NSPCs can be successfully encapsulated within HA-t hydrogels. NT2 cells were used to initially evaluate long term (52week) viability and neurite outgrowth, but are not a good representation of healthy neuronal cells.

NSPCs remain viable in 3D, and extend neurites after only 3days in a matrix with the optimum stiffness. The rate of neurite extension was surprising, and a first for this laboratory. No literature has been found with equal or higher neurite outgrowth rates. Additionally, the author knows of no study incorporating p4 mouse NSPCs within in a 3D HA-t hydrogel system. Of equal novelty is the encapsulation of rat DRGs within HAMC, which demonstrates that materials functionalised RGD modulate the adhesion of DRG neurites. Significant repeats of these data are required before adequate conclusions can be drawn. Nonetheless, HAMC and HA-t are excellent contenders for future encapsulation studies, and possess properties suitable for the study of neural network formation.

Chapter 8

Conclusions and Future Perspectives

8.1 Conclusions

Cell Fate Commitment

Neural tissue engineering has steadily transformed into a field with genuine clinical significance. This thesis set out to evaluate the role of the hydrogels in neural tissue engineering. Indeed, it is ineffective to simply summarise the aims and results of this thesis again. Rather, the concluding chapter will discuss the important aspects of the data presented herein in a larger context. As with much scientific data, it can be challenging to separate the meaningful results from the irrelevant.

A common differentiation protocol – containing dbcAMP, $\text{IFN}\gamma$ – was acquired personally from Professor Molly Shoichet at the University of Toronto, and was widely used in this thesis to generate stable populations of neurons. This was established in Chapter 6, where the effect of the NCAM mimetic peptides on differentiation was demonstrated successfully. Additionally, the cytotoxicity of these factors in 3D (or indeed the lack thereof) was investigated in Chapter 7.

In light of the data presented in this thesis, the NCAM mimic FGL may initiate neurogenesis by activating Erk/MAPK via FGFr. What is certain is that FGL appeared to increase the number of β III-Tubulin positive NSPCs. Rat DRGs also respond positively to FGL-based peptides, and novel analytical techniques developed by the author can shed a quantitative light on DRG activity. What is more surprising, is the effect of the cyclised form of FGL and the novel PSAm-SG₅-FGL on differentiation. Cyclising peptides such as RGD appear to alter the way that a cell interacts with it, possibly by enhancing cell binding. Data presented in Chapter 6 may allow us to augment neural cell fate commitment by

8.1 Conclusions

supplementing culture medium or functionalising culture surfaces with FGL. Of additional importance in Chapter 6 is the conformational modelling of the mimetic peptides, the first of its kind in the literature.

Of more interest is the very significant effect of a novel peptide (PSAm-SG₅-FGL) on differentiating NSPCs. Polysialic acid *in vivo* is thought to be an important negative regulator of cell-cell interactions. Interestingly, however, when modified by PSA, NCAM influences a vast set of cellular behaviours, including axonal outgrowth and fasciculation, cell migration, synaptic plasticity, and adult and embryonic neurogenesis (Bonfanti, 2006). Whether PSAm-SG₅-FGL binds to FGFR, activating downstream mediators such as MAPK, remains to be investigated. What is certain is that this is the first example of a peptide mimic of PSAm-NCAM with some evidence of action on neural stem cells *in vitro*. Some important conclusions with respect to cell fate commitment are outlined in the following list:

- Computational modelling of peptides confirms loop-like structure of FGL;
- Novel quantitative techniques based on DRG neurite area and fluorescence suggest that FGL affects rat DRG adhesion;
- FGL and related mimetic peptides significantly affect the differentiation of mouse NSPCs;
- Novel peptides appear to affect several complex cellular behaviours such as neurite outgrowth; and
- Functionalised FGL can bind to HAMC hydrogels and affect neurite outgrowth of rat DRGs.

