Glycosaminoglycan (GAG) Functionalised Electrospun Poly(lactic-co-glycolic acid) (PLGA) Scaffolds for the Propagation and Differentiation of Mouse and Human Embryonic Stem Cells

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences

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School of Materials
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Abstract
Embryonic stem (ES) cells have the capacity to form any cell type. However, their propagation and differentiation is limited by current two dimensional (2D) culture techniques which offer little flexibility in terms of surface structure and functionalisation with bioactive molecules. The aim of the current work was to produce a novel scaffold that could manipulate ES cell behaviour using both architectural and biological cues. Electrospinning is a flexible technique that creates nonwoven meshes that mimic the fibrous architecture of the ECM. Initial work focused on investigating the suitability of electrospun poly(lactic-co-glycolic acid) (PLGA) meshes for 2D and three dimensional (3D) culture of mouse ES cells, with the hypothesis that the fibrous architecture would assist in maintaining pluripotency. The study also sought to functionalise the scaffolds with biologically active molecules. Heparan sulphate proteoglycans (HSPGs) reside at the cell surface and within the ECM where they mediate growth factor binding, assist cell attachment and stabilise the ECM. Furthermore, ES cells modulate their own microenvironment by controlling the composition of heparan sulphate (HS), regulating the binding of growth factors such as fibroblast growth factor (FGF) family members. Therefore, we aimed to immobilise HS and heparin (a highly sulphated structural analogue of HS) on the fibre surface in a form that was freely accessible for protein/cell interactions and that retained its biological activity.
Electrospinning parameters were optimised to produce microfibre electrospun meshes with an average fibre diameter of 570nm. Cell morphology, proliferation and pluripotency were monitored using an Oct4-GFP reporter cell line and results compared with flat spin coated films. To investigate the potential for 3D culture, spinning parameters were altered to increase fibre diameter to >3µm with infiltration assessed using pro-migratory E-cadherin^-/- ES cells. Scaffolds were coated with plasma polymerised allylamine (ppAm) to enable non-covalent immobilisation of HS/heparin. Ligand binding assays with the link module of TSG-6 and anti-heparin/HS antibodies were used to probe HS/heparin presentation on the fibre surface. The biological activity of the immobilised HS/heparin was analysed by testing the ability of coated scaffolds to rescue the neural differentiation capacity HS deficient EXT1^-/- ES cells. Finally, human ES cells were cultured on the surface of ppAm scaffolds +/- HS in both unconditioned and mouse embryonic fibroblast (MEF) conditioned media for 5 days.
Both microfibre meshes and flat spin coated films supported the attachment, growth and pluripotency of mouse ES cells. Cells adopted distinct morphologies, with mouse ES cells aggregating in rounded colonies on microfibre scaffolds and demonstrating increased spreading on spin coated films. Fibres >3µm created a thicker mesh with potential for 3D culture supporting the infiltration of E-cadherin^-/- ES cells. ppAm enabled non-covalent immobilisation of HS/heparin in a form that was free to participate in protein interactions and which presented essential sulphation motifs within the HS/heparin chains. Bound HS was biologically active and functioned in synchrony with FGF4 to enhance neural differentiation of EXT1^-/- ES cells. The constructs also supported the attachment and growth of human ES cells, with HS functionalised scaffolds demonstrating a slight increase in compatibility during culture in unconditioned media.
The successful functionalisation of electrospun meshes with HS/heparin creates a highly versatile scaffold for ES cell culture and differentiation. The architecture of the meshes can be manipulated to either serve as a fibrous substrate for maintenance of pluripotency or support the formation of complex cell interactions present in vivo. The immobilisation of HS provides an extra dimension of versatility, as the scaffold can be tailored with specific HS species, potentially enabling the differential regulation of growth factor binding.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMAC</td>
<td>2-aminoacridone</td>
</tr>
<tr>
<td>bA-Link_TSG-6</td>
<td>Biotinylated link module of TSG-6</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>CS-6S</td>
<td>Chondroitin-6-sulphate</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulphate</td>
</tr>
<tr>
<td>EBs</td>
<td>Embryoid bodies</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ES cells</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>E-SEM</td>
<td>Environmental SEM</td>
</tr>
<tr>
<td>EXT</td>
<td>One of the exostosin family of genes</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion tyrosine kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GPI anchor</td>
<td>Glycosylphosphatidyl anchor</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HFIP</td>
<td>Hexafluoroisopropanol</td>
</tr>
<tr>
<td>HME</td>
<td>Hereditary multiple exotoses</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>IdoA</td>
<td>Ilduronic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
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</tr>
<tr>
<td>IHH</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>KSR</td>
<td>Knockout Serum Replacement</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NDST</td>
<td>N-deacetylase/N-sulphotransferase</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural progenitor cell</td>
</tr>
<tr>
<td>PCL</td>
<td>poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEO</td>
<td>poly(ethylene oxide)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>P&lt;sub&gt;L&lt;/sub&gt;LA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>P&lt;sub&gt;D&lt;/sub&gt;LA</td>
<td>Poly(D-lactic acid)</td>
</tr>
<tr>
<td>P&lt;sub&gt;D,L&lt;/sub&gt;LA</td>
<td>Poly(D, L-lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>P&lt;sub&gt;D,L&lt;/sub&gt;PGA</td>
<td>Poly(D, L-lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>P&lt;sub&gt;L&lt;/sub&gt;PGA</td>
<td>Poly(L-lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PMMA-g-PEO</td>
<td>Poly(methacrylate)-graft-poly(ethylene oxide)</td>
</tr>
<tr>
<td>ppAm</td>
<td>Plasma polymerised allyl amine</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RGD</td>
<td>α5β1 integrin binding sequence – Arginine-Glycine-Aspartic acid</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high pressure liquid chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SSEA</td>
<td>Stage specific embryonic antigen</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
</tr>
<tr>
<td>Tg</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TRA</td>
<td>Tumour recognition antigen</td>
</tr>
<tr>
<td>TSG-6</td>
<td>The product of tumor necrosis factor-stimulated gene-6</td>
</tr>
<tr>
<td>UA</td>
<td>An unspecified uronic acid</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus G</td>
</tr>
<tr>
<td>WCA</td>
<td>Water contact angle</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>2-OST</td>
<td>2-O-sulphotransferase</td>
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<tr>
<td>3-OST</td>
<td>3-O-sulphotransferase</td>
</tr>
<tr>
<td>6-OST</td>
<td>6-O-sulphotransferase</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
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Introduction

Pluripotent, self-renewing ES cells hold a great degree of potential in the field of tissue engineering. Their capacity to form any cell type of the adult body makes them excellent candidates for the treatment of diseases such as diabetes and Parkinsons. However, ES cells are derived from a 3D in vivo environment, surrounded by cell: cell and cell: ECM interactions. The standard two 2D culture in which ES cells are maintained does not adequately replicate this microenvironment and differentiation towards certain lineages is often dependent on the formation of embryoid bodies (EBs) in suspension culture; a method which produces a heterogeneous population of cell types and highlights the need for the formation of sophisticated and complex 3D interactions.

Biomaterial scaffolds offer an excellent opportunity to provide the support and environmental cues for either ES cell propagation or differentiation into mature cell phenotypes. The many techniques available create highly porous scaffolds, each with a unique structure for cell attachment and growth. Electrospinning is a highly versatile method of fabricating fibrous meshes which replicate the architecture of the ECM the cells would experience in vivo. Flexibility is provided by the capacity to electrospin a wide variety of natural and synthetic polymers (either together or separately) to easily create scaffolds with different fibre architecture and to modify meshes post-spinning with bioactive ligands. Electrospun PLGA meshes have demonstrated potential in the culture and differentiation of mesenchymal stem cells (MSCs) (Xin et al., 2007), whilst polyamide meshes support the maintenance of a more in vivo like cell morphology of fibroblasts (Nur et al., 2005; Schindler et al., 2005).

Synthetic polymers such as PLGA and polyamide lack the biological cues provided by the natural ECM. In order to enhance the biocompatibility of synthetic polymers, natural polymers are often incorporated into the polymer blend, adsorbed onto the surface or covalently attached. Traditional ECM molecules utilised in this way include collagen and fibronectin. However, HS (a glycosaminoglycan (GAG)) is a highly structured molecule that can differentially regulate growth factor binding, assist in migration and stabilise ECM. Mouse ES cells express unusually low sulphated forms of HS with differentiation towards neural (Johnson et al., 2007) and mesodermal (Baldwin et al., 2008) lineages accompanied by, and dependent on, the generation of specific sulphation motifs within the HS chains. In addition, HS stabilises and facilitates the activity of FGF2, a growth factor fundamental in the propagation of human ES cells. Despite the diverse functionality and key role of HS in ES cell culture, the combination of a biomaterial scaffold with HS and ES cells has yet to be explored.
1.1 Embryonic Stem Cells

1.1.1 Deriving and Defining Embryonic Stem Cells

ES cells are derived from the inner cell mass (ICM) of the pre-implantation embryo or blastocyst. The blastocyst arises from sequential cleavage of the fertilised egg without any visible cell growth until the 8 (mouse) or 8-16 (human) cell stage at which point compaction occurs forming the morula (Chuva de Sousa Lopes and Mummery, 2006). As development progresses, the blastocyst forms with an outer trophectoderm layer and the ICM attached to one end of the structure (Chuva de Sousa Lopes and Mummery, 2006). Cells exposed to the blastocoelic cavity on the periphery of the ICM differentiate to form the primitive endoderm whilst the undifferentiated cells within form the epiblast which subsequently gives rise to the embryo proper and is the structure from which ES cell are derived (Chuva de Sousa Lopes and Mummery, 2006). An overview of mouse blastocyst formation and subsequent development of the embryo is described in Figure 1.

**Figure 1. Mouse blastocyst formation and development.** The compacted morula forms the blastocyst, creating two cell types; the cdx2 expressing extraembryonic trophectoderm and the ICM. The ICM consists of apolar, undifferentiated cells that express pluripotent markers Oct3/4 and Nanog (Niwa, 2007). Cells of the ICM exposed to the blastocoelic cavity differentiate to form the extraembryonic primitive endoderm (characterised by Gata6 expression) which remains in close contact with the undifferentiated epiblast (Chuva de Sousa Lopes and Mummery, 2006; Niwa, 2007). During implantation, progressive development of the blastocyst creates the egg cylinder. The trophectoderm forms the extraembryonic ectoderm and the primitive endoderm differentiates into parietal endoderm and visceral endoderm (Chuva de Sousa Lopes and Mummery, 2006). Whilst the parietal endoderm forms over the trophectoderm, the visceral endoderm encapsulates the epiblast contributing to the subsequent development of the primitive ectoderm (Chuva de Sousa Lopes and Mummery, 2006). Diagram is reproduced from Niwa 2007.
ES cells are derived following culture of the blastocyst as a whole (Evans and Kaufman, 1981) or isolation of the epiblast by immunosurgery (Martin, 1981; Thomson et al., 1998). The resulting ES cells are pluripotent, and have the capacity to form all three germ layers of the developing embryo, as well as the extraembryonic lineages of the trophectoderm and primitive endoderm (Niwa, 2007).

Growing in tight heaped colonies, ES cells are characterised by a rounded morphology and a high nucleus: cytoplasm ratio. ES cells have a high proliferation rate, with mouse ES cells doubling approximately every 12 hours (Niwa, 2007). During proliferation, ES cells remain pluripotent due to their capacity to self-renew. The combination of high proliferation rate and self-renewal means that, in the right culture conditions, ES cells could represent an infinite stock of cells for tissue engineering applications. These combined characteristics also contribute to the clonogenic capacity of ES cells; in optimal conditions a single ES cell can divide to form a homogenous population of cells (Amit et al., 2000).

The pluripotency of cultured ES cells can be assessed by *in vitro* differentiation into EBs the morphology of which is reminiscent of early embryonic development and leads to the formation of cell types from all three germ layers (Evans and Kaufman, 1981; Itskovitz-Eldor et al., 2000; Martin, 1981). When injected into immunodeficient mice, ES cells also form teratomas comprised of an array of different cell types (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). The phenotype of ES cells can be easily assessed by monitoring stem cell specific markers, including stage-specific antigen (SSEA)-3, SSEA-4 and tumour recognition antigens TRA1-60 and TRA1-81 on human ES cells (Stewart et al., 2006; Thomson et al., 1998). In comparison to human ES cells, mouse ES cells do not express the above markers but are characterised by expression of SSEA-1 (Stewart et al., 2006).

Despite the differences in surface antigens, both human and mouse ES cells express the same transcription factors essential for maintenance of pluripotency. These include Oct4 and Nanog, the expression of which can be determined using polymerase chain reaction (PCR) analysis, flow cytometry or immunocytochemistry.

### 1.1.2 Pluripotency and Self Renewal

The pluripotent state of both mouse and human ES cells is sustained by a core regulatory network comprised primarily of transcription factors Oct4, Sox2 and Nanog. Each is expressed within the ICM and epiblast and loss of any one component leads to a co-ordinated down regulation of all elements, leading to ES cell differentiation (Fong et al., 2008). Due to the inherent differences between mouse and human ES cells, the downstream targets of the network are not wholly comparable (Boyer et al., 2005; Loh et al., 2006). However, both human and mouse ES cells share a dependence on this core network for maintenance of the pluripotent state.

The expression levels of Oct4 are key to its regulatory activity. In mouse, loss of Oct4 expression leads to differentiation into trophectoderm, whilst over expression causes differentiation into cells resembling primitive endoderm (Niwa, 2007; Niwa et al., 2000; Stewart et al., 2006). In contrast, loss of Nanog leads to differentiation into primitive endoderm like cells and over expression in mouse ES cells maintains the pluripotent state independent of LIF/STAT3 pathway (Mitsui et al., 2003; Niwa, 2007; Stewart et al., 2006). In a simplified model, it was proposed that Oct4 prevents trophectodermal differentiation by inhibiting transcription factors such as Cdx2 (Niwa, 2007; Niwa et
al., 2005), whilst Nanog prevents primitive endoderm expression by repression of transcription factors such as Gata6 (Mitsui et al., 2003; Niwa, 2007). The opposing effects of Nanog and Oct4 on primitive endoderm differentiation can be linked to the autoregulation and feedback mechanisms involved in the transcriptional network. Oct4 partners with Sox2, forming a heterodimer that binds enhancers and modulates the expression of a number of genes including FGF4 (Rodda et al., 2005) and Nanog (Loh et al., 2006; Rodda et al., 2005; Stewart et al., 2006). The Oct4:Sox2 heterodimers and Nanog also regulate their own expression, creating a feedback loop which helps to maintain appropriate expression levels (Chew et al., 2005; Loh et al., 2006; Niwa, 2007). At normal expression levels within an ES cell, Oct4:Sox2 activates Nanog expression. In a model proposed by Pan et al. 2006, the heterodimer acts as a repressor of Nanog at high concentrations resulting in differentiation to primitive endoderm (Pan et al., 2006).

In both mouse and human ES cells, the three main transcription factors can behave as both activators and repressors of gene expression (Boyer et al., 2005; Loh et al., 2006). As well as activating their own expression and that of genes involved in the maintenance of pluripotency, the transcription factors also act to repress genes implicated in developmental processes (Boyer et al., 2005; Loh et al., 2006).

### 1.1.3 Culture of Mouse ES Cells

First derived on STO fibroblasts (Evans and Kaufman, 1981; Martin, 1981) in teratocarcinoma conditioned media (Martin, 1981), the culture of mouse ES cells has become gradually more defined with improved understanding of their biology. The requirement for a feeder layer has been removed as cells can be successfully maintained on gelatinised TCP in media supplemented with either foetal calf serum (FCS) or Knockout Serum Replacement (KSR, a defined synthetic substitute) (Ward et al., 2002). Until recently, the key to mouse ES cell pluripotency and self-renewal has been Leukaemia inhibitory factor (LIF), a member of the IL6 family of cytokines. LIF signalling is mediated via LIFRβ and gp130 receptor binding, leading to signalling through STAT3 phosphorylation and activation (Matsuda et al., 1999). An additional factor, bone morphogenic protein (BMP) 4, has come to the forefront of mouse ES cell culture. When combined with defined N2B27 media supplemented with LIF, addition of BMP4 removes the need for serum and maintains ES cell pluripotency by activating Id genes which in turn prevent neural differentiation (Ying et al., 2003a). In addition to activating Id genes, BMP4 also functions by blocking ERK phosphorylation and signalling via the MAPK pathway, events upstream of mouse ES cell differentiation (Qi et al., 2004; Ying et al., 2008). The refinement of the signalling mechanisms involved in maintaining mouse ES cell pluripotency as led to the proposed ‘ground state’ model of ES cell self renewal (Ying et al., 2008). By combining low concentrations of inhibitors of FGF receptor (FGFR) and ERK signalling with defined N2B27 media, mouse ES cell pluripotency was maintained but with reduced cell viability. However, viability was restored by the addition of a GSK3 inhibitor which was hypothesised to compensate for the loss of the pro-proliferative signalling of the MAPK/ERK pathway (Ying et al., 2008). Further refinements led to the production of 3i media that sustained ES cells independently of the LIF/STAT3 pathway and BMP4 (Ying et al., 2008). Therefore, Ying et al. (2008) proposed that ES cells inherently self-maintain their pluripotent phenotype unless stimulated by exogenous factors that result in down regulation of the core transcriptional network and subsequent differentiation. In comparison, signalling via LIF/STAT3 and BMP4 buffer the ES cells.
against exogenous stimulation in undefined culture conditions (i.e. endogenous FGF4 signalling via ERK) (Ying et al., 2008).