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Manipulating and Characterising Hydrogel Properties

Chapter 4 hypothesised that the macroscopic properties (for example the storage modulus and viscosity) and microscopic properties (such as the mass transfer of solutes including glucose) could not only be measured and assessed, but also controlled carefully to suit the requirements of the encapsulated cell. In this chapter we highlight for the first time, data suggesting that differentiating neural precursors stabilise their ECM – in this case, a mechanically optimised HA-t hydrogel (subsection 4.3.3). It is generally accepted that developing neurons can alter their extracellular environment to suit their requirements, usually by reducing stiffness via proteolytic enzymes. The data presented here was – to the best knowledge of the author – the first example of differentiating neurons stabilising their substrate. This stabilisation is probably due to the formation and proliferation of neurites that serve as ‘scaffolding’, holding the hydrogel together. It is important to note that no immunocytochemistry was carried out to confirm this, although previous encapsulation data (not shown) highlight the presence of neurite bundles similar to those found in subsection 7.3.1. An essential micro-characterisation technique is SANS. Chapter 5 reviewed important models of polymeric characterisation that were used on this occasion to yield some useful structural data.

Hyaluronan hydrogels are formed when a functionalised macromer reacts with a reactive crosslinker. Hypothetically, reducing the concentration of either the macromer or the crosslinker will reduce the stiffness of the hydrogel. Indeed, this was confirmed in Subsection 4.3.2, where both methods predictably reduce the storage modulus of the material. The predominant theory surrounding crosslinking

8.1 Conclusions

kinetics suggests that this reduction in stiffness is a function of the crosslinking density, however the data collected here propose that the crosslinking density is not affected by a reduction in macromer or crosslinker concentration. Chapter 5 offers the first insight into the microscopic nature of fully crosslinked HA-t and the effects of reducing the macromer concentration. Future studies could include the introduction of encapsulated cells to investigate the cell–HA interaction and the effects of differentiation on properties such as crosslinking density and mesh size. Important conclusions from Chapter 4 and Chapter 5 can be summarised below:

- HA-t hydrogels can be optimised mechanically to suit developing neuronal precursor cells;
- Differentiating neural precursor cells stabilise a synthetic HA-t based ECM; and
- SANS confirms that stiffness is not necessarily a product of crosslinking density, but of meshsize and polymer swelling.

8.1 Conclusions

Three Dimensional Cell Culture and Hydrogel Functionalisation

One of the original aims of this thesis was to study the effect of functionalised hydrogels on encapsulated neural precursor cells. The functionality in this case was linear FGL, modelled computationally in Subsection 6.3.1. HAMC hydrogels have been used extensively by Shoichet *et al* for neuronal cell encapsulation, including the NSPCs (Mothe et al., 2013) and DRGs (Stanwick et al., 2012); no literature has been presented to date where the functionality of FGL was successfully demonstrated within HA. It was hypothesised that FGL would modulate neural adhesion and neurite outgrowth.

A complicating factor in this experiment was the mechanical stability of HAMC, which prevented immunocytochemistry that included numerous wash steps; HAMC is inherently weak due to the lack of covalent bonding. Although Chapter 7 presents the first example of an FGL functionalised HAMC gel, there was little statistical evidence that FGL increased neurite outgrowth. Statistical evidence suggests a role of FGL in adhesion, although this remains largely hypothetical.

Due to time constraints and problematic peptide synthesis methodologies, the outcomes of Chapter 7 are limited. Assessing neural functionality in functionalised hydrogels is a non-trivial exercise, and the difficulties that accompany these techniques should not be underestimated. It is clear that the initial objectives of this final experimental chapter were optimistically undertaken. However, the data does demonstrate the ability of HA-t and HAMC to support live neural cell cultures. Important conclusions from these experimental data can be evaluated as following:

- HA is a viable substrate for 3D encapsulation and long term culture of human,

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mouse and rat neuronal cells;

- Mouse NSPCs extend neurites into soft HA-t hydrogels after 3days, significantly faster than other published and non-published studies; and
- Functional HAMC hydrogels viably support rat DRGs, promoting adhesion rather than neurite outgrowth

In summary, HA hydrogels can be optimised chemically and mechanically to form stable hydrogels that support the long term culture of human, mouse and rat neuronal cells. Modifying these materials with peptides that mimic NCAM yield yet further control over complex mechanisms such as differentiation and neurite outgrowth. Rheology and SANS provide a highly sensitive platform from which to characterise the microstructural and macrostructural properties of HA. Contrary to existing literature, stiffness is not necessarily a function of crosslinking density, rather implicating hydrogel mesh size and swelling.

8.2 Future Perspectives

It is a well established fact that doctoral research can be as endless as the student intends it to be, and that has certainly been realised in this thesis. With time, much more could be accomplished. The aim of these few short pages is to outline the further work that would be carried out if Chronos would allow.