The effect of exogenous stimulation on ES cell pluripotency appears to extend to integrin engagement. Hayashi et al. (2007) determined that mouse ES cells express a repertoire of integrins for fibronectin ($\alpha_5\beta_1$, $\alpha_V\beta_1$) and laminin ($\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_7\beta_1$) binding. However, growth on laminin and fibronectin led to focal adhesion tyrosine kinase (FAK) signalling and differentiation (Hayashi et al., 2007). As binding to gelatin and collagen I was not mediated by integrin binding, no differentiation occurred on these substrates (Hayashi et al., 2007).

Despite these recent developments, mouse ES cells are mainly and successfully maintained in media supplemented with FCS/KSR and LIF on gelatinised TCP. In stark contrast to mouse ES cells, human ES cells cannot be maintained by LIF (Thomson et al., 1998) and suppression of BMP signalling promotes human ES cell pluripotency (Xu et al., 2005).

1.1.4 Culture of Human ES Cells

As with mouse ES cells, human ES cells were originally derived on a feeder layer of MEFs providing a surface for attachment and secreting an unknown combination of factors into the surrounding media (Thomson et al., 1998). If human ES cells are to be applied therapeutically it is necessary for a defined, xeno-free environment (free from feeder layers and animal derived products) to be determined. The necessity for an animal-product free culture environment was highlighted by Martin et al. (2005) who demonstrated the incorporation of non-human sialic acid Neu5Gc by human ES cells cultured on a MEF feeder layer.

1.1.4.1 A Suitable Substrate

As well as secreting supporting factors into the media, MEFs provide a matrix of proteins and glycans, including fibronectin, collagen I, collagen IV and laminin, for human ES cell attachment, proliferation and self-renewal (Braam et al., 2008). The MEF feeder layer was initially replaced by matrigel, a heterogeneous mix of ECM molecules including collagen IV, HS proteoglycans (HSPGs) and laminin (Kleinman et al., 1982; Xu et al., 2001). Matrigel has subsequently supported the successful culture of human ES cells in a variety of different media compositions (Levenstein et al., 2006; Li et al., 2005b; Lu et al., 2006; Ludwig et al., 2006; Xu et al., 2005). However, matrigel is produced by mouse Engelbreth-Holm-Swarm (EHS) tumours and represents an undefined, animal derived product in human ES cell culture.

In order to refine the culture substrate, studies have maintained human ES cells in a variety of different media on TCP coated with ECM substrates such as collagen IV, fibronectin and laminin. A number of studies have successfully used laminin as a substrate (Li et al., 2005b; Xu et al., 2001) with the integrin dimer $\alpha_6\beta_1$ specific for laminin binding detected on the cell surface (Xu et al., 2001). After assessing integrin expression of human ES cells, Meng et al. (2009) refined the adhesive substrate to defined laminin-derived peptide sequences. The peptides were specific for HS and $\alpha_\nu\beta_3$ and $\alpha_6\beta_1$ integrin binding, with the HS (specifically syndecan-1) binding peptide required at higher levels. Although Xu et al. (2001) found fibronectin inferior to laminin, a number of studies have successfully maintained human ES cells on fibronectin (Amit et al., 2004) and in some cases have found laminin to be less adequate compared to fibronectin (Baxter et al., 2009). The
The ability of fibronectin to support human ES cell growth is re-enforced by the expression and interaction of fibronectin binding integrins α5β1 (Baxter et al., 2009). In comparison, Ludwig et al. (2006) determined that a combination of collagen IV, fibronectin, laminin and vitronectin was necessary to support cells in fully defined TeSR1 media.

The conflicting results described above could be due to batch-to-batch variations in protein preparations, differences in media composition or variations in the origin of the proteins. Equally, they may also represent an inherent difference between human ES cell lines. Differences within cell lines can also be established as one line is grown in different labs. Regarding substrate, Amit et al. (2004) determined that although human fibronectin supported human ES cell proliferation, bovine fibronectin did not sustain human ES cells under certain conditions (Amit et al., 2004). After determining functional expression of αVβ5 (vitronectin), α5β1 (fibronectin) and α6β1 (laminin) integrins, Braam et al. (2008) reported the ability of matrigel, laminin, fibronectin, collagen IV and vitronectin to support human ES cell culture in the presence of MEF conditioned media. However, when media was changed to TeSR1, only vitronectin and matrigel could support cell attachment and growth.

The ability of matrigel to sustain human ES cells in a variety of media compositions may due to its qualities as a cell-secreted, pre-assembled ECM (Kleinman et al., 1982). The organisation and architecture of the molecules are in an optimal configuration for ES cell interaction. In comparison, the coating of TCP with specific ECM molecules is dependent on protein adsorption to the culture surface and may vary between TCP products. The proteins may not be adsorbed in a manner which enables effective ligand interaction and are not part of a highly ordered structure in the same way as the native ECM.

### 1.1.4.2 Culture Media

The first step towards a more defined environment occurred with the replacement of FCS with synthetic KSR (Amit et al., 2000). Developed for mouse ES cell culture, serum replacement is more defined, largely free of animal derived products and does not suffer from the batch-to-batch variations associated with FCS. However, despite dramatically enhancing cloning efficiency, this substitution required the addition of relatively low concentrations (4ng/ml) of FGF2 to maintain proliferation of the clonally derived human ES cells (Amit et al., 2000). From this study onwards, FGF2 has been a key factor in the development of a defined culture environment and is present in all subsequent media compositions. Xu et al. (2001) partially bypassed the need for a MEF feeder layer by successfully culturing hES cells on human fibronectin in MEF conditioned media supplemented with FGF2 (8ng/ml). However, higher concentrations of FGF2 (100ng/ml) are sufficient to support human ES cell self-renewal and proliferation in unconditioned, KSR supplemented media (Levenstein et al., 2006; Xu et al., 2005). Levenstein et al. (2008) proposed that the dramatic increase in FGF concentration necessary in unconditioned media may be partially attributed to the lack of stabilising HSPGs normally secreted by MEFs. The study identified 5 species of MEF secreted HSPGs including perlecan, agrin and syndecan-4 and proposed the secreted products not only stabilised FGF-2 in the media but also assisted receptor binding (Levenstein et al., 2008). However, although addition of heparin/HS enhanced human ES cell culture at lower FGF2 concentrations they did not match growth in MEF conditioned media,
suggesting optimal FGF2 signalling required a specific repertoire of MEF secreted HSPGs (Levenstein et al., 2008). In contrast, Furue et al. (2008) successfully applied heparin to a defined human ES cell culture system, maintaining human ES pluripotency at levels equivalent to growth on MEF feeder layers. The study highlighted the potential of heparin/HS in human ES cell propagation, reporting a dose dependent increase in colony formation and FGF receptor (FGFR) phosphorylation with added heparin. The optimal media composition contained 100ng/ml heparin and 10ng/ml FGF2, a dramatically reduced concentration compared to previous studies. The differing results could be partially attributed to different media compositions. Whilst Levenstein et al. used DMEM/F12 media, Furue et al. applied a base medium, ESF, previously developed for mouse ES cell culture. A recent study by Eisellrova et al. (2009) indicated that although human ES cells express all four FGF receptors (FGFR, with FGFR1 expression predominant) FGF2 signals primarily through FGFR2. The study also highlighted the expression of endogenous FGF2 by human ES cells and proposed FGF2 functioned by primarily promoting cell adhesion and survival rather than directly maintaining pluripotency (Eisellrova et al., 2009). This is supported by Furue et al., who reported enhanced FGFR phosphorylation with added heparin in the absence of exogenous FGF2 (Furue et al., 2008).

The concentration of FGF2 in unconditioned media can be reduced when combined with other factors, including TGFβ1 (Amit et al., 2004; Ludwig et al., 2006) and the BMP inhibitor noggin (Xu et al., 2005). Amit et al. (2004) supported human ES cells in media supplemented with KSR, 4ng/ml FGF2 and 0.12ng TGFβ1. The two factors were also fundamental elements of the defined media TeSR1, which also substituted KSR with a supplement of lipids, minerals and cloned growth factors creating a completely defined, animal free culture medium in which two new human ES cell lines were derived (Ludwig et al., 2006). In a study that brings to mind the hypothesised ‘ground state’ of mouse ES cells, Xu et al. (2005) highlighted the need to suppress pro-differentiation signals such as BMP4 to sustain ES cell pluripotency. Addition of noggin to unconditioned media reduced the required concentration of FGF2 to 40ng/ml. The study also revealed that MEF conditioned media promotes a lower level of BMP signalling compared to unconditioned media, suggesting MEFs act to either remove the signalling agent or to secrete inhibitors into the media.

Despite progressive steps towards a defined culture environment, human ES cells are still routinely cultured with MEF-conditioned media or unconditioned media supplemented with high concentrations of FGF2. Indeed, in many laboratories precious stock cultures of human ES cells are maintained on MEFs with KSR and low FGF2 levels. The inability to depart from reliance of MEFs is in part due to a lack of understanding of the molecular mechanisms maintaining human ES cells and also an inability to fully replicate the results presented in the above papers (Baxter et al., 2009).
1.1.5 Differentiation

1.1.5.1 Embryoid Body Formation

ES cells can be effectively differentiated into a heterogeneous population of cells by EB formation. ES cells are typically cultured in suspension without the presence of LIF (in the case of mouse ES cells), resulting in the formation of cell aggregates that develop into a highly ordered 3D structure composed of cells from all three germ layers, reminiscent of early embryonic development. An overview of EB formation is depicted in Figure 2.

Figure 2. EB formation. ES cells aggregate in suspension culture. The outer cells flatten, forming tight junctions and form an endoderm layer equivalent to the visceral endoderm of the developing embryo (Li et al., 2003). The visceral endoderm cells secrete components such as laminin leading to the formation of a basement membrane composed of collagen IV, nidogen and dystroglycan (Colognato and Yurchenco, 2000). Apolar cells within attached to the deposited basement membrane become pseudostratified forming a polarised population of cells equivalent to the primitive ectoderm (Li et al., 2003). Cells not attached to the basement membrane undergo apoptosis creating an inner cavity (Li et al., 2003), whilst the polarised primitive ectoderm further differentiates to produce cells from all three germ layers. Figure reproduced from (Li et al. 2003).

In mouse, the outer visceral endoderm layer is induced by signalling downstream of FGFR (possibly signalling via FGF4) which stimulates the expression of GATA4 and GATA6 (Li et al., 2004; Li et al., 2003). This in turn leads to expression and secretion of the key basement membrane component, Lamnin-1. Laminin secretion is stimulated by both FGF2 and β1 integrin signalling (Li et al., 2004; Li et al., 2003; Li et al., 2002a) and regulates the expression and distribution of basement membrane component dystroglycan (Li et al., 2002a). Complete loss of laminin leads to the failure of basement membrane formation which can only be rescued by the addition of exogenous laminin to the media (Li et al., 2004; Li et al., 2003; Li et al., 2002a). It is proposed by Li et al. (2002a) that laminin interacts with the apolar cells within the EB via a distinct heparin binding sequence and assembles into a lattice structure which forms the basis of the basement membrane (Li et al., 2002a). It would appear that assembly of the basement membrane is co-ordinated by the apolar cells within the EB, as ES cells express laminin receptors and can co-ordinate basement membrane assembly in the absence of the visceral endoderm layer (Henry et al., 2001; Li et al., 2004). The co-ordinated behaviour of basement membrane assembly between the two cell types (visceral endoderm and pre-primitive ectoderm cells) is partly responsible for the highly organised 3D structure of the EB (Li et al., 2004).
Sachlos et al. (2008) recently assessed EB structures derived from human ES cells by scanning electron microscopy (SEM). After 3 days, the aggregates of cells had deposited fibrous ECM coating with individual cells still discernable. By 7 days of culture, individual cells could not be identified as they were had encapsulated in an outer ECM plaque composed primarily of collagen I. A large proportion of ES cell differentiation protocols involve EB formation and stimulation with particular exogenous factors in order to enhance the differentiation of a particular cell phenotype within the heterogeneous population. Sachlos et al. enhanced the neural-inducing effect of retinoic acid by treating EBs with collagenase which loosened the outer ECM and degraded the collagen IV within the basement membrane enabling enhanced diffusion of retinoic acid (RA) into the centre of the EB.

EB formation has been employed to produce many cell types including dopaminergic neurons (Yan et al., 2005), motoneurons (Li et al., 2005a), cardiomyocytes (Kehat et al., 2004) and insulin secreting cells (Hori et al., 2002). The enhancement of a particular cell phenotype is dependent on the substrate on which the EBs are cultured, the media composition and the timing of treatment with particular morphogens/growth factors. For instance, in human ES cells, motoneurons (Li et al., 2005a) and dopaminergic neurons (Yan et al., 2005) are developed using the same basic differentiation protocol involving EB formation and culture, creating neural rosettes characteristic of neuroectoderm. However, whilst treatment of the developing neuroectoderm with RA enhances the formation of motoneurons (Li et al., 2005a), treatment with FGF8 leads to the development of dopaminergic neurons (Yan et al., 2005). Timing is also an essential factor and gives an indication of the developmental windows that occur in vivo. For instance, exposure to FGF8 before Sox1 expression primarily creates midbrain dopaminergic neurons, whilst late exposure (after Sox1 expression) enhances forebrain dopaminergic neural development (Yan et al., 2005).

Transplantation of differentiated cells into animal models has had a degree of success, illustrating the therapeutic potential of ES cells. Cardiomyocytes derived from human ES cells have partially rescued the hearts of atrio-ventricular blocked pigs, displaying integration into the host tissue and a potential pace making activity (Kehat et al., 2004). Streptozotocin induced diabetic mice were also partially rescued by renal grafting of insulin producing clusters derived from mouse ES cells (Hori et al., 2002). However, if cells are to be used therapeutically it is essential that all ES cells reach a definitive cell phenotype as any undifferentiated cells present within the culture have the potential to form teratomas in the host tissue (Hori et al., 2002). Cell survival at the graft site also compromises the therapeutic potential of transplanted cells. However, this can potentially be overcome with the use of a scaffold or matrix that enhances cell survival and inhibits initial inflammatory responses (Uemura et al., 2010). Uemura et al. used matrigel combined with neural progenitor cells (NPCs) to increase cell survival and proliferation at the graft site. However, the neurite extension and migration observed was not recapitulated in vivo (Uemura et al., 2010).

1.1.5.2 Monolayer culture

An excellent example of the differentiation of ES cells in a monolayer culture is the protocol reported by Ying et al. (2003b) for the neural differentiation of mouse ES cells. By utilising a Sox1-GFP reporter cell line it was possible to follow and quantify the neural differentiation of ES cells seeded and cultured on gelatin in defined, serum free N2B27 media. Sox1 expression within the
population peaked to 75% after 4 days of culture and then steadily declined after 8-10 days as differentiation progressed. The cells developed a network of extended neural processes that were positive for the neuronal marker βIII tubulin. It was also possible to manipulate the developing cells towards specific cell phenotypes, as replating cells on fibronectin at days 6-8 followed by treatment with Sonic hedgehog (SHH) and FGF8 enhanced the development of tyrosine hydroxylase positive (dopaminergic) neurons. Autocrine/paracrine FGF4 signalling via FGFRs was determined as the primary signalling pathway inducing neural differentiation, reinforced by the exogenous addition of FGF4 increasing the proportion of Sox1 positive cells. In comparison, addition of FGF2 had no effect. Inhibition of FGF4 by BMP4, FGFR inhibitors and dominant negative FGFR induced the formation of non-neural cells. The inhibition of neural differentiation by BMP4 is a keystone of the protocol for mouse ES cell self-renewal first presented by Ying et al. (2003a) and described in section 1.1.3. Further research revealed that FGF4 signals specifically through the MAPK/ERK1/2 pathway and only a small signalling window of at least 1 hour (optimal 9 hours) is necessary for induction towards the neuronal phenotype (Stavridis et al., 2007).