Firstly, existing data would be expanded greatly by conducting further experiments, chiefly on the functionalisation of TCP and HA-t hydrogels with NCAM mimetic peptides. There are some areas of work which would require experimental repeats to ensure statistical significance. The most important of these include the 2D and 3D functionalisation experiments. Specificity is extremely important in tissue engineering. For any significant conclusions to be made, the direct effect of FGL on well known receptors (such as FGFr) must be accurately established, namely by PCR and western blotting for phosphorylated FGFr and the resulting downstream cascade (e.g. Erk and MAPK). An important addition to this data would include the regulatory effect of FGL on several mouse NSPC genes.

The importance of repeating experiments is essential, as discussed above. Possibly of more relevance, however, is the translation of knowledge into clinically viable techniques and treatments. As can be seen in the list below, this translation could begin with a feasibility study into the functionality of neural networks. Functionality is essential, without which the vast majority of the techniques developed in this thesis would not reach the clinic. Functionality could be tested in a few ways, for example by multi-electrode array analysis or calcium imaging of encapsulated neurons. Of final interest is the use of human cells. It is well known that human and

8.2 Future Perspectives

animal cell types can differ significantly, and much of the experimental work would need to be carried out again with primary human cells, or induced pluripotent stem cells. The disadvantages of using human cells (e.g. cell sourcing, medical ethics) prevented their use in this thesis. The following list outlines a possible plan for research.

1. Confirm the nature of the interaction between the NCAM mimetic peptides and developing neuronal cells
 - (a) Repeat 2D experiments in 3D using HA-t hydrogels rather than TCP
 - i. Conjugate FGL to HA-t;
 - ii. Confirm functionalisation of HA-t by FTIR and immunocytochemistry; and
 - iii. Conduct neuronal cell encapsulation study, investigate neurite outgrowth of mouse NSPCs.
 - (b) Confirm the exact FGL–NSPC binding mechanism in 2D and 3D
 - i. Probe for phosphorylated FGFr1/2, Erk, and MAPK to confirm theoretical FGL–FGFr interaction. Western blotting and PCR are suitable techniques.
 - (c) Complete a genotyping assay of the mouse NSPC, highlighting the influence of FGL on specific gene expression
 - i. Use PCR to compare the regulation of differentiation genes (e.g. Notch, Pax6, Syntaxin–1a and neurofilament).
2. Translating the knowledge gained in (1) into clinically relevant techniques

8.2 Future Perspectives

- (a) Attempt the generation of functional neural networks in 3D by using mimetic peptides such as FGL and PSAm-SG₅-FGL
 - i. Generate stable populations of terminally differentiated neurons in 3D; and
 - ii. Demonstrate network functionality by multi electrode array (MEA) analysis and calcium imaging.
- (b) Conduct experiments on the formation of functional neural networks of primary human (rather than mouse) neuronal precursors or stem cells
 - i. Attempt the generation of a functional human neural network using techniques established above; and
 - ii. Reproducibly introduce injury or disease states (e.g. stroke or traumatic brain injury), and measure up-regulation of affected genes by PCR.

The future of this work is extremely promising. Not only will it shed light on very specific developmental mechanisms, but it may lead to clinically relevant approaches to neurological injury or disease. The importance of specificity has been discussed at length, but must not be understated: for tissue engineering to be clinically relevant, it must be specific on a molecular level. Functionalised hydrogels are useful, but we need to truly understand how and why they are useful, to which receptors they bind, and which genes they regulate. Therein lies the future of this work.

Appendix A

Rat Dorsal Root Ganglion

Neurite Outgrowth

Quantification

Measuring average neurite length is a relatively straightforward procedure, with results dependent on the number of repeats and samples taken per repeat. The author developed a robust method to repeatedly measure the area of DRG neurite outgrowth, using this as a potential tool for assessing the effect of certain peptides on neurite outgrowth. of

1. Upload the *.oib* file to FIJI and deselect the DAPI channel (if more than one fluorophore is present) during upload by selecting split channels.
2. Set measurements by Analyse > Set Measurements > Select Area. Others can be ignored.