1.1.6 ES Cell Microenvironment

1.1.6.1 Cell: Cell contact

Cell: cell contact in both human and mouse ES cells is mediated by E-cadherin, the expression of which is promptly downregulated upon differentiation with concomitant up-regulation of N-cadherin (Eastham et al., 2007; Spencer et al., 2007). E-cadherin participates in homophilic interactions with neighbouring cells and is anchored to the actin cytoskeleton via β-catenin (Cavallaro and Christofori, 2004). The loss of E-cadherin and upregulation of N-cadherin is a defining characteristic of the epithelial to mesenchymal transition (EMT) event necessary to allow epiblast cells to enter the primitive streak (Cavallaro and Christofori, 2004). The loss of E-cadherin from ES cells has been studied in order to gain an insight into the molecular mechanisms underlying EMT which is also associated with the metastasis of cancer cells (Eastham et al., 2007; Spencer et al., 2007).

E-cadherin−/− embryos are unable to progress past the blastocyst stage of development and E-cadherin−/− ES cells demonstrate a defective ability to contribute to chimeric embryos (Larue et al., 1996). E-cadherin−/− ES cells lack the cell: cell contacts of wildtype ES cells, growing as single cells with a polarised actin arrangement rather than in tight colonies (Eastham et al., 2007; Larue et al., 1996; Spencer et al., 2007). Despite the morphological change, E-cadherin−/− ES cells retain a pluripotent phenotype in standard culture conditions and have enhanced motility (Eastham et al., 2007; Spencer et al., 2007). The phenotype is fully reversible with reconstitution of E-cadherin restoring normal ES cell cortical actin and the formation of ES cell colonies (Eastham et al., 2007; Larue et al., 1996; Soncin et al., 2009; Spencer et al., 2007).

A recent study by Soncin et al. (2009) revealed that inhibition of E-cadherin, either by gene targeting, RNAi or use of a peptide inhibitor, enabled ES cells to remain pluripotent in the absence of LIF. β-catenin−/− ES cells displayed the same phenotype indicating that removal of the E-cadherin-β-catenin complex enabled ES cells to maintain pluripotency independent of the LIF/STAT3 pathway. Further analysis identified an alternative self renewal pathway via the TGFβ pathway. E-cadherin−/− ES cells were maintained in serum free media supplemented with FGF2 (to
stimulate proliferation) and Activin-A and Nodal, both of which are members of the TGFβ superfamily and were required to maintain pluripotency. This suggests that separate pathways able to support self-renewal exist within the mouse ES cell, with particular pathways brought to the forefront according to the cellular context.

1.1.6.2 ECM

The primary ECM the developing ICM encounters during early development is the basement membrane deposited by the primitive endoderm and its derivatives. The basement membrane is mainly composed of collagen IV, laminin, nidogen/entactin and perlecan (Li et al., 2003), the structure and function of each in forming the basement membrane is reviewed by LeBleu et al. (2007). The basement membrane forms an intricate, stable network for cell attachment. The two major components are collagen IV and laminin, which polymerise to form separate networks linked by nidogen/entactin and perlecan. The structure of each component and the resulting network is depicted in Figure 3.

Collagens (specifically reviewed by Gelse et al. (2003) and Koshnoodi et al. (2008)) are formed from a triple helix comprised of 3 polypeptide chains (α chains) and are formed from three of the same (homotrimer) or from two or more different chains (heterotrimers). The majority of collagens, such as collagen I, arrange to form dense fibrils which provide tensile strength and have load bearing properties. Collagen IV (α1α1α2, α3α4α5 and α5α5α6) is a non-fibrillar collagen and is found exclusively in basement membranes with the α1α1α2 form dominant in early embryonic development. Rather than forming fibrils, its flexible structure combined with self-binding domains at both the N- and C- terminus enable it to form the network depicted in Figure 3. The central triple helical collagenous domain, is composed of a repeating Gly-X-Y sequence with X-Y either proline and hydroxyproline or lysine and hydroxylsine. The flexibility is provided by numerous interruptions of the Gly-X-Y sequence along the chain length. The N-terminal 7S domain is also comprised of Gly-X-Y repeats and enables four separate molecules to interact. In contrast, the globular C-terminal NC-1 domain is cysteine and lysine rich and mediates head to head interactions via hydrophobic and hydrophilic interactions. The network is further stabilised by lateral interactions along the helical collagenous domain.

Laminins are heterotrimeric glycoproteins comprised of α, β and γ chains, with laminin-1 (α1β1γ1) the most prominent isoform during early development (Colognato and Yurchenco, 2000). The three chains tightly associate through a central α-helical coiled-coiled domain (Colognato and Yurchenco, 2000). N-terminal short arms of each chain extend from the coiled region and mediate self-self binding essential for polymerisation. The α chain has an extended C-terminus, forming the G domain comprised of 5 globular domains (LG modules) which house binding sites for heparin and integrins and is largely responsible for mediating cell adhesion (Colognato and Yurchenco, 2000).
Figure 3. Basement membrane components and structure. Laminin 1 and collagen IV assemble separate meshes within the basement membrane through homotypic interactions. Laminin polymerises via short arms and primarily binds cell surface molecules such as α6β1 integrins through the globular (G) domain on the α chain (Colognato and Yurchenco, 2000; Li et al., 2003). Collagen IV molecules primarily associate through the 7S (4 molecule interaction) and NC1 domains (interaction of two molecules) (Khoshnoodi et al., 2008). Perlecan and nidogen interweave and bind both laminin and collagen IV, connecting the two networks providing extra stability. ECM components are based on figures from Li et al. (2003), Colognato and Yurchenco (2000) and LeBleu et al. (2007). The basement membrane image is taken from Molecular Biology of the Cell (Alberts et al., 2008).

Other basement membrane components such as nidogen/entactin and perlecan appear to link and stabilise the laminin and collagen IV networks. Nidogen/entactin is a glycoprotein comprised of three globular domains, the second of which (G2) binds the collagenous domain of collagen IV whilst the third domain (G3) binds to laminin. Perlecan, a HSPG, can integrate into the basement membrane through its core protein and also via the three HS chains attached to the N-terminus. The core protein can bind both nidogen/entactin and the collagenous domain of collagen IV, whilst the HS chains can potentially interact with both laminin (G domain) and collagen IV (NC-1 domain).

Fibronectin (as reviewed by Leiss et al. (2008)) is also a crucial component of the ECM and has been frequently used as an adhesive substrate for human ES cells, as described in section 1.1.3. Fibronectin is secreted as a disulfide bonded dimer and contains binding sites for fibronectin (self-binding sites) collagen IV, heparin and fibrin. Unfolding of the compact dimer is mediated by adhesion to the major fibronectin binding integrin α5β1 via a distinct RGD binding sequence and an adjacent synergy region. Extension of the fibronectin dimer reveals the binding sites described above and enables the self-self binding necessary for the assembly of multimeric fibrils. Fibronectin-integrin binding leads to the formation of focal adhesion complexes, Rho activation and cytoskeletal reorganisation. The reorganised cytoskeleton enhances cell contractility and aids the extension of the fibronectin molecules and therefore fibril formation. The two heparin binding
regions at opposing ends of the fibronectin molecule bind HS chains on cell surface HSPGs syndecan-2 and syndecan-4. Both interactions enhance the formation of focal adhesion complexes and aid the extension of the fibronectin molecule.

The formation of the basement membrane is a fundamental step in EB formation and guides polarisation and subsequent differentiation of epiblast cells. As discussed above, the production of ECM components by MEFs and integrin mediated binding to basement membrane components laminin and collagen IV and also fibronectin appear to be fundamental in the proliferation and self renewal of human ES cells. In contrast it appears that integrin binding to fibronectin and laminin negatively regulates pluripotency of mouse ES cells (Hayashi et al., 2007). However, mouse ES cells can be successfully maintained on gelatin (denatured collagen I) suggesting other adhesion mechanisms are employed.

1.1.6.3 Integrin Binding

Cell attachment to the ECM is primarily mediated by integrin binding and is comprehensively reviewed by Arnaout et al. (2007). Integrins consist of an α and β subunit, with 19 α and 24 β subunits in mammals pairing to form 24 integrin receptors. Integrins can exist at the cell surface in an open and closed configuration, with the switch to an open, ligand binding conformation regulated by the internal cytoplasmic domains. Integrins function as bidirectional communication molecules, with ‘inside out’ signalling mediating receptor interaction and ‘outside in’ ligand binding stimulating the sequential formation of focal complexes, focal adhesions and fibrillar adhesions. Both receptor activation and initial anchorage to the actin skeleton involves interaction with talin, which binds integrin cytoplasmic domains and stimulates a conformational change into the active configuration in response to internal cell signalling events. Once integrin activation occurs, talin links the integrin to the actin cytoskeleton and recruits paxillin, forming complexes within the lamellipodia (Cukierman et al., 2002; Morgan et al., 2007). The development of focal adhesions, characterised by actin filament bundles (stress fibres), strengthens cell attachment and involves the incorporation of numerous proteins including vinculin and FAK (Cukierman et al., 2002; Morgan et al., 2007). Focal adhesions can form centrally located fibrillar adhesions, formed by integrin movement towards the centre of the cell. The contractile forces involved in forming fibrillar adhesions have been implicated in the deposition of fibronectin around the cell (Cukierman et al., 2002; Morgan et al., 2007). However, integrins do not function alone in governing cell adhesion and migration. Cell surface HSPGs, particularly syndecan-4, have been shown to play a prominent role in focal adhesion formation (Dovas et al., 2006; Woods et al., 2000) and persistent migration (Bass et al., 2007). However, this is just one of the many roles HSPGs play in the cell microenvironment which will be addressed in the following section.

1.2 HSPGs

HSPGs consist of HS chains attached to and branching out from central core proteins and are fundamental elements of the cell microenvironment, residing at the cell surface and within the ECM. The composition of the HS chains are tightly regulated by a set of core regulatory enzymes, with structure and sulphation patterns demonstrating tissue and cell type specific variations. The importance of these structural variations is demonstrated by the tissue-specific morphological phenotypes observed in studies using knock-out mice. Indeed, developing blastocysts which
completely lack HS are unable to develop past gastrulation. The fundamental importance of HS may be due to its wide variety of functions. Key ECM proteins such as laminin and fibronectin possess HS/heparin binding domains which, as well as supporting cell attachment (Woods et al., 2000), enable the stable incorporation of HSPGs into the ECM. Within the context of the ECM, HSPGs provide structural stability (Henry et al., 2001) and also sequester growth factors, creating reservoirs which are ‘tapped’ upon ECM degradation and remodelling, assisting processes such as angiogenesis (Iozzo, 2005; Knox and Whitelock, 2006). One of the most explored aspects of HS is its role in mediating signalling of growth factors, where the structure and sulphation patterns within HS chains have been implicated in modulating receptor binding at the cell surface.

### 1.2.1 Structure and Biosynthesis

**Figure 4. HSPGs: Syndecans, glypicans and perlecan.** Syndecans and glypicans reside at the cell membrane participating in growth factor binding and the formation of morphogen gradients. Syndecans are transmembrane proteins presenting up to 5 GAG chains and are heavily implicated in cell adhesion and migration. Glypicans are attached to the membrane by a GPI anchor with up to three HS chains attached. Both can be proteolytically cleaved and shed from the cell surface. Perlecan is secreted into the surrounding ECM and has a core protein with a modular structure. Each domain shares homology with distinct molecules, such as laminin (domain III). Three GAG chains can be attached to domain I with a possible fourth chain attached on domain V. Adapted from Hacker et al. (2005).

HSPGs reside at the cell surface in the form of glypicans and syndecans, as depicted in Figure 4. The family of glypicans consists of 6 members each with cysteine rich globular ectodomains attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor. A maximum of three HS chains extend out from the molecule attached to the C-terminus below the globular domain (Esko and Lindahl, 2001; Hacker et al., 2005). Syndecans (-1,-2,-3 and -4) are Type I transmembrane proteins, possessing a cytoplasmic domain which facilitates endocytosis and has the capacity to associate with the cell cytoskeleton (Esko and Lindahl, 2001; Hacker et al., 2005). Carrying up to 5 GAG chains, syndecans can present chondroitin sulphate (CS), dermatan sulphate (DS) and HS. However, they predominantly present HS (Esko and Lindahl, 2001; Hacker et al., 2005). Both molecules have cleavage sites, specifically the GPI anchor in glypicans, enabling the HSPGs to be shed from the cell surface and into the surrounding microenvironment (Esko and Lindahl, 2001; Hacker et al., 2005). In addition to shedding of cell surface HSPGS, cells also secrete non-
membrane bound HSPGs into the surrounding ECM, the most notable of which is perlecan (see section 1.2.2 for more detail).

HS is a non-branched, linear GAG composed of alternating N-acetylglucosamine (GlcNAc) and uronic acid (UA) (either glucuronic (GlcA) or iduronic acid (IdoA)) residues with distinct sulphation patterns at the disaccharide level and within the chain as a whole. Attachment, elongation and sulphation are conducted by a large repertoire of enzymes situated in the Golgi and the synthesis and structural modifications of HS are reviewed Lamanna et al. (2007) and Esko et al. (2001). HS attachment is mediated by a tetrasaccharide linker (glucuronic acid, galactose, galactose, xylose) attached to specific serine residues within the core protein. Elongation of the HS chain is initiated by attachment of a critical GlcNAc residue, committing the site to HS assembly. Alternate addition of GlcA and GlcNAc residues is catalysed by the exotisin glycosyltransferases EXT1 and EXT2 which function as a hetero-oligomeric complex (Lin et al., 2000; Stickens et al., 2005). The primary modification that occurs during chain elongation is N-deacetylation and N-sulphation of the GlcNAc residues by GlcNAc N-deacetylase/sulfotransferase enzymes (NDSTs). NDST activity plays a crucial role in patterning the HS chain, creating distinct regions of continuous N-sulphation (S domains) flanked by regions of alternating N-sulphated and N-acetylated disaccharides (transition zones). These modified areas are separated by sections of non modified, GlcNAc residues. Within the S domains and transition zones, further modifications occur including epimerization of GlcA to IdoA, 6-O- and 3-O-sulphation of GlcNAc/GlcNS and 2-O-sulphation of the UA residues. These sequential modifications further enhance the hierarchical structure of the HS chains. Specific sulphation motifs within these regions are essential in providing specificity for ligand binding and for mediating molecular interactions (Figure 5).
Figure 5. HS modification and domain structure. Disaccharides of GlcA and GlcNAc are sequentially modified to create specific sulphation motifs and domains within the HS chain. Modifications include de-N-acetylation/N-sulphation, 6-O-sulphation and 3-O-sulphation of the GlcNAc residue. The GlcA residue can be epimerised to IdoA with both forms potentially harbouring 2-O–sulphation. Highly sulphated S domains are characterised by contiguous N-sulphation with high levels of 2-O, 6-O and, more rarely, 3-O sulphation. S-domains are flanked by transition zones which have alternating N-sulphated and N-acetylated GlcNAc residues and have lower levels of sulphation. Sulphated regions are separated by non sulphated domains (NA domains). The structural modifications of HS are reviewed by Lamanna et al. (2007) and Esko and Lindahl (2001) from which these diagrams are derived.

The importance of HS chains and also specific sulphation motifs is illustrated by mouse knockout studies targeting enzymes within the biosynthetic pathway. Loss of either EXT1 or EXT2 in homozygous mutant mice completely removes all HS, creating an embryonic lethal phenotype with impaired extraembryonic tissue and mesoderm formation (Lin et al., 2000; Stickens et al., 2005). Hereditary multiple exostoses (HME, also referred to as hereditary multiple osteochondroma) is a skeletal disorder in humans characterised by a short stature and ectopic bone growth creating cartilage capped protrusions (exostoses/osteochondromas) and is thought to be caused by a reduction in HS synthesis due to heterozygous mutations in genes encoding either EXT1 or EXT2 (Zak et al., 2002). The phenotype manifests due to irregular chondrocyte proliferation and is linked to impaired signalling of growth factors such as Indian hedgehog (IHH) and FGF during development (Lin et al., 2000; Stickens et al., 2005; Zak et al., 2002).

Homozygous and heterozygous mutation in EXT1 and EXT2 highlight a fundamental role of HS during development. However, abrogation of enzymes involved in further HS modifications underscores the importance of intricate and specific sulphation motifs within the HS chain. Complete loss of 2-OST activity in Hs2st/- mice causes death at birth due to renal agenesis and abnormalities of the eye and skeleton (Bullock et al., 1998). Characterisation of HS from Hs2st/- MEFs revealed complete loss of 2-O-sulphation, which was partially compensated for by an increase in N-sulphation (creating larger S domains) and an increase 6-O-sulphation (specifically
within the S-domains) (Merry et al., 2001). Hs2st\(^{−/−}\) HS had a reduced affinity for both FGF1 and FGF2 but this was not translated into reduced biological activity (Merry et al., 2001). However, the severe phenotype and death of Hs2st\(^{−/−}\) mice illustrates the importance of 2-O-sulphation within the HS chain for normal development.

Unlike 2-O sulphation, NDST and 6-OST activity is governed by multiple isoforms. Loss of a single isoform creates distinct phenotypes reflecting the expression profile and specificity of the targeted enzyme. Habuchi et al. (2007) investigated the loss of HS 6-OST-1 (HS6ST-1) from homozygous mutant mice and reported a late embryonic lethal phenotype due to impaired angiogenesis within the placenta. Poor vascularisation was linked to impaired Wnt2 binding which in turn reduced Wnt2 induced VEGF signalling. A significant reduction in UA-GlcNS(6S) and GlcNAc(6S) residues in both the kidney and lung reflected the high expression of HS6ST-1 within these tissues. In comparison, the trisulphated disaccharide IdoA(2S)-GlcNS(6S) was not significantly reduced reflecting the specificity of the enzyme towards UA-GlcNS disaccharides.

HS chains at the cell surface are also post-synthetically modified by 6-O-endosulfatases. In mammals, Sulf-1 and Sulf-2 function co-operatively to selectively remove 6-O-sulphation primarily within the S domains, which subsequently modulates growth factor binding (Narita et al., 2006; Uchimura et al., 2006). The two enzymes have a hydrophilic domain residing between the N and C terminus that mediates high affinity binding to the UA(2S)-GlcNS(6S) and UA-GlcNS(6S) residues (Frese et al., 2009). both Sulf1 and Sulf2 demonstrate high specificity towards UA(2S)-GlcNS(6S) and UA-GlcNS(6S) with higher activity observed for the trisulphated disaccharide (Lamanna et al., 2008). Within Sulf1\(^{−/−}\)/Sulf2\(^{−/−}\) MEFs this activity is translated into a marked increase in both disaccharides as well as an increase in the monosulphated UA-GlcNAc(6S) indicating in vivo activity towards S domains and transition zones of the HS chain (Lamanna et al., 2006; Lamanna et al., 2008). The loss of Sulf1 and Sulf2 in MEFs also had an impact on expression of biosynthetic enzymes, indicating a close interplay between components of the HS biosynthetic/modification network. Sulf1\(^{−/−}\)/Sulf2\(^{−/−}\) MEFs had increased expression of Hs2st1 which resulted in an increase in total 2-O-sulphation (Lamanna et al., 2008). This dynamic regulation of enzymes within the HS biosynthetic/modification pathway is also supported by the compensatory increase in total 6-O and N-sulphation in Hs2st\(^{−/−}\) MEFs (Merry et al., 2001).

HS architecture is therefore regulated by a complex network of enzymes, each differentially expressed in order to produce chains with specific macromolecular structure and intricate sulphation epitopes. Both the domain structure and specific sulphation motifs are essential for developmental processes as illustrated by the gross morphological phenotypes of knockout mice. However this is not surprising considering the many roles HSPGs play in mediating cell adhesion, binding growth factors and integrating into the ECM.

### 1.2.2 Functional Significance

#### 1.2.2.1 Cell Adhesion

Of the four mammalian syndecans (syndecan-1,-2,-3 and -4), the ubiquitously expressed syndecan-4 appears to play a prominent role in cell adhesion (Woods et al., 2000) and migration (Bass et al., 2007) and is reviewed by Morgan et al. (2007). The most well characterised syndecan-mediated cell attachment mechanism is to fibronectin, which possesses an α5β1 integrin binding
motif (RGD) and two heparin binding domains at opposing ends of the molecule. Upon attachment, syndecan-4 relocalises to focal adhesions, the formation of which is dependent on a direct interaction between the HS (and not CS) chains and the heparin binding domain of fibronectin (Woods et al., 2000). The role of syndecan-4 in attachment and migration appears to be primarily mediated through the binding of PKCα by the cytoplasmic domain, which in turn mediates the activity of Rac1 and RhoA GTPases (Bass et al., 2007; Dovas et al., 2006). RhoA is directly implicated in the formation of focal adhesions and actin stress fibres (Dovas et al., 2006). Prior to RhoA activation, Rac1 activity is required to form membrane protrusions and transient focal complexes (Bass et al., 2007). Syndecan-4 binding co-ordinates Rac1 activity to the leading edge of the membrane and is essential for persistent migration in response to substrate topography (Bass et al., 2007). As with integrins, syndecans appear to be bi-directional signalling molecules (Bass et al., 2007). Binding of syndecan-4 activates and localises Rac1 (outside in) which in turn co-ordinates the formation of membrane protrusions and persistent migration (inside out) (Bass et al., 2007). Although co-operative binding of syndecan-4 with α5β1 integrin is the most well characterised, other partnerships include αVβ3 /αVβ5 and syndecan-1 binding to vitronectin and α2β1 /α6β4 binding with syndecans to laminin.

1.2.2.2 Role in ECM

The most prominent and well characterised HSPG residing within the ECM is perlecan, reviewed by Iozzo (2005) and Knox et al. (2006). The protein core of perlecan is comprised of 5 domains, each with a definitive structure resembling molecules in the ECM and at the cell surface. Three GAG chains (predominantly HS) attach to domain I, while a fourth is hypothesised to attach to domain V. The core protein domains are homologous to the lipoprotein (LDL) receptor (domain II), laminin domain IV (domain III) and neural cell adhesion protein N-CAM (domain IV). As described in section 1.1.6, perlecan plays an essential role in stabilising basement membranes during EB formation (Smyth et al., 1999) and assists in the assembly of laminin networks on the surface of ES cells (Henry et al., 2001).

The activity of perlecan has been implicated in a number of processes including angiogenesis and chondrogenesis. Perlecan can act as both an activator and inhibitor of angiogenesis. The HS chains enable binding of growth factors such as FGF2 and VEGF-A, therefore perlecan residing within the ECM can sequester FGF2 unless cleaved by matrix metalloproteinases, the expression of which occurs during vascular remodelling. The cleavage of perlecan domain V yields endorepellin which has three laminin like globular domains. Endorepellin negatively regulates angiogenesis independently of HS chains, interacting with α2β1 and stimulating a signalling cascade which causes focal adhesion disassembly and cytoskeleton disruption.

1.2.2.3 Growth Factor Binding

HS is implicated in the binding of a large array of growth factors including VEGF (Jakobsson et al., 2006), Wnt (Ai et al., 2003; Hacker et al., 2005), and BMPs (Takada et al., 2003). The most well characterised signalling interaction is the HS mediated binding of the family of FGFs to their receptors (reviewed by Harmer (2006) and Dvorak et al. (2005)). The mammalian FGFRs are encoded by four genes, creating four main receptors (FGFR1, 2, 3 and 4) of which there are numerous splice variants each with specific ligand binding characteristics and expressed in a tissue
specific manner. All FGFRs consist of three extracellular Ig-like domains (IgI, IgII and IgIII) which
bind both FGF (IgII and IgIII) and HS (IgII). A single transmembrane helix anchors the receptors
into the plasma membrane and intracellular tyrosine kinase domains transphosphorylate upon
receptor dimerisation. Receptor activation leads to the recruitment of multiple protein complexes,
which facilitate signalling via pERK/MAPK, PI3K-Akt and PLCγ-PKC pathways. The dimerisation
essential for receptor signalling is induced by ligand binding. Although FGFs can weakly bind
FGFRs, HS is necessary to dramatically enhance affinity and create a stable ternary complex for
robust signalling.

Analysis of crystal structures of the signalling complex suggest one HS/heparin molecule functions
to dimerise FGF molecules, which in turn bridge a dimeric receptor complex (Pellegrini et al.,
2000). However, an opposing interpretation suggests that two FGF:FGFR:heparin/HS heterotrimers dimerise via interactions between FGFRs (Schlessinger et al., 2000). The latter
interpretation is supported by Pye et al. (1999), who concluded that monomer complexes of FGF2
and HS were sufficient for FGFR signalling with no dimerisation of the unbound FGF2-HS complex
observed. This hypothesised interaction is also supported by the diversity in size of functional HS
units. If single HS chains function to dimerise FGF, these would have to be of sufficient length to
bridge the two molecules. However, HS sequences as short as a tetrasaccharide are sufficient to
promote binding of FGF-1 to FGFR2 IIIb (Ostrovsky et al., 2002).

Binding specificity of HS chains towards both FGF and FGFRs is thought to be mediated by
specific sulphation motifs within the S-domain although this is contested (Kreuger et al., 2005) and
is a highly contentious area. It would appear that HS binding to FGF2 is dependent on N-sulphation
and 2-O-sulphation with the minimum binding sequence defined as UA-GlcNS-UA-GlcNS-IdoA2S
(Maccarana et al., 1993), with enrichment of IdoA2S within the HS chain correlating with high
affinity binding (Turnbull et al., 1992). However, although able to bind FGF2, the above sequence is
not capable of promoting FGFR activation and a minimum decassacharide length is necessary for
FGF2 receptor binding (Pye et al., 1998). The study by Pye et al. determined that increases in 6-O-
sulphation, in particular the trisulphated IdoA(2S)-GlcNS(6S) species, directly correlated with
increased biological activity. The requirement of 6-O-sulphation for receptor, but not FGF2, binding
is supported by the study of Guimond et al. (1993) which determined 6-O-desulphated, but not 2-O-
desulphated HS functioned as a competitive inhibitor of FGF2 binding. The study concluded that
FGF binding required 2-O-sulphation, whilst receptor binding required both 6-O-sulphation and 2-
O-sulphation. The presence of the appropriate motifs enabled interaction with both growth factor
and receptor, promoting the formation of an active signalling complex. By stripping HS of 6-O-
sulphation, the receptor binding activity was removed. However, the binding of FGF2 remained
intact resulting in 6-O-desulphated HS acting as an inhibitor of signalling by sequestering FGF2
away from the receptor. The requirement for both sites in the HS chain for receptor activation
reinforces the ability of HS chains to act as both activating and inhibitory factors in growth factor
signalling and highlights the versatility of HS in modulating cellular response to the surrounding
microenvironment. The required sulphation motifs are also specific for different FGF/FGFR
combinations and by expressing certain HS compositions, cells can manipulate which signalling
events occur at the cell surface. As with FGF2 activity, an increase in 6-O-sulphation, particularly in
trisulphated disaccharide IdoA(2S) GlcNS6S, correlated with an increase in biological activity of FGF1 (Pye et al., 2000).

HS regulates the stability and tissue distribution of growth factors in order to create morphogen gradients, as reviewed by Hacker et al. (2005). Mutation studies in drosophila reveal that GAGs are required for the diffusion of hedgehog (Hh) in the wing. Loss of GAGs, due to mutations in the biosynthetic pathway, result in increased concentrations of Hh at the source cell. Drosophila glypicans have also been implicated in decapentaplegic (Dpp) signalling, a BMP homologue. Dpp signals in a concentration dependent manner and loss of glypicans Dally and Dally-Like prevent Dpp traversing the anterior-posterior boundary of wing discs.

The generation and importance of specific sulphation motifs in HSPGs have also been implicated in ES cell culture and differentiation, the significance of these changes in mediating ES cell behaviour will be discussed in the following section.

1.2.3 ES Cell Culture and Differentiation

Mouse ES cells express unusually low sulphated HS with low levels of contiguous N-sulphation (Johnson et al., 2007). However, differentiation appears to be dependent on the generation of specific sulphation epitopes within the HS chains. Johnson et al. (2007) monitored neural differentiation of mouse ES cells expressing a GFP reporter gene under the control of Sox1 (a transcription factor specifically upregulated during neural differentiation). Sox1-GFP expressing neural progenitor cells (NPCs) had increased expression of HS biosynthesis enzymes including NDST4, 3-OST and 6-OST-2/-3 which correlated with increases in 6-O and 2-O sulphation. In addition, increased binding of the ScFv antibody RB4EA12, which preferentially binds HS rich in GlcA/IdoA-GlcNS(6S) (see section 2.1 for more detail concerning ScFv antibodies) was observed as was decreased FGF2 binding (Johnson et al., 2007). The generation of specific sulphation epitopes during mouse ES cell differentiation was further reinforced by Baldwin et al. (2008). Progressive mesodermal differentiation of mouse ES cells was characterised by the transient generation of HS with high affinity binding to ScFv antibody HS4C3 (Baldwin et al., 2008). Weak binding of HS4C3 is observed to oligosaccharides with N-, 2-O and 6-O-sulphation, with high affinity binding characterised by additional 3-O-sulphation (Ten Dam et al., 2006). Isolation and culture of ES cells presenting HS with high affinity HS4C3 binding, together with mesodermal markers Brachyury (Bry) and VEGF receptor foetal liver kinase 1 (Flk1), led to significantly increased haemangioblast progenitors and endothelial colonies (Baldwin et al., 2008).

These results suggest that the low sulphated HS expressed by mouse ES cells may limit the signalling of pro-differentiation factors, a theory which supports the “ground state” model of mouse ES cell pluripotency proposed by Ying et al. (2008). This hypothesis is also supported by the phenotype of EXT1<sup>−/−</sup> mouse ES cells. Despite lacking endogenous HS, EXT1<sup>−/−</sup> ES cells maintain a pluripotent state (expressing Nanog and Oct4) and have characteristic ES cell morphology in culture. However, EXT1<sup>−/−</sup> cells undergo impaired EB formation, do not differentiate in the absence of LIF (Kraushaar et al., 2010; Lin et al., 2000) and cannot undergo neural differentiation in conditions proposed by Ying et al. (Johnson et al., 2007). It has been recently reported that this phenotype can be replicated by culturing wild type cells in the presence of a specific inhibitor of FGFRs and that EXT1<sup>−/−</sup> cells have a reduced response to exogenous FGF2 indicating defective

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signalling via this pathway (Kraushaar et al., 2010). However, differentiation and FGFR signalling could be rescued by the addition of exogenous heparin to the media (Johnson et al., 2007; Kraushaar et al., 2010) underlining the essential role of HS in transmitting signals essential for ES cell differentiation.

A recent study by Lanner et al. (2009) provided further evidence for the essential role of HS sulphation in ES cell differentiation. Mouse ES cells deficient in NDST1 and 2 and cells treated with NaClO₃ (a specific inhibitor of PAPS synthetase) also demonstrated impaired differentiation (Lanner et al., 2009). Cells treated with NaClO₃ demonstrated impaired FGF2 and FGF4 signalling and had enhanced Nanog expression compared to control cultures. Intriguingly, NDST1⁻/-/2⁻/- cells demonstrated attenuated FGF4, but not FGF2 signalling indicating a specific requirement for N-sulphation in FGF4 binding in vivo. However, the removal of NDST activity does not only remove N-sulphation, but also has significant effects on 2- and 6-O-sulphation which may also influence growth factor binding (Holmborn et al., 2004).

Although the HS profile of human ES cells has not yet been elucidated, MEF secreted HS appears to play an essential role in sustaining human ES cell proliferation and self renewal. As mentioned previously, MEFs secrete a range of HSPGs into the media including perlecan, agrin, syndecan-4 and glypicans -1 and -4 (Levenstein et al., 2008). The presence of secreted HSPGs within MEF conditioned media is essential for stabilising and assisting the binding of FGF2. Addition of HS and heparin to unconditioned media enhanced culture of human ES cells in unconditioned media supplemented with 40ng/ml FGF2 (Levenstein et al., 2008). However, cells were only sustained for three passages indicating the necessity of other MEF secreted factors in human ES cell self renewal. The study also observed a dose dependent increase in FGF2 binding with increasing concentrations of HS/heparin and the ability of conditioned media to support human ES cell culture was undermined by heparinase treatment (Levenstein et al., 2008).

HSPGs possess diversity in structure and in function, playing essential roles in development and in governing the ES cell phenotype. However, despite this functional significance little research has focused on stably incorporating fully functional HS/heparin into biomaterial scaffolds for tissue engineering applications. Indeed, there is no research currently evident that combines HS with biomaterials for ES cell culture, despite the wide array of scaffolds currently under development.