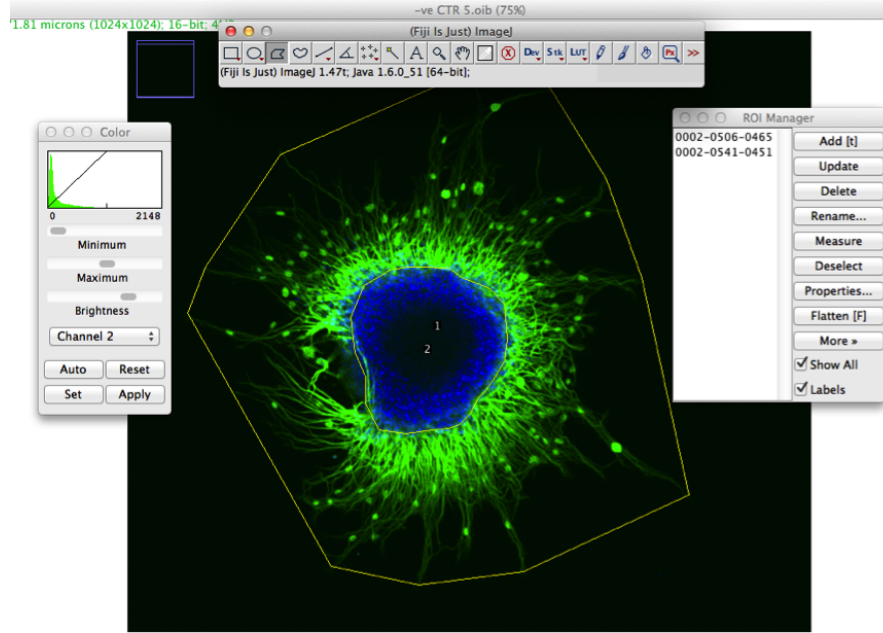


Figure A.1: Using the polygon tool outline the entire DRG and the inner cell mass defined by the DAPI positive border. Measure the areas by uploading to the ROI manager. The Colour threshold may need to be adjusted to reveal each neurite.

3. Using the polygon tool, trace around the whole DRG, moving between the longest adjacent neurites. Ensure that the final segment is joined to the first. Press “T” to upload to ROI Manager. The reader is referred to figure A.1
4. Repeat for each sample repeat, in this case $n = 9$. In ROI manager: Select/highlight all > Measure.
5. Repeat the entire process for the area surrounding the inner cell mass, tracing around the outermost DAPI positive cells. Measure area in a similar manner.
6. Copy to excel worksheet and subtract the entire DRG area from the inner cell mass to give an approximate value for pure neurite extension. This method reduces DRG size bias. The mean neurite extension value can now be calculated, or the raw data can be analysed by statistical software.

Appendix B

Rat Dorsal Root Ganglion Fluorescence Quantification

To assess the corrected total cell fluorescence, the following method was adapted and modified slightly from (Burgess et al., 2010), and subsequently adapted by (Gavet and Pines, 2012; Potapova et al., 2011) to quantify cell fluorescence.

1. Upload the *.oib* file to FIJI and deselect the DAPI channel during upload by selecting split channels.
2. Set measurements by Analyse > Set Measurements. Select area, integrated density, and mean grey value. Others can be ignored.
3. Adjust threshold by Image > Adjust > Threshold. Select default, Over / Under. Adjust the desired channel till all neurites are included clearly and the majority of the inner cell mass is excluded.
4. Using the wand tool, select the whole DRG. Press “T” to upload to ROI

Manager. Repeat with three background controls around the DRG.