1.3 Biomaterials

The 2D culture conditions used to propagate and maintain cells are an artificial system that does not represent the in vivo microenvironment from which the cells are derived. In the native microenvironment, cells are surrounded and governed by the complex architecture and bioactive cues of the ECM as well as extensive cell: cell contacts. An array of biomaterial scaffolds have been developed in an attempt to better replicate this 3D environment, to either create an effective biomedical implant or enhance the propagation and differentiation of stem cells into organised tissues composed of mature adult cell types.

1.3.1 Cell Attachment to 3D scaffolds

Cukierman et al. (2001) cultured fibroblasts on 3D fibronectin matrices and proposed the formation of an adhesion complex distinct from focal complexes, focal adhesions and fibrillar adhesions (all
observed in 2D culture), known as the ‘3D matrix adhesion’. Dependent on a 3D environment, integrin α5β1, matrix pliability and ECM components (primarily fibronectin), the 3D matrix adhesion is characterised by structures composed of α-actinin, paxillin and phosphorylated vinculin (Berrier and Yamada, 2007; Cukierman et al., 2001). In addition to the formation of 3D matrix adhesions, fibroblasts demonstrated increased attachment, migration and proliferation within the matrices, where they also adopted a more in-vivo like spindle morphology (Cukierman et al., 2001).

A series of studies on electrospun polyamide (average fibre diameter 180nm) have also highlighted the differences between 2D and 3D culture. Fibroblasts on the scaffold surfaces adopted an elongated, bipolar morphology with thinner actin fibres running in parallel along the cell (Schindler et al., 2005). This is in contrast to the criss-crossing networks of stress fibres formed by cells cultured in 2D (Schindler et al., 2005). Cells on the nanofibrillar scaffold also demonstrated more diffuse vinculin staining and less well defined localisation of phosphorylated FAK (Schindler et al., 2005). These morphological differences were linked to a selective upregulation of Rac (Nur et al., 2005). A study by Nur et al. (2005) highlighted the preferential formation of membrane ruffles on the scaffold as opposed to stress fibres and focal adhesions. Rac was upregulated on the 3D scaffolds, localised to lamellipodia and membrane ruffles, with activity associated with an increase in proliferation (Nur et al., 2005). To further confirm improved cell behaviour in response to the in vivo-like architecture of 3D/electrospun scaffolds, T47D breast epithelial cells formed duct like tubular structures and spheroids when cultured on the meshes. In contrast, these cells formed a monolayer in flat 2D conditions (Nur et al., 2005). The effect of 3D culture conditions on cell signalling was further reinforced by an upregulation of Rac, PI3K and Nanog in mouse ES cells cultured on electrospun polyamide meshes (discussed in section 1.3.9) (Nur et al., 2006).

Although the above studies are referred to as 3D, infiltration into the meshes was not assessed and cells may have been responding to the fibrous topography of the scaffolds. However, this appears to be sufficient to induce changes in cell attachment, morphology, proliferation and cell signalling.

### 1.3.2 Scaffold Overview

Several techniques, combined with an array of natural and synthetic materials, have been used to synthesise artificial cell matrices for optimal cell infiltration, propagation and/or differentiation. Such scaffolds aim to replicate the architecture the cells would experience within their native 3D microenvironment and the different methods employed have provided a range of scaffold architectures, from fibrous electrospun meshes to macroporous polymer sponges.
Figure 6. Biomaterial overview: scaffold architecture. The various methods employed to create biomaterial scaffolds for cell culture result in a high degree of structural diversity. All images were generated by SEM except for A which was generated using a cryo-SEM. A) Fmoc-FF hydrogel with average fibre diameter 56nm (Jayawarna et al., 2006). B) Polyacrylonitrile and gelatin cryogel with pore sizes 50-100μm (Jain et al., 2009). C) Poly (D,L-Lactide) (PDLA) scaffold produced by supercritical CO₂. Average pore diameter 300μm (Barry et al., 2005). D) PLA scaffolds formed from particle leaching (Mooney et al., 1997). E) Nanofibrous scaffold formed from a phase separation technique using poly(L-lactic acid) P(LA). Pore diameter ranged between 250-420μm, whilst the textured fibres ranged between 50-500nm in diameter (Woo et al., 2003). An electrospun PLGA mesh with fibre diameter between 500-800nm and 91.63% porosity (Li et al., 2002b).

The most distinct category of scaffold is the hydrogels. Hydrogels are formed from a network of nanoscale fibres which create a highly hydrated environment for cell culture. Physical hydrogels rely on the aqueous environment for self assembly of small molecules and polymer entanglements, which are governed by hydrogen bonding as well as ionic and hydrophobic interactions (Peppas, 2004). For instance, the self assembly/stacking of Fmoc-dipeptides (specifically Fmoc-phenylalanine-phenylalanine (Fmoc-FF)) at pH 8 forms a nanofibrillar environment which supports the encapsulation and culture of chondrocytes (Jayawarna et al., 2006) (Figure 6, A). The physical properties of the gels are governed primarily by gel concentration (Peppas, 2004). In comparison, chemical hydrogels are formed by covalent crosslinks between polymer chains (Peppas, 2004). Crosslinking can be mediated by groups present on the polymer chain or by small molecule crosslinking agents and in certain cases can be stimulated by UV light (Elisseeff et al., 2005; Gerecht et al., 2007). The degree of crosslinking largely governs the physical properties of the gel, with the molecular weight and crosslinking density determining the swelling properties (Peppas, 2004). The properties of hydrogels determine the diffusion of oxygen, nutrients, growth factors and waste product as well as fibre density, all of which can have a direct impact on the survival and growth of cells within the gels (Brandl et al., 2007; Peppas, 2004).

Hydrogels are largely unique in their dependency on a hydrated environment for both the formation and maintenance of the nanofibrillar structure. Other techniques enable the formation of dry polymer scaffolds, synthesised using either (or both) natural and synthetic polymers each of which impart distinct structural topographies. Cryogels are formed by polymerisation at sub-zero temperatures (Jain et al., 2009). During the polymerisation process, ice crystals form and expand, creating a network of interconnecting pores within the scaffold (Jain et al., 2009) (Figure 6, B). A
supercritical CO$_2$ method also produces a porous scaffold suitable for cell culture (Barry et al., 2006; Barry et al., 2005). Above a certain critical temperature and pressure, supercritical CO$_2$ possesses the properties of CO$_2$ in both its gas and liquid form and diffuses into the polymer matrix (Barry et al., 2006). A sudden reduction in pressure reduces the solubility of CO$_2$ causing expansion, forming pores within the polymer foam which become fixed in shape once the temperature and pressure are returned to normal (Barry et al., 2006). This process creates a highly porous structure (85% porosity) with an average pore diameter of 300µm (±160µm) into which cells (e.g. fibroblasts) migrate and grow (Barry et al., 2005) (Figure 6, C). The addition of salt particles to polymer solutions and subsequent salt leaching has also been effective in creating a porous (average pore size 250µm) synthetic scaffold that has shown a high degree of promise in stem cell tissue engineering (Mooney 1997) (Figure 6, D).

The porous structures described above have demonstrated a high degree of biocompatibility and are successful in creating 3D culture environments. However, with the exception of hydrogels, the above scaffolds do not replicate the fibrillar architecture of the ECM, in particular that of specialist architectures such as the basement membrane. Woo et al. (2003) created interconnected porous scaffolds with a nanofibrillar surface texture by combining paraffin spheres with a phase separation technique (Woo et al., 2003) (Figure 6, E). The nanofibrous scaffolds demonstrated greater protein adsorption and osteoblast attachment compared to solid walled controls (Woo et al., 2003). Electrospinning is another well established technique for creating fibrous scaffolds suitable for cell culture (Figure 6, F). Demonstrating extensive versatility, a vast range of synthetic and natural polymers can be electrospun into a variety of structural conformations. This technique will be described in detail in the following section.

### 1.3.3 Electrospinning

Electrospinning of polymers creates non woven, porous fibre meshes with high surface area: volume ratios (Huang et al., 2003). The principles, mechanisms and applications of electrospinning are reviewed by Huang et al. (2003). As depicted in Figure 7 the polymer solution is fed through a blunted metal capillary at a specified flow rate. The application of a high voltage to the polymer solution via the metal capillary causes electrostatic charge repulsion to occur. As the charge repulsion competes with the surface tension of the polymer solution, the polymer distorts to form a conical shape known as the Taylor cone (Deitzel et al., 2001). Once the charge repulsion overcomes the surface tension, a charged, stable jet erupts and travels towards a grounded collector (Deitzel et al., 2001). As the jet travels, the surface charge repulsion destabilises the jet causing it to whip and elongate, stretching the discharged polymer (Deitzel et al., 2001; Fridrikh et al., 2003). By the time the jet reaches the collector, the solvent has evaporated leaving a dry mesh of polymer fibres.

Important parameters mediating the quality and dimension of the deposited fibres include solution concentration, choice of solvent, flow rate, voltage, viscosity and surface tension. All of which can be adjusted to either avoid the formation of beads or increase/decrease fibre diameter. Viscosity is dependent on the combination of polymer and solvent and can be increased by increasing solution concentration (Fong et al., 1999). Increasing solution viscosity in this manner can overcome the problem of beading and assist the formation of smooth fibre deposits (Fong et al., 1999). Surface
tension serves to negatively regulate fibre formation, with high surface tension promoting the formation of beads (Fong et al., 1999). However, this can be overcome by adjusting the solvent and increasing solution viscosity (Fong et al., 1999). The dielectric constant of the solvent also assists in determining fibre morphology. Solvents with a higher dielectric constant (i.e. higher polarity) increase the charge repulsion within the solution, enhancing whipping instability/elongation forces and creating fibres with a lower average diameter (Son et al., 2004). Addition of salts to polymer solutions also increases solution conductivity contributing to the formation of thinner fibres (Son et al., 2004).

Figure 7. Electrospinning apparatus. The basic electrospinning set up consists of a syringe pump that feeds the polymer solution at a specified flow rate, a high voltage source that is applied to the blunted metal capillary and a grounded collector (in this case a rotating mandrel). Basic parameters that can be altered to manipulate the structure of the mesh include polymer concentration, polymer flow rate, applied voltage, speed of mandrel rotation and the working distance (distance between capillary tip and the collector).

Flow rate has to be above a certain level in order to meet the rate of removal (Deitzel et al., 2001) and can be increased to maximise average fibre diameter (Kumbar et al., 2008; Pham et al., 2006). Increasing polymer concentration is also an effective way of increasing average fibre diameter (Deitzel et al., 2001; Ekaputra et al., 2008; Kumbar et al., 2008; Pham et al., 2006). The effect of applied voltage on fibre diameter is more variable. Previous research has demonstrated a general decrease in fibre diameter with increasing applied voltage due to larger elongation forces (Deitzel et al., 2001). However, others argue that the higher applied voltage increases the amount of fluid present in the polymer jet, leading to the deposition of larger fibres (Huang et al., 2003).

Excellent examples of standard electrospun meshes include those created by Li et al. (2002b) and Matthews et al. (2002). Li et al. electrospun PLGA in a solvent combination of tetrahydrofuran and dimethylformamide, creating a defect-free fibre mesh with fibre diameter ranging between 500-800nm (Li et al., 2002b). Scaffolds were highly porous with pore diameter ranging between 2-465um and a porosity of 91.63% (Figure 6, F). Matthews et al. (2002) electrospun collagen I into fibres ranging from 100-730nm characterised by the 67nm banding present in native collagen.
Aligned fibres could also be created by spinning onto a mandrel rotating at 4500rpm. The ability to form meshes from natural and synthetic polymers contributes to the versatility of the electrospinning method. The advantages and disadvantages of synthetic and natural polymers in scaffold design, with a focus on electrospinning, will be discussed in the following section.

### 1.3.4 Natural Polymers

Scaffolds fabricated from natural polymers derived from the ECM provide structural support and the necessary biological cues for cell attachment and growth. However, glycosylated proteins such as fibronectin and laminin are difficult to derive and highly expensive compared to collagen and gelatin. Considering the quantities necessary for sufficient scaffold production, ECM proteins such as collagen have taken precedence in scaffold design. In addition, easily derived and widely available polysaccharides such as hyaluronic acid (HA, an ECM nonsulphated GAG), alginate (found in brown algae), and chitosan (formed from chitin present in fungi and crustaceans) have also been used in scaffold synthesis.

Collagen I gels have supported the differentiation of human ES cells into hepatocytes (Baharvand et al., 2006), while addition of either fibronectin or laminin to semi-interpenetrating collagen I gels supported the survival and growth of encapsulated EBs (Battista et al., 2005). The addition of either fibronectin or laminin was effective in directing EB differentiation towards endothelial and cardiac tissue lineages respectively, supporting the formation of organised, 3D structures (Battista et al., 2005). As described previously, collagen I was successfully electrospun by Matthews et al. (2002) with the fibres exhibiting the 67nm banding present in native collagen (Matthews et al., 2002). The electrospun meshes supported the attachment and growth of smooth muscle cells. Shields et al. (2004) electrospun collagen II, creating a fibrous mesh (fibre diameter 70nm - 2.74 µm) that supported chondrocyte culture. In addition to collagen, fibronectin coated electrospun HA meshes supported the attachment and infiltration of fibroblasts (Ji et al., 2006). Phase separation techniques using gelatin (Liu and Ma, 2009) and a mixture of alignate and chitosan (Li et al., 2010) have also yielded promising scaffolds for cell culture, with alignate/chitosan scaffolds supporting human ES cell culture (Li et al., 2010) see section 1.3.9).

Scaffolds produced by ECM molecules such as collagen and gelatin have a number of advantages, including creating an environment that provides the cells with ECM molecules in a conformation more similar to that found in vivo. In addition, they have the potential to be degraded and remodelled by the cells themselves. However, all of the above scaffolds, with the exception of the collagen gels, required crosslinking to maintain scaffold integrity during cell culture. Crosslinking can be mediated by exposure to gluteraldehyde vapour (Matthews et al., 2002; Shields et al., 2004) or soaking in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl and N-hydroxy-succinimide the combination of which covalently couples carboxyl and amino groups (Liu and Ma, 2009). In the case of electrospun HA meshes, the HA was thiolated and a poly(ethylene glycol)-diacylate (PEGDA) crosslinking agent added to the as-spun fibres (Ji et al., 2006). The need to crosslink the scaffolds may alter the architecture and the biological activity of the meshes (Liu and Ma, 2009). In addition, although use of natural polymers provides the cells with ligands for attachment, it is difficult to discern whether cells are responding to the architecture or more specifically to the array of biological motifs they are presented with.
In comparison to natural polymers, synthetic polymers have a high degree of reproducibility and can provide structural stability with tailored degradation rates (Dong et al., 2009). Synthetic polymers also demonstrate versatility in the incorporation of whole proteins/polysaccharides, enabling hybrid scaffolds to be formed (Ghasemi-Mobarakhe et al., 2008; Lee et al., 2009; Li et al., 2006b). In addition, synthetic polymers can be modified with particular ECM motifs, encouraging cell attachment and potentially creating a more defined culture environment (Kim and Park, 2006; Yoon et al., 2004).

### 1.3.5 Synthetic Polymers

Synthetic polymers are largely characterised by a carbon backbone and are composed of repeating monomer units with different structural variations. Linear, biodegradable polyesters have been successfully used to create a range of biomaterial scaffolds. Polyesters contain an ester group within the repeating monomer unit and including poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and the copolymer poly(lactic acid-co glycolic acid) (PLGA). The characteristics and degradation of the above polyesters, particularly in the form of electrospun meshes, is reviewed by Dong et al. (2009).

One of the defining characteristics of polymers is their degree of crystallinity. Crystallinity occurs in regions of the polymer matrix where polymer chains exist in a highly ordered, thermodynamically favourable, aligned formation (Stevens, 1999). Polymers with a low tendency towards crystallinity are regarded as amorphous and have a high degree of rotational freedom (Stevens, 1999). The degree of crystallinity within a polymer network is reflected in the glass transition temperature (Tg), which is the temperature at which the heated polymer has increased rotational freedom and more motion, accompanied by reduced stiffness (Stevens, 1999).

PGA and PLA degrade into glycolic acid and lactic acid respectively, products that can be metabolized by the body. PGA has a high degree of crystallinity and is therefore not readily soluble in most solvents (Shalaby, 1994). It also has high strength and as a hydrophilic polymer has a high degradation rate (Shalaby, 1994). PLA is characterised by a methyl side group that contributes to its higher hydrophobicity and slower degradation rate. The PLGA monomer exists in two forms, an L- and D-Lactide which combine to form three PLA stereoisomers (expressed for clarity as PDLA, PLLA, PLLA) with different degradation rates and physical properties. Whilst PLLA is semi-crystalline, the mixed polymer of PDLA is largely amorphous.