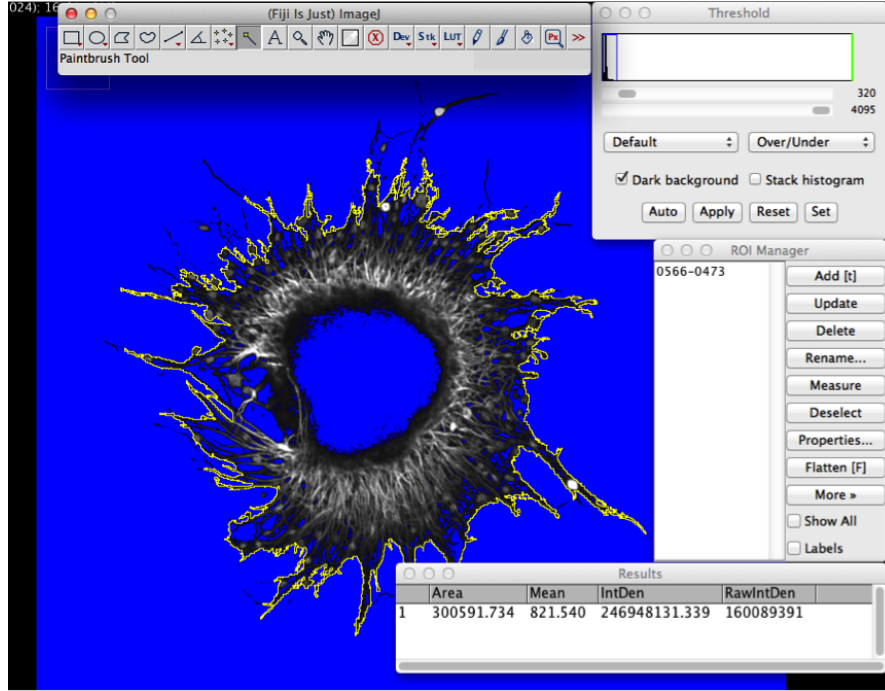


Figure B.1: Thresholding an individual fluorescence channel to highlight the area of interest, in this case the neurites. Care should be taken not to exclude any detail.

5. Select/highlight all > Measure.
6. Copy to excel worksheet. Using the following formula, calculate the corrected total cell fluorescence (CTCF), where $\int \rho$ is the integrated density A_c is the area of the selected cell or explant, $\bar{X}F_b$ is the mean fluorescence of all background readings

$$CTCF = \int \rho - (A_c \bar{X}F_b) \quad (B.1)$$

7. Average the three blank values (displayed here as a mean grey/fluorescent value) in each image, and plug into equation above and repeat with each

Appendix C

List of Publications

C.1 Journal Articles

1. Putter, P.J., Cui, Z.F. The Development of Functional Hydrogels for Neural Tissue Engineering: A Review. *Manuscript in preparation*.

C.2 Conference Papers

1. Putter, P.J., Szele, F.G., and Cui, Z.F. Three Dimensional Neuronal Cell Culture: Mimicking the Native Extracellular Matrix (poster, see C.1). *The British Neuroscience Association conference*, 2013.
2. Putter, P.J. Hydrogels and Tissue Engineering: Making you Nervous for all the Right Reasons (poster, see C.2). *Polymeric and Self-Assembled Hydrogels*, 2012.

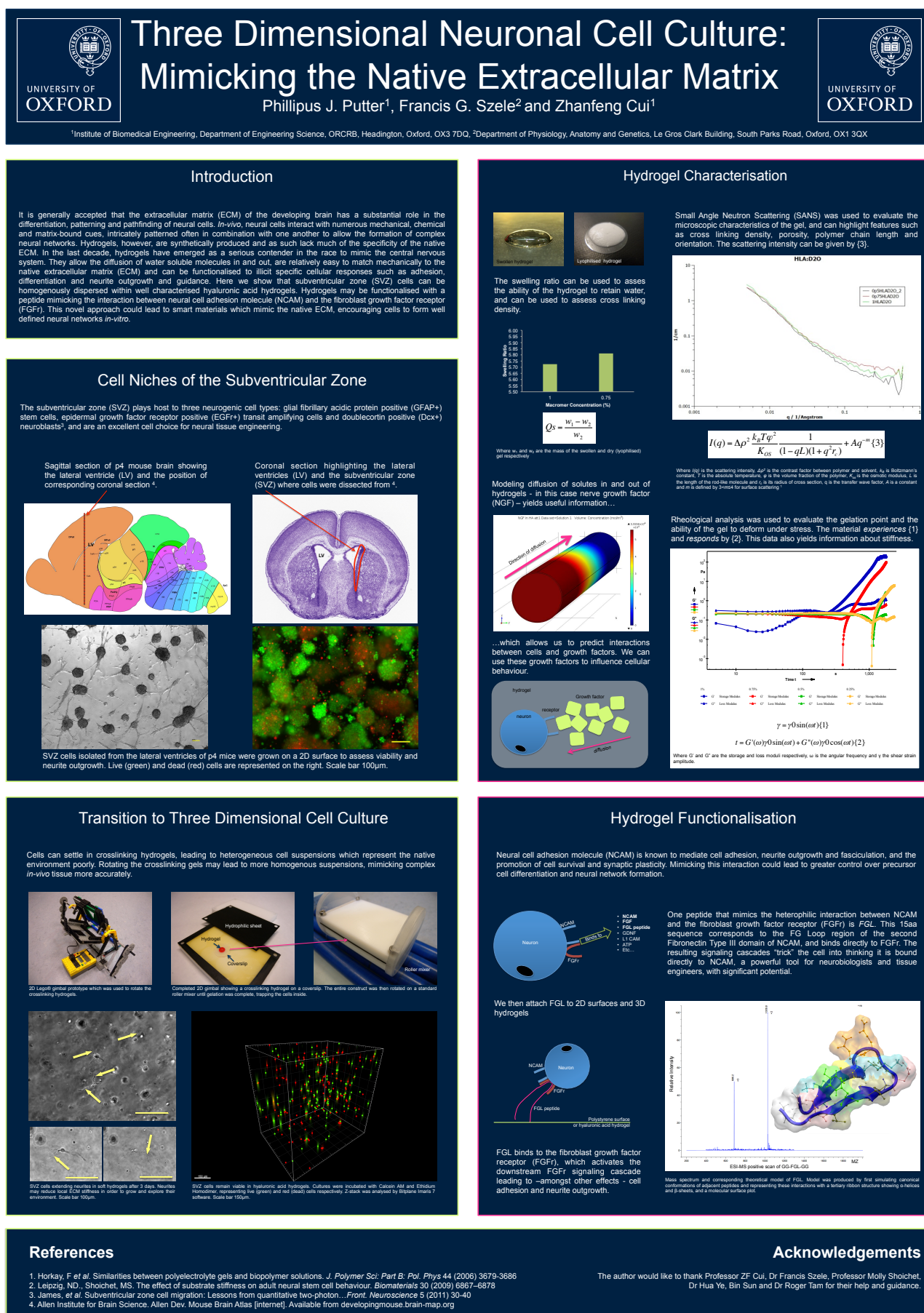
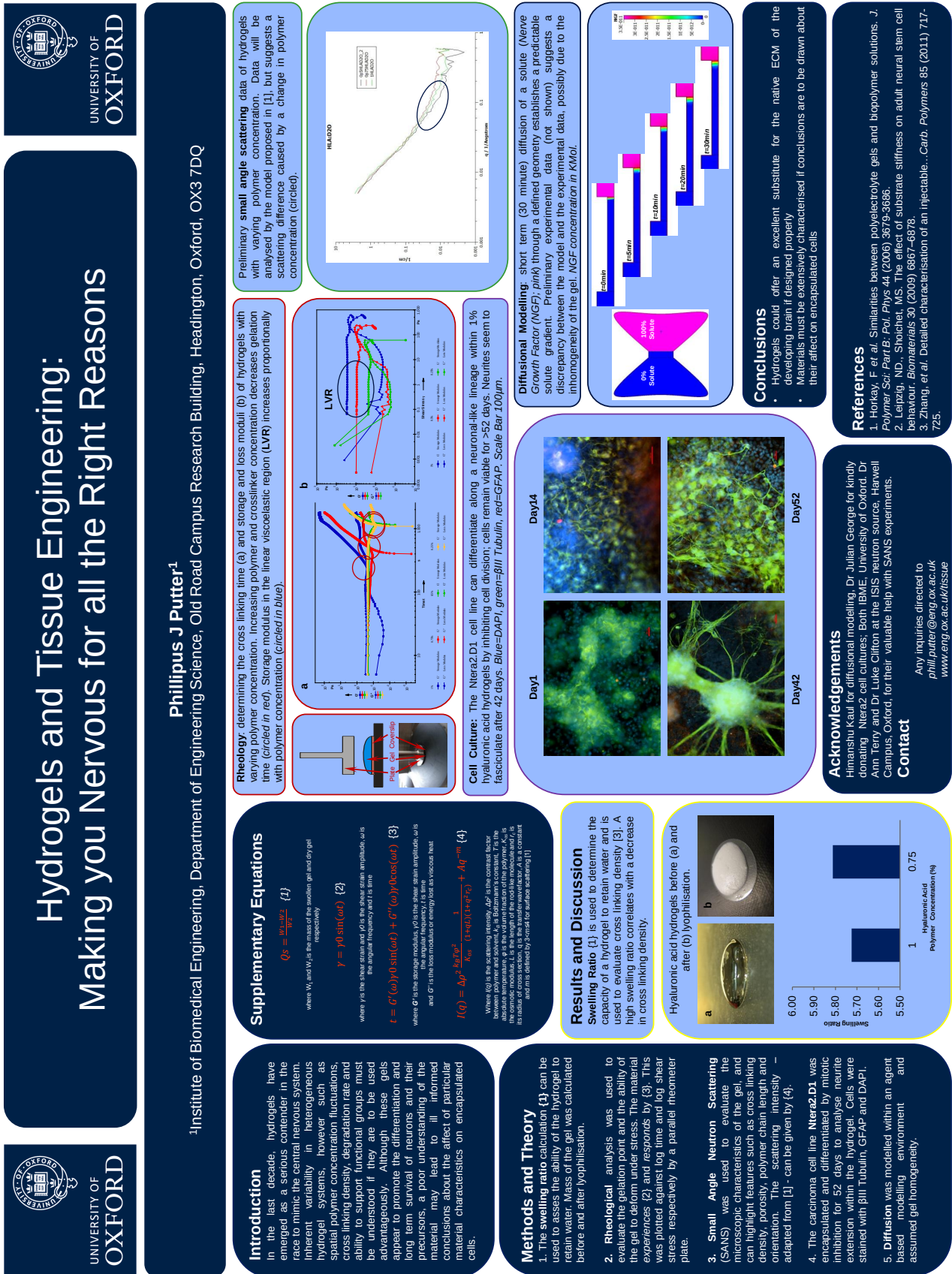
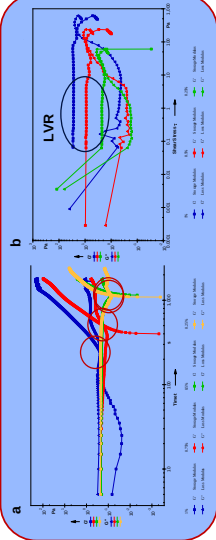