#### 1.3.5.1 Degradation

PLGA is an amorphous copolymer of PLA and PGA and with specific properties reflecting the ratio of the two components. PLGA has several properties that give it an advantage over alternative polymers such as PCL. Most notably, the degradation rate can be tailored by altering the ratio of PLA and PGA. Increasing PGA increases degradation whilst increasing the proportion of hydrophobic PLA decreases the degradation rate (Caticker et al., 2000). This can be fine tuned by using either PDLA or PLLA, as PLLA possesses a higher degree of crystallinity and exhibits slower degradation. The degradation rate of polymers such as PLGA is an important consideration when combining them with a cellular environment, either in vitro or in vivo. Degradation has to be adjusted so that scaffolds can provide sufficient support for cell growth. In addition, accumulation of...
Degradation products in a localised area can create an acidic environment counter active to cell survival.

Degradation occurs through hydrolysis of the ester linkages, leading to chain scission and the production of low molecular weight fragments (Shalaby, 1994). Two mechanisms of degradation recorded for PLGA are bulk and surface degradation (Vey et al., 2008). Bulk degradation has been observed in PLGA films incubated in PBS and is characterised by an initial loss of average molecular weight as water diffuses into the amorphous regions of the polymer and stimulates chain scission (Vey et al., 2008). At this point the degraded products are too large to diffuse out of the polymer and remain trapped creating a highly acidic environment which stimulates autocatalysis of the degradation process (Vey et al., 2008). As degradation progresses, the oligomers reduce in size until they are able to diffuse out into the surrounding environment stimulating a sudden decrease in pH (Vey et al., 2008). Surface erosion occurs at the surface of the polymer and is characterised by a loss of mass but not molecular weight. The buffering action of the surrounding PBS and the ease of diffusion removes the effect of autocatalysis (Vey et al., 2008).

The architecture of the polymer material can have dramatic effects on the mode and rate of degradation. Films of polymer appear to be dominated by bulk degradation (Caticker et al., 2000). However, fibrous structures have a higher surface area and are therefore more exposed to surface erosion. However, below a certain fibre diameter the degradation products can diffuse more easily out of the material and remove the autocatalysis of bulk erosion therefore reducing degradation rates.

A degradation model of PLGA (10:90) electrospun meshes was proposed by Zong et al. (2003). Electrospinning reduced the Tg of PLGA to 39°C causing thermally induced crystallization within the amorphous fibres at 37°C, forming lamellar stacks. Chain scission targets the amorphous regions leading to increased mobility, which in turn causes reorganisation of the chains and an increase in crystallinity. As degradation continues, accelerated mass loss occurs causing amorphous regions to disappear, the lamellar stacks to collapse and the disintegration of fibre integrity. Degradation of electrospun fibres can lead to a number of morphological changes. PLGA fibres have demonstrated a ‘melted’ morphology in culture as the Tg is further lowered during degradation, causing increased mobility and fibre ‘melting’ to reduce surface tension. Also, fibre breakage occurs at weak points, exposing the broken tips to the surrounding media and leading to fibre erosion. Examples of the morphological changes of fibres in culture conditions are displayed in Figure 8.
Figure 8. Morphology of degraded electrospun meshes. SEM images of electrospun A) P,LAT fibres after 10 weeks at 37°C in PBS (Cui, 2008) and Electrospun PLGA (10:90 PGA:PLA) after 21 days at 37°C in PBS (Zong et al., 2003).

1.3.5.2 Protein Adsorption

Cell attachment to polymers such as PLGA and PCL is hindered by their high degree of hydrophobicity and is largely dependent on adsorbed proteins, either deposited from supplementary serum or following coating with specific ECM components. The influence of wettability and particular surface chemistries on cell attachment was addressed by Webb et al. (1998). Glass slides were treated with silane coupling reagents to produce surfaces presenting quaternary amine, amine, methyl, thiol and oxidised thiol groups. The surface chemistries exhibited different water contact angles (WCA) at pH 7.5, with oxidised thiol possessing the lowest (7.3°) and methyl groups exhibiting the highest (92°). In serum-free conditions without any pre-bound protein, fibroblasts demonstrated greatest attachment to quaternary amine (WCA 22.7°) functionalities. Lower attachment to amine (WCA 36.7°) and thiol (WCA 59.7°) surfaces were observed, with the lowest level of attachment to oxidised thiol and methyl. It would appear that cells do not attach well to highly hydrophobic (methyl groups) or highly hydrophilic (oxidised thiol) surfaces, but do attach to surfaces with WCAs ranging between 20° – 60°. The study also hypothesised that the significantly higher attachment to quaternary amine functionalised surfaces may be partially due to electrostatic interactions between the positively charged surface and negatively charged molecules (such as polysaccharides) on the outside of the cell. Adsorption of serum and bovine serum albumin (BSA) improved attachment, with amine functionalised surfaces demonstrating the greatest improvement in comparison to quaternary amines. However, the lowest attachment was observed on methyl and thiol surfaces. The attachment profiles observed in the presence of adsorbed proteins reflect a combination of the surface chemistry, which governs the adsorption process, and the composition and conformation of the adsorbed protein layer. However, cell attachment to different surface chemistries appears to be cell type dependent. Further experiments revealed that osteoblasts demonstrated greater attachment to thiol functionalised surfaces in the presence of serum, in contrast to the preference for quaternary amines demonstrated by fibroblasts (Webb et al., 2000).

The different binding properties of the two cell types could be due to differences in substrate adhesion requirements mediated by individual integrin expression profiles (Webb et al., 2000). The functionalised surface chemistries would each have adsorbed a protein layer from the serum that varied in composition and conformation. Kesewolsky et al. (2003) analysed the adsorption of fibronectin to surfaces functionalised with methyl, hydroxyl, amine and carboxylic acid groups.
Equal density of adsorbed fibronectin did not equate to equal functional presentation of motifs within the molecule essential for integrin binding (Keselowsky et al., 2003). Despite adsorbing the most fibronectin, methyl surfaces demonstrated the lowest degree of functional fibronectin binding. In comparison hydroxyl groups demonstrated highest cell adhesion at the lowest fibronectin densities (Keselowsky et al., 2003). The architecture of the surface can also mediate protein adsorption. Taking into account the increased surface area, Woo et al. 2003 observed increased protein adsorption on nanofibrillar phase-separated scaffolds compared to smooth walled structures. As well as binding a greater amount, the nanofibrillar surface also selectively bound different proteins from the serum demonstrating a preference for adsorption of fibronectin and proteins <120 kDa (Woo et al., 2003).

In order to improve cell attachment and create a more defined environment, biomaterial scaffolds have been modified in a number of ways including the incorporation of specific proteins within the scaffold structure, attachment of specific integrin-binding motifs and surface modification by procedures such as plasma polymerisation. These modifications will be discussed in the following section.

1.3.6 Scaffold Modification

1.3.6.1 Inclusion of Natural Polymers and Ligands

By combining synthetic materials with natural polymers, it is possible to increase scaffold stability whilst providing the necessary biological cues for cell attachment and proliferation. In addition, the combination can enhance scaffold structure and alter the mechanical properties of the material. For instance, the filling of a phase separated P(PLA) nanofibrous scaffold with a crosslinked fibrin gel enhanced chondrocyte proliferation and created a scaffold with higher mechanical strength more appropriate for cartilage engineering (Zhao et al., 2009). Increasing the ratio of gelatin in poly(acrylonitrile) cryogels created holes (5-20µm) within the larger pores of the scaffold enhancing interconnectivity (Jain et al., 2009). Increasing amounts of gelatin also created a more spongy and elastic structure which compensated for the hydrophobicity of poly(acrylonitrile) and encouraged the attachment of fibroblasts (Jain et al., 2009). By encapsulating alginate in PDLA scaffolds fabricated by super critical CO₂, Kanczler et al. (2010) were able to release VEGF and BMP at different rates from the two fractions.

PLGA gelatin and elastin polymer blends have been successfully electrospun by Li et al. (2006b), creating a stable mesh without the need for chemical crosslinking. Hydrated fibres adopted a more fibrous morphology, with fibres separating into bundles of smaller fibrils which were enhanced with increasing concentrations of gelatin and elastin relative to PLGA. PLGA and blended scaffolds supported the growth of rat cardiac myoblasts. Electrospinning a blend of PCL and gelatin reduced WCA and increased the viability and neurite extension of C17.2 nerve stem cells (Ghasemi-Mobarakeh et al., 2008). However, the incorporation of gelatin led to weaker mechanical properties and increased the degradation rate (Ghasemi-Mobarakeh et al., 2008). PCL/Collagen electrospun blends also demonstrated greater water affinity and adsorption compared to pure PCL scaffolds. As with PCL/gelatin blends, PCL/collagen scaffolds demonstrated increased degradation (Lee et al., 2009). However, the inclusion of collagen enhanced mechanical elongation properties and conveyed better scaffold recovery after elongation (Lee et al., 2009). In addition, PCL/Collagen
scaffolds enhanced the adhesion, proliferation and expression of osteogenic genes of a pre-osteogenic cell line (Lee et al., 2009).

In some cases it may be desirable to retain all the properties of the synthetic polymer whilst still enhancing cell attachment and growth. By immobilising specific peptide sequences isolated from ECM proteins such as fibronectin, it is possible to create a defined environment for mediating cell attachment and proliferation. The integrin binding sequence RGD isolated from fibronectin has been successfully applied to a wide array of scaffolds. Zhou et al. (2009) incorporated Fmoc-RGD into Fmoc-phenylalanine-phenylalanine (Fmoc-FF) peptide gels enabling dermal fibroblasts to spread and form a 3D network of cells within the gel (Zhou et al., 2009). To confirm spreading was mediated by and dependent on the RGD ligand, results were compared with gels incorporating Fmoc-RGE, within which cells adopted a rounded morphology with a poorly organised actin cytoskeleton (Zhou et al., 2009). In addition, spreading within the Fmoc-RGD gels was inhibited by α5β1 integrin blocking antibodies (Zhou et al., 2009). Silva et al. also functionalised self assembling hydrogels using amphiphilic molecules presenting the laminin-derived epitope IKVAV (Silva et al., 2004). The hydrogels were composed of fibres 5-8 nm in diameter and supported the neurite outgrowth and neuronal differentiation of NPCs (Silva et al., 2004). By using PLGA functionalised with a terminal amine group attached by a poly(ethylene glycol) (PEG) linker, Park and colleagues functionalised porous scaffolds formed from gas foaming/salt leaching (Yoon et al., 2004) and electrospun meshes (Kim and Park, 2006) with RGD. The ligand was orientated to the surface enhancing cell attachment, proliferation (Kim and Park, 2006) and differentiation (Yoon et al., 2004).

Common synthetic polymers such as PLGA and PCL do not possess the functional groups necessary for covalent protein/peptide immobilisation. Researchers can overcome this by using modified co-polymers as described above or by altering the surface chemistry. Altering the surface chemistry of a polymer can in itself enhance biocompatibility and one versatile method to achieve this is plasma polymerisation.

### 1.3.6.2 Plasma Polymerisation

Plasma polymerisation is an efficient method of altering surface chemistry without affecting the properties or architecture of the scaffold. The plasma polymerisation of monomers such as allyl amine (ppAm) (Barry et al., 2005; Chu et al., 2006; Shard et al., 2004), acrylic acid (Alexander and Duc, 1999; O’Toole et al., 1996) and allyl alcohol (O’Toole and Short, 1997; Whittle et al., 2000) can coat a surface with amine, carboxylic acid and alcohol groups respectively. Plasma polymerisation is described in detail by Morosoff (1990) and the basic apparatus is depicted in Figure 9. The monomer is introduced to the reactor and flows through at a steady rate throughout the process. This enables any by-products and unreacted monomer to be continuously removed. A ‘glow discharge’ is stimulated by exposing the monomers to an electric field, created by charged coils or electrodes. The transferred energy creates free electrons which perpetuate the formation of more electrons as well as ions and free radicals. These react with each other, forming oligomer species and also react with the substrate surface. An adherent coating will initially be formed as the polymerising plasma forms covalent bonds with the substrate surface. The plasma polymerised layer then grows vertically, during which it will be further modified by the plasma forming a highly
The layer is also built by the adsorption of oligomer species which are covalently incorporated into the layer by further plasma fragmentation.

The plasma polymerisation process is dependent on a number of parameters including monomer flow rate, frequency at which the glow discharge is generated, the excitation power and the geometry of the reactor. During the plasma polymerisation it is necessary to optimise parameters to ensure retention of monomer functionality. High plasma power creates a more stable insoluble plasma polymerised layer (Alexander and Duc, 1999; Chu et al., 2006). However, the benefits are compromised by a loss of monomer functionalities (Alexander and Duc, 1999; O’Toole et al., 1996; O’Toole and Short, 1997) as a higher power input increases the interaction of energetic species with monomer molecules.

Surface grafting can also be conducted on a substrate surface and is distinct from plasma polymerisation as no deposited layer is formed. Instead, the application of a glow discharge to nonpolymerising components such as oxygen and nitrogen chemically modifies the substrate surface (plasma etching). This in itself can cause a dramatic increase in hydrophilicity and increase biocompatibility (Prabhakaran et al., 2008). However, monomer units can become covalently attached, or grafted, to the active sites created on the surface by introducing monomer vapour after plasma etching.

Air plasma treatment of electrospun PCL (p-PCL) fibres dramatically increased oxygen containing groups on the scaffold surface without altering scaffold morphology causing a significant increase in hydrophilicity (Prabhakaran et al., 2008). Air plasma treatment also increased biocompatibility as p-PCL scaffolds supported the growth of Schwann cells to a greater extent than PCL/collagen blended meshes, making plasma treatment a more cost-effective alternative (Prabhakaran et al., 2008). Grafting of acrylic did not alter the porosity, pore size, fibre dimensions or mechanical characteristics of electrospun PLA, PGA and PLGA meshes, yet increased mesh hydrophilicity and enhanced fibroblast proliferation on the surface (Park et al., 2007). Plasma polymerisation of allyl

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**Figure 9. Plasma polymerisation apparatus.** The monomer is introduced to the plasma polymerisation chamber at low pressure and in this case is continually flowed through, with excess monomer and reaction by-products collected by a liquid nitrogen cold trap. The glow discharge is initiated by charged coils at a particular radio frequency (RF) (typically 13.56 MHz) and power. Image reproduced from Whittle et al. (2000).
amine onto glass significantly improved attachment and maintained functionality of hepatocytes (Dehili 2006) and also increased attachment of neuronal cells to polysiloxane (Harsch et al., 2000).

The increased biocompatibility of plasma treated surfaces could be due to the increased hydrophilicity. However, the dramatic change in surface chemistry also alters the adsorbed protein layer. Whittle et al. (2004) analysed the adsorption of IgG, vitronectin and collagen II onto plasma polymerised allyl alcohol, acrylic acid and octa-1,7-diene (octadiene) layers (Whittle et al., 2002). Each surface demonstrated different binding patterns with each protein. Most notably, although low adsorption of collagen II was observed on acrylic acid, the acrylic acid surfaces adsorbed the most vitronectin both in pure solutions and selectively from FCS (Whittle et al., 2002). Zhang et al. (2007) reported an increase in fibrinogen and BSA adsorption with increasing amine functionalities and concluded it was due to stronger electrostatic interactions (Zhang and Feng, 2007).

The enhanced adsorption of treated surfaces has been exploited by a number of researchers. Most notably, is the coating of poly(L-Lactic acid)-co-poly(ε-caprolactone) scaffolds with collagen I (Chan et al., 2009) and ppAm coated PLGA spheres with fibronectin (Bible et al., 2009) which enhanced the attachment of MSCs and neural stem cells respectively. The modified surfaces can also be used for the covalent attachment of molecules such as collagen on ammonium plasma treated PDLLA films (Yang et al., 2002) and oxygen plasma treated polyethersulfone electrospun meshes (Shabani et al., 2009) which enhanced the cell attachment in both instances.

Heparin was covalently anchored to PLGA/dexamethasone scaffolds formed from gas foaming and phase separation coated with plasma polymerised acrylic acid (Park et al., 2009). Scaffolds were pre-loaded with TGFβ and the scaffold was designed to allow dual release of TGFβ by heparin and dexamethasone from the PLGA polymer for chondrogenic differentiation of MSCs (Park et al., 2009). However, scaffolds without pre-loaded TGFβ supported enhanced chondrogenic differentiation compared to loaded scaffolds (Park et al., 2009). Although the study analysed the release of dexamethasone, it neglected to ascertain the binding and biological capacity of the covalently bound heparin (Park et al., 2009). The covalent attachment of heparin may have altered the heparin molecule and led to reduced activity. For instance, the heparin may have bound in a conformation that was sufficient to bind TGFβ but not to facilitate receptor activation, therefore sequestering the growth factor away from the cell. Considering the importance of the sulphation motifs of HS and heparin in mediating their biological activity (discussed in section 1.2.2), covalent modification may have a detrimental effect on their function.

**1.3.6.3 Growth Factors**

As discussed above, Park et al. engineered PLGA scaffolds for the release of dexamethasone and TGFβ for enhanced chondrogenic differentiation. Although the inclusion of heparin did not enhance TGFβ signalling, the incorporation of dexamethasone within PLGA led to an initial quick discharge during the first 10 days followed by a slow, continuous release for the rest of the 30 day culture period (Park et al., 2009). The degradation of synthetic polymers in culture has enabled them to be used as drug and growth factor delivery vehicles. Kanczler et al. (2010) create d porous scaffolds composed of alginate and PLA using supercritical CO2. By combining two materials, the study aimed to create a quick release profile of VEGF from the alginate phase to stimulate angiogenesis in combination with a slower release of BMP4 from PLA for stimulation of osteogenesis. VEGF
showed an increased release compared to BMP-2 within the first 7 days of culture, with BMP-2 release eventually accelerating between days 7 and days 28. Both growth factors were bioactive, with independent release profiles that supported the osteogenic differentiation and implantation of MSC loaded scaffolds.

Electrospinning has also been utilised to create growth factor loaded meshes for tissue engineering. Valmikinathan et al. (2009) successfully incorporated neural growth factor (NGF) into PCL/BSA fibres, with sustained release of bioactive NGF over 28 days. Sahoo et al. (2009) compared polymer blending (electrospinning of a blended polymer/growth factor solution) and co-axial spinning for incorporating FGF2 into PLGA fibres. The co-axial method involved a central solution of FGF2, around which was an isolated shell solution of PLGA. Both are simultaneously electrospun and by keeping the two solutions separate, the growth factor is protected from the harsh solvents used to dissolve the polymer. Whilst the blended solution produces a random distribution of FGF2, the co-axial approach creates fibres with an outer shell of PLGA and an inner core of FGF2. The incorporation of FGF2 led to enhanced attachment and proliferation of rabbit MSCs. However, despite different distributions, little difference was observed between the two conditions.

Rather than incorporate growth factors into the polymer scaffold, Fan et al. (2007) and Nur et al. (2008) covalently tethered growth factors to poly(methyl methacrylate)-graft-poly(ethylene oxide) (PMMA-g-PEO) films and electrospun polyamide scaffolds, respectively. Tethering of epidermal growth factor (EGF) via the N terminus to PMMA-g-PEO films led to elevated ERK phosphorylation and more sustained signalling in MSCs compared to soluble EGF. Tethered EGF signalling via the ERK/MEK pathway also led to a more spread phenotype and provided resistance against pro-apoptotic signalling by FasL, results that could not be replicated by soluble EGF. The study concluded that the enhanced signalling of tethered EGF was due to an increase in the local concentration of EGF at the cell surface and the prevention of ligand-receptor internalisation, which also compartmentalised cell signalling to the cell surface. Nur et al. covalently bound FGF2 to amine modified polyamide electrospun meshes. The covalent attachment of FGF2 dramatically increased its stability in culture, with approximately 20% remaining after 48 hours at 37°C. Signalling via FGFR and ERK phosphorylation, the tethering of FGF2 enhanced cell spreading and proliferation of fibroblasts on the scaffold surface. The modified scaffolds also supported neurite outgrowth of rat cerebellar granule neurons and supported the enhanced attachment and colony formation of human ES cells. Unfortunately, the study did not assess the level and persistency of FGF2 signalling compared to soluble FGF2. However, it could be hypothesised that the increase in stability, inhibition of endocytosis and increased local concentration of FGF2 lead to increased signalling and enhanced cellular responses.

1.3.7 GAG Incorporation

Rather than incorporate growth factors directly into scaffolds, researchers have also attempted to use heparin/HS for enhanced growth factor activity or biocompatibility. As mentioned previously, Park et al. (2009) covalently attached heparin to PLGA scaffolds coated with plasma polymerised acrylic acid. Unfortunately, the heparin did not function to enhance the activity of TGFβ-1, most likely due to the covalent immobilisation inhibiting its capacity to facilitate receptor binding. This is
supported by a study by Ho et al. (2009) in which heparin was covalently attached to chitosan-alginate scaffolds. Although the immobilised heparin bound and released bioactive FGF2, the binding efficiency of heparin was reduced by the covalent immobilisation to chitosan via heparin carboxylic acid groups.

Few studies have incorporated heparin/HS or sulphated GAGs with electrospun matrices. Zhong et al. (2005) electrospun collagen I with chondroitin-6-sulphate (CS-6S) creating meshes with fibre diameter ranging between 100-600nm. In order to maintain scaffold stability it was necessary to crosslink the mesh with gluteraldehyde vapour. Crosslinked scaffolds containing CS-6S enhanced fibroblast proliferation compared to non-crosslinked and collagen only controls. Luong-Van et al. (2006) electrospun a blend of PCL and heparin, creating a homogenous distribution of heparin within the fibre mesh, which was released in a sustained, bioactive manner over 14 days of culture. Interestingly, increasing heparin concentrations led to a decrease in fibre diameter due to the increased charge density. In a similar study, Casper et al. (2005) electrospun low molecular weight heparin (LMWH) with poly(ethylene oxide) (PEO) and PLGA which created fibre meshes with a uniform dispersion of LMWH. Although LWMH initially rapidly leached out of the scaffold, incorporation of a five-starred PEG molecule led to greater retention and enhanced growth factor binding. In a later study, domain I of perlecan and heparin-BSA-biotin complexes were covalently attached to crosslinked collagen and gelatin electrospun meshes (Casper et al., 2007). Although the immobilised molecules appeared to have the capacity to bind FGF2, the biological and receptor binding activity was not assessed (Casper et al., 2007).

As mentioned previously, HA had been successfully incorporated into electrospun meshes that, once crosslinked and fibronectin coated, supported the culture of fibroblasts (Ji et al., 2006). Due to its high molecular weight, HA has also been used to facilitate the electrospinning of gelatin (Li et al., 2006a). In addition, thiolated HA has been used to produce PEG-diacrylate crosslinked hydrogels that support the survival and growth of fibroblasts (Zheng Shu et al., 2004). Incorporation of thiolated heparin combined with growth factors FGF and VEGF led to sustained growth factor release over 40 days, with growth factor retention increasing with increasing heparin concentrations and HA:heparin:VEGF constructs supporting in vivo angiogenesis to the greatest extent (Pike et al., 2006).

1.3.8 Infiltration

Hydrogels have the advantage that cells can be encapsulated during hydrogel preparation, enabling a homogeneous distribution of cells within the scaffold. Pre-fabricated scaffolds do not have this advantage and in many cases cell migration or cell seeding into the scaffold centre is difficult to achieve. Interpenetrating pores are needed, typically larger than the cell, to allow cell infiltration.

Surface modification of the scaffold can enhance biocompatibility and cell infiltration. Barry et al. (2005) reported enhanced cell attachment and infiltration into porous PLGA scaffolds coated with plasma polymerised allyl amine. Zelzer et al. (2008) proposed the formation of a plasma polymerised allyl amine-hexane gradient. Although the method was not applied to a 3D scaffold, fibroblasts preferentially attached to areas with a higher composition of allyl amine. If this could be
translated into 3D, a higher concentration of allyl amine in the scaffold centre may enhance cell infiltration.

One of the drawbacks of electrospun matrices is the lack of cell infiltration, creating a topographically distinct but essentially 2D culture environment. As the fibres are deposited on top of one another a dense meshwork is created that does not encourage cell infiltration. A number of studies have attempted to overcome this using a variety of techniques. Nam et al. (2007) incorporated salt crystals into PCL fibre meshes using a sheath surrounding the electrospinning capillary. The scaffolds had a delaminated layer structure with caps of 100-200µm created by salt deposits and cell infiltration occurred from the edge inwards. However, the scaffold was not uniform in structure and there was little evidence of cell infiltration between the delaminated areas. Tzenana et al. (2008) electrospun sheets of PCL fibres into a water bath. The sheets were removed and layered on top of each other before being placed in a vacuum oven. The vacuum oven treatment led to the evacuation of trapped water, which stretched and expanded the mesh creating a looser 3D scaffold, enabling the infiltration of myoblasts and human ES cells. Another approach is to co-electrospin water soluble fibres such as PEO or gelatin, the leaching of which would leave behind a looser network of synthetic fibres (Ekaputra et al., 2008). Ekaputra et al. (2008) electrospun nanometer PCL/collagen fibres with gelatin and PEO, with little/no cell infiltration observed. However, when fibres in the micrometer range (1.61µm) were electrospun in conjunction with electrosprayed heparasil (a commercially available hydrogel of heparin and HA), cells infiltrated into the full thickness of the mesh. The incorporation of heparasil reduced the volume density of the fibres and provided a biodegradable element available for cellular remodelling. Even without heparasil, a degree of infiltration was observed in meshes with a larger average fibre diameter of 1.29µm. Enhanced migration into meshes composed of larger fibres was also reported by Pham et al. (2006). By increasing flow rate and solution concentration the study produced fibres 5µm in diameter, increasing pore size and enabling infiltration of rat MSCs, with full perfusion achieved in conjunction with a bioreactor. The increase in pore size with increasing fibre diameter is in agreement with the mathematical model proposed by Eichhorn and Sampson (2005) which concluded that the number of fibre contacts and distance between crossings is a function of fibre diameter. Larger fibre diameters decrease the number of, and increases the distance between, the fibre crossings creating a larger pore size.

1.3.9 Application to ES Cell Culture

ES cells have been combined with a wide array of biomaterials, either to support propagation or to enhance differentiation into more mature, organised structures. Hydrogels have demonstrated promise in both respects. Li et al. (2006c) developed a RGD-functionalised hydrogel which also incorporated enzyme-cleavable peptide sequences. The efficiency of human ES cell colony formation on the gel surface was enhanced with increasing amounts of RGD. However, the study did not encapsulate the cells and they were only cultured for 5 days with no long term passaging. Gerecht et al. (2007) encapsulated human ES cells in UV crosslinked HA scaffolds. Cells survived the encapsulation procedure and were removed from the scaffold, still in an undifferentiated state, using hyaluronidase digestion. However, the cells needed to be seeded at high concentration, >5 x 10^6 cells/ml, in order to support colony formation and maintain cell viability and growth. This is not ideal if the scaffolds are applied to ES cell propagation. In addition, as with Li et al. (2006), the
study did not assess long term maintenance of human ES cells within the scaffolds. Hydrogels have also been applied to ES cell differentiation and have supported the formation of 3D structures and more mature cell phenotypes. Hwang et al. (2006) encapsulated mouse EBs in PEG-based hydrogels, enhancing differentiation towards more mature chondrogenic phenotypes compared to 2D culture conditions. Encapsulation of disassociated mouse EBs in PuraMatrix (a commercially available self-assembling peptide hydrogel) also enhanced osteogenic differentiation (Garreta et al., 2006).

Scaffolds derived from natural polymers have also been applied to ES cell culture. For instance, Li et al. successfully cultured pluripotent human ES cells in chitosan-alginate scaffolds in unconditioned media for 21 days. The volume of the scaffold meant cells did not need to be subcultured for 21 days, during which time pluripotency was maintained at equivalent levels to control cultures on MEFs.

The lack of endogenous adhesive ligands makes the culture of ES cells on synthetic materials more complex. Gao et al. (2010) assessed the attachment of human ES cells to PLGA, both in 2D and 3D (scaffolds formed by phase separation) conditions. The study coated PLGA films with collagen I, collagen IV, laminin and fibronectin, with laminin providing the best adhesive substrate for human ES cells. The preferential binding of human ES cells to laminin-coated PLGA was also translated to 3D culture, indicating that the scaffold preparation procedure had not altered the surface chemistry of the scaffold. PLGA 3D scaffolds have successfully enhanced the differentiation of human ES cells into 3D tissue structures. Most notable is the work conducted by Levenberg and colleagues. Using a 50:50 blend of PLGA and P, sponges formed from salt leaching with a 250-500um pore size successfully supported the differentiation of human ES cells into neurons, cartilage and liver cell phenotypes (Levenberg et al., 2003). Cells induced towards a neural lineage formed ductular structures resembling the embryonic neural tube (Levenberg et al., 2003). Immature capillary networks formed through the structures (with the exception of RA treated samples) and 2 week old constructs were permeated with host blood vessels when transplanted into immunodeficient mice (Levenberg et al., 2003). In a subsequent experiment, Caspi et al. (2007) created 3D vascularised cardiac tissue by a tri-culture of human umbilical vein endothelial cells (HUVECs), human ES cells and human embryonic fibroblasts. However, in both experiments scaffolds were pre-treated with either fibronectin or matrigel.

Smith et al. (2009) highlighted the advantage of a nanofibrous substrate in the differentiation of human ES cells. Phase separation of P,LA scaffolds under different parameters produce both nanofibrous and solid walled scaffolds. Enhanced osteogenic differentiation of mouse ES cells was observed on the nanofibrous architecture compared to smooth walled scaffolds, with differential requirements for supplements and growth factors observed between the two surfaces.

Electrospun meshes have been applied to the culture and differentiation of ES cells. In further studies using electrospun polyamide scaffolds, Nur et al. (2006) cultured mouse ES cells on gelatinised meshes. Cells formed colonies on the cell surface of electrospun scaffolds but failed to attach to flat films, highlighting the differential effects of surface topography on cell attachment and proliferation. The effect of the topography on ES cell behaviour was also demonstrated by enhanced proliferation compared to flat gelatinised coverslips. In support of previous research by
the same group, the study linked the increased proliferation with significantly increased Rac GTPase activity. The study also observed enhanced PI3K activity on the electrospun scaffolds which was linked to an increase in Nanog expression compared with flat 2D controls. Although cells were cultured with MEFs and long term culture was not assessed, the study highlights the potential of electrospun meshes in governing or enhancing the culture of mouse ES cells.

ES cells have been differentiated towards a number of cell phenotypes on electrospun meshes, including adipocytes (Kang et al., 2007), cardiomyocytes (Fromstein et al., 2008) and neurons (Carlberg et al., 2009; Xie et al., 2009). Kang et al. (2007) reported enhanced adipogenic differentiation of mouse ES cells on PCL meshes with the formation of spherical lipid laden cells surrounded by flattened non-adipogenic cells, reminiscent of adipocyte cell architecture in vivo. The ability of the fibre architecture to enhance differentiation was further supported by adipocytic differentiation without hormonal induction. Fromstein et al. (2008) compared polyurethane thermally induced phase separated (TIPS) scaffolds with electrospun meshes for the differentiation of cardiomyocytes from mouse ES cells. Polyurethane meshes were comprised of fibres ranging between 2-10µm in length and created a scaffold 70µm thick. In comparison, TIPS scaffolds were 1000µm thick and composed of interconnected pores (21µm) with a rough textured surface. Cells adopted different morphologies on the two scaffolds. Cells on the TIPS scaffold adopted a rounded morphology, whilst cells on the electrospun meshes elongated along the fibres and adopted spread morphology more characteristic of cardiomyocytes. Electrospun meshes demonstrated enhanced cell viability, as the thicker TIPS scaffolds restricted nutrient diffusion to the scaffold centre. However, despite the differences in morphology, both scaffolds supported cardiomyocyte contraction.

Due to the potential application of electrospun scaffolds in nerve regeneration, ES cells have been differentiated towards a neural lineage on random polystyrene (Carlberg et al., 2009) and aligned PCL (Xie et al., 2009) fibres. Human ES cells differentiated on random polystyrene meshes in N2B27 media supplemented with EGF and FGF2 formed neurons positive for Map2 and βIII tubulin, and also dopaminergic marker TH (Carlberg et al., 2009). In comparison, 2D cultures consisted of a large population of GFAP positive astrocytes with a lower proportion of Map2, βIII tubulin and TH positive neurons (Carlberg et al., 2009). Xie et al. (2009) differentiated mouse EBs on both aligned and random fibres. On both orientations, EBs remained in a rounded morphology on the scaffold surface as cells differentiated into neurons, oligodendrocytes and astrocytes. However, during the differentiation process, the differentiating cells migrated away from the main EB body, a behaviour which was enhanced on aligned meshes. Both migration and neurite extension were guided by the direction of fibre alignment with enhanced neurite elongation observed on aligned fibres.