Figure C.1: Poster presented at the British Neuroscience Association, London, 2013.



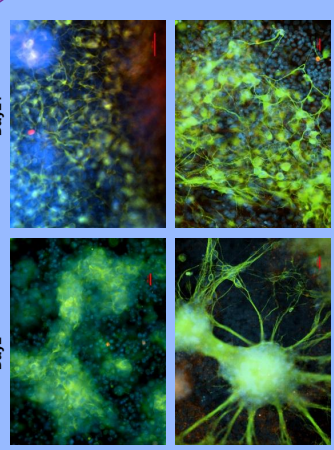
Rheology

determining the cross linking time (a) and storage and loss moduli (b) of hydrogels with varying polymer concentration. Increasing polymer and crosslinker concentration decreases gelation time (circled in red). Storage modulus in the linear viscoelastic region (LVR) increases proportionally with polymer concentration (circled in blue).



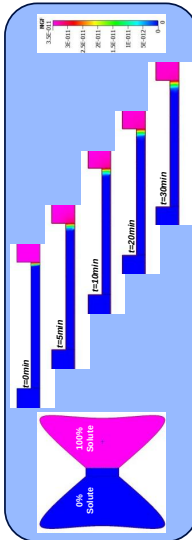
Cell Culture

The Ntera2.D1 cell line can differentiate along a neuronal-like lineage within 1% hyaluronic acid hydrogels by inhibiting cell division; cells remain viable for >52 days. Neurites seem to fasciculate after 42 days. Blue=DAPI, green=βIII Tubulin, red=GFAP. Scale Bar 100µm.



Diffusional Modelling

short term (30 minute) diffusion of a solute (Nerve Growth Factor (NGF), pink) through a defined geometry establishes a predictable solute gradient. Preliminary experimental data (not shown) suggests a discrepancy between the model and the experimental data, possibly due to the inhomogeneity of the gel. NGF concentration in Kmol.



Conclusions

- Hydrogels could offer an excellent substitute for the native ECM of the developing brain if designed properly
- Materials must be extensively characterised if conclusions are to be drawn about their affect on encapsulated cells

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Figure C.2: Poster presented at the Polymeric and Self-Assembled Hydrogels conference, London, 2012.

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