1.4 Conclusion

Considering the versatility and established nature of electrospinning, it is surprising that so few studies have combined electrospun scaffolds with ES cells. This could be due to the lack of observed infiltration into the scaffold structure. However, electrospun meshes can still provide a nanofibrous meshwork for cell culture and can alter the adhesion dynamics and signalling of attached cells. Progress has also been made in creating 3D meshes for cell culture, by increasing
fibre diameter and therefore pore size or by creating looser meshes by layered hydrospinning. Considering the reliance of ES cells on ECM cues, both in standard culture conditions and during EB formation, the structure of the meshes may enhance either ES cell propagation or differentiation.

The potential combination of heparin/HS with a biomaterial scaffold has yet to be fully realised, particularly in electrospun scaffolds. Covalent modification can adversely affect the highly organised structure and sulphation motifs within the GAG chain, reducing or inhibiting its biological function. Electrospinning mechanisms have aimed to, or resulted in, the leaching of heparin from the fibre mesh, with no successful immobilisation on the scaffold surface yet reported. The combination of HS/heparin on electrospun fibres in a fully functional form may provide architectural and biological cues for enhanced ES cell culture or differentiation. Considering the structural diversity of HS, the scaffold could potentially be tailored to differentially regulate growth factor binding, replicating the control occurring naturally in the cell microenvironment.

1.5 Aims and Objectives

The initial aim of the study was to assess the ability of electrospun PLGA meshes to support the culture of mouse ES cells, with the hypothesis that the fibrous architecture would assist in maintaining pluripotency as reported previously (Nur et al., 2006). In order to mimic the architecture of the native ECM, the study aimed to create meshes with an average fibre diameter of ~500nm, similar dimensions as previous electrospun PLGA scaffolds (Li et al., 2002b; Xin et al., 2007) and the research of Nur et al. (2006).

PLGA was selected as it is a highly versatile polymer, with a tailorable degradation rate and the ability to be electrospun with ECM molecules such as elastin and gelatin (Li et al., 2006b). Electrospun PLGA meshes have also been successfully applied to the culture and differentiation of MSCs (Xin et al., 2007), with their application to ES cell culture remaining to be explored. As the degradation of PLGA generates acidic by-products that may be toxic to ES cells, the study aimed to conduct a preliminary assessment of the degradation of electrospun PLGA meshes in standard ES cell media. Scaffolds were incubated for 30 days, with fibre integrity assessed using SEM and pH of the surrounding media monitored.

The suitability of electrospun meshes for culture of mouse ES cells was compared with flat spin coated PLGA films with a focus on cell morphology, proliferation and pluripotency. Initial research focused on the culture of mouse ES cells during a seven day culture period with cell morphology assessed using standard and variable pressure SEM and actin staining. Proliferation over the seven day culture period was assessed using a Hoescht DNA assay.

The ability of electrospun and spin coated scaffolds to sustain ES cell pluripotency was assessed by passaging the mouse ES cells on the scaffolds over a 17 day culture period. An Oct4-GFP reporter cell line was utilised, enabling quantitative analysis of pluripotency using flow cytometry. Results were further reinforced by Nanog antibody staining and cells were counted at each passage enabling the population doubling time to be calculated.

The study also aimed to investigate the potential of electrospun PLGA scaffolds to support 3D culture of mouse ES cells. In accordance with previous research, the fibre diameter of the
electrospun scaffolds was increased by altering spinning parameters such as polymer concentration, applied voltage and flow rate according to the hypothesis that increasing fibre diameter would increase pore size and therefore enable infiltration of ES cells into the mesh (Eichhorn and Sampson, 2005; Pham et al., 2006). The ability of electrospun scaffolds to support ES cell infiltration was assessed using pro-migratory E-cadherin−/− mouse ES cells, which were visualised using SEM.

In order to functionalise the scaffolds and provide biological as well as architectural cues for ES cell culture, scaffolds were coated with plasma polymerised allyl amine with the aim to non-covalently immobilise heparin/HS on the fibre surface (Mahoney et al., 2004). The immobilisation of heparin/HS was regarded as a novel functionalisation strategy, which had not been previously achieved with electrospun scaffolds. In addition, it was hypothesised that the heparin/HS displaced by the fibres would be presented to the cells in a form more reminiscent of their native state, attached to core proteins at the cell surface and within the ECM. It was also hypothesised that by functionalising the scaffolds with particular HS species it would be possible to mimic the modifications which occur within the native HS chain during ES cell differentiation (Baldwin et al., 2008; Johnson et al., 2007), enhancing or directing cell fate towards certain lineages.

Although plasma polymerised allylamine had been previously used to treat surfaces for the non-covalent immobilisation of GAGs (Mahoney et al., 2004), the approach had not yet been applied to 3D, degradable substrates. X-ray photoelectron spectroscopy (XPS) and WCA were used to confirm the alteration of the scaffold surface chemistry after treatment with plasma polymerised allylamine and immobilised HS was quantified and analysed using reverse phase high pressure liquid chromatography (RP-HPLC).

One of the primary aims was to immobilise heparin/HS in a form that was free to interact with proteins and that retained and presented the sulphation epitopes essential for mediating differential binding of growth factors such as FGF2. In order to confirm the accessibility of immobilised heparin, a bA-Link_TSG-6 binding assay previously used to assess binding of heparin to allyl amine coated plates (Mahoney et al., 2004; Marson et al., 2009) was adapted for the coated electrospun meshes. To assess the presentation of sulphation epitopes within the HS chain, phage display anti-heparin/HS antibodies specific for distinct sulphation epitopes were also utilised.

It was also necessary to confirm the bound HS was retained during culture and not lost into the culture medium as reported by previous studies (Casper et al., 2005; Luong-Van et al., 2006). In order to assess this, radiolabelled 3H HS was immobilised on the scaffold surface. Scaffolds were incubated in neural differentiations media (N2 B27) and PBS for 10 days and the presence of 3H HS detected at selected time points.

It was critical to ensure the immobilised HS retained its biological activity. This was assessed using EXT1−/− mouse ES cells which lack endogenous HS and as a consequence have impaired differentiation (Lin et al., 2000). However, their ability to differentiate can be rescued by the addition of exogenous HS/heparin (Johnson et al., 2007). Therefore, EXT1−/− ES cells were stimulated towards a neural lineage on allylamine coated scaffolds with and without immobilised HS. The ability of the immobilised HS to facilitate growth factor binding was also investigated by adding
exogenous FGF4 to the media and assessing whether the bound HS and FGF4 worked in concert to enhance neural differentiation on the scaffold surface.

The final aim of the study was to apply the HS functionalised scaffolds to the culture of human ES cells with the hypothesis that the immobilised HS would assist the binding of FGF2 and maintain pluripotency. Human ES cells were cultured on allylamine coated scaffolds with and without bound HS in unconditioned and MEF conditioned media. Pluripotency was assessed after 5 days of culture by antibody staining for Oct4.
2 Materials and Methods

2.1 Methods of Analysis

2.1.1 High Pressure Liquid Chromatography

RP-HPLC analysis conducted in this research was first introduced by Deakin and Lyon (2008). HS preparations are digested into disaccharides using a combination of three enzymes, heparinases I, II and III and then labelled at the reducing end with an AMAC (2-aminoacridone) fluorophore. The AMAC labelling method is ideal as it attaches to all major sulphated GAG species and there is no clean up necessary after labelling, reducing loss of sample. The labelled sample is applied to a column with a silica stationary phase and a 3.5\(\mu\)m pore size, saturated with 0.1M ammonium acetate. One applied, the column is exposed to increasing concentrations of methanol with a shallow and sharp gradient which elutes the disaccharides into 8 distinct peaks from high to low sulphation. As efficiency of AMAC labelling is not 100% for each species, the peaks produced by Deakin and Lyon were compared with an alternative method of analysis (SAX-RP-HPLC) and the total fluorescence of each raw peak corrected accordingly. The only significant contaminant of the profile is an AMAC related peak at 24.5-25minutes. However, this peak largely remains distinct and easily identifiable from disaccharide peaks, with the exception of lower (<100ng) GAG concentrations.

The above method has several advantages including relatively simple labelling procedure with little loss of sample. Despite the contaminant AMAC peak, relatively small amounts of GAG (~10ng) can be analysed. As well as determining disaccharide composition, the total quantity of GAG can be estimated by treating a known amount of GAG alongside an analytical sample and comparing total fluorescence.

2.1.2 HS specific antibodies

Phage-display derived ScFv antibodies specific for distinct HS sulphation motifs are produced by a phage “bio-panning” method, as described by Smits et al. (2006). In brief, DNA encoding antibodies are cloned into a suitable vector together with vesicular stomatitis virus G (VSV-G) protein, a protein on the phage coat, creating a fusion protein. When the phage assembles within a bacterial host, the fusion protein is incorporated into the phage coat, displaying the antibody on the outside surface. A large library of phage-display antibodies can be assembled and incubated with immobilised GAGs. Phages that display antibodies specific for the particular GAG species presented will bind and be successfully recovered. In order to increase antibody specificity, the recovered population of phages are expanded by bacterial reinfection, collected and then exposed to the GAG species once more. This process is repeated a number of times before phages are individually selected and further analysed for GAG binding by ELISA and immunohistochemistry.

Ligand specificity is determined by assessing antibody binding affinity to HS preparations from particular tissue sources and following sequential modification of HS/heparin (e.g. N-desulphation followed by N-acetylation). HS4C3 is an example of an ScFv that has a fully defined epitope, the identification of which was assisted by HS4C3 binding a rare, highly sulphated IdoA(2S)-GlcNS(3S,6S) epitope found within the antithrombin binding motif of heparin (Ten Dam et al.,
The ScFv antibodies have been successfully employed to demonstrate the tissue restricted expression of particular HS epitopes in rat kidney (van Kuppevelt et al., 1998), skeletal muscle (Jenniskens et al., 2000) and human lung (Smits et al., 2004).

In addition to assessing HS distribution in tissue sections, ScFv antibodies have been used to assess HS expression profiles of differentiating ES cells using flow cytometry (Baldwin et al., 2008; Johnson et al., 2007). Flow cytometry was also utilised to assess the differential binding of RB4E12 and EV3C3 to MEFs lacking 6-O-endosulfatases Sulf1 and Sulf2 (Lamanna et al., 2006). In this current study, ScFv antibodies combined with a biotinylated secondary antibody were used in a microtitre assay modified from the binding studies discussed in the following section.

### 2.1.3 Microtitre Assay

The use of ppAm treated 96 well plates for a heparin/HS microtitre assay system was first introduced by Mahoney et al. (2004). Incubating ppAm plates with increasing concentrations of heparin in PBS overnight ionically immobilised the unmodified molecules in a conformation freely available to interact with a number of heparin binding proteins including the Link module of TSG-6 (product of tumour necrosis factor stimulated gene 6) (Mahoney et al., 2004). Oligosaccharides as small as a decassacharide could be successfully immobilised and the ionic interaction, although disrupted by high salt strengths at 1M NaCl and above, was stable at 300-500mM NaCl (Mahoney et al., 2004). By using biotinylated Link_TSG-6 (bA-Link_TSG-6) it was possible to assess protein interactions using an avidin conjugated alkaline phosphatase, the reaction of which with the development reagent (p-nitrophenylphosphate) elicited a dose dependent colorimetric change (recorded as absorption at 405nm) (Mahoney et al., 2004). The assay was subsequently used to assess the binding of selectively mutated forms of the TSG-6 Link module, contributing to the identification of the heparin binding domain distinct from the previously identified HA binding site (Mahoney et al., 2005).

GAGs have a restricted ability to immobilise onto surfaces in a functional state without covalent modification of the molecule. The ppAm 96 well plates enable unmodified GAGs to be immobilised in a stable, functional form and can therefore be used as “GAG arrays” allowing the interaction of specific proteins with immobilised GAGs to be easily assessed (Marson et al., 2009). The plates were further modified by investigating the optimal coating for functional heparin immobilisation. By introducing octadiene into the plasma polymerisation process it was possible to alter the total percentage of ppAm (Marson et al., 2009; Robinson et al., 2008). It was observed that although increasing percentages of ppAm bound increasing quantities of heparin (as determined by XPS), the functionality of the bound heparin was compromised (as determined by bA-Link_TSG-6 binding) (Marson et al., 2009; Robinson et al., 2008). Optimal functional binding was observed at 60-90% ppAm with a drop in functionality at 100% ppAm (Marson et al., 2009; Robinson et al., 2008).

The plates have demonstrated extensive versatility in the range of GAGs that can be immobilised on the surface, with optimal immobilisation of specific GAG species dependent on the ppAm content of the coating. For instance, DS and heparin bound most efficiently to 90% ppAm whilst CS-6S optimally bound at 100% ppAm (Marson et al., 2009). In the recent study by Marson et al. (2009), despite a slight drop in maximal binding with certain species, 100% ppAm was chosen as
the optimal coating as it bound a broader range of GAGs in a functional conformation, including HA, DS, C-6-S and various forms of selectively desulphated heparin (Marson et al., 2009).

2.1.4 SEM

SEM analysis produces high (nm scale) resolution topographical images of the sample surface. Images are formed by scanning the specimen with a high resolution electron beam. The interaction of the beam with the sample creates signals such as secondary electrons, back scattered electrons and characteristic X-rays (Wellls, 2008). Images are primarily formed from secondary electrons, which possess an energy of 1-10eV (maximum 50eV) (Wellls, 2008). The topographical detail is derived from the angle of electron beam incidence on the specimen surface, the secondary emission rate, penetration effects at sharp edges and variations in efficiency of SE collection (Wellls, 2008). Coating a specimen with a conductive material, such as gold, prevents charging of the specimens and increases secondary electron emission rate (Wellls, 2008). Standard SEM functions at high vacuum so gaseous molecules do not interfere with the electron beam or the produced signals. As a consequence, all biological samples must be dehydrated before analysis. However, this leads to distortion/cracking of the sample and the images may not be a true representation of cells in culture. Environmental SEM (E-SEM) enables hydrated samples to be visualised by lowering the chamber pressure. In order to achieve this, the beam column is separated by pressure limiting apertures which divide the column into distinct pressure zones (Donald, 2007). As the beam progresses through the column, the pressure is steadily increased until it reaches the sample chamber (Donald, 2007). Due to the lower vacuum, the incident beam is partially scattered and broadened before it meets the sample causing a reduction in image quality (Donald, 2007). However, this can be overcome by reducing the working distance and ensuring pressure is kept at a minimum (Donald, 2007).

2.1.1 WCA

When a water droplet is placed on a surface, the shape adopted is a reflection of the balance between cohesive forces within the liquid and adhesive forces between the liquid and the solid surface. A hydrophilic surface is characterised by spreading of the droplet and a low WCA. Hydrophobic surfaces have a high contact angle as the water droplet remains rounded, displaying minimal contact with the surface. Wetting is analysed using Young’s equations (applicable to flat surfaces) and Wenzel and Cassie-Baxter equations (applicable to rough/textured surfaces) (Whyman et al., 2008). In the Cassie-Baxter model, air is trapped beneath the water droplet in the textured surface, causing an increase in contact angle. In the Wenzel model, water penetrates into the textured surface. Droplets can change from a Cassie-Baxter state to the Wenzel model by water penetration within the centre of the droplet (‘nucleation’) which grows until the droplet has adopted a Wenzel state, which is reflected by a lower WCA (Ishino and Okumura, 2006; Whyman et al., 2008)

2.1.2 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy passes mid-infrared light (wave number 4000-400cm\(^{-1}\)) through a sample and records which wavelengths have been absorbed by the material (Young and Lovell, 1991). Vibrations or bond deformations (such as stretching and bending) between atoms are dependent on the molecules involved and absorbance occurs when the IR
frequency match that of the vibrational frequency of a particular bond deformation (Young and Lovell, 1991). Analysing absorbance across a range of frequencies generates an absorbance, or transmittance spectra. As different materials or compounds are comprised of characteristic atomic interactions, the spectrum is unique to that material and adsorption peaks can be allocated to particular bonds (Young and Lovell, 1991). FTIR spectrometers analyse absorbance across a range of frequencies in seconds and only require a small amount of material (Young and Lovell, 1991).

2.1.3 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) continuously measures the weight of a material on a highly sensitive thermobalance as the temperature is gradually increased (Stevens, 1999). Samples are heated in either air or an inert atmosphere (Stevens, 1999). Weight loss occurs due to evaporation of residual water, with higher temperatures inducing weight loss from polymer decomposition (Stevens, 1999). If a polymer has been processed in a particular solvent, TGA can be used to measure the loss of the solvent during heating, with maximum loss typically occurring at the boiling point of the solvent in question.

2.1.4 X-Ray Photoelectron Spectroscopy

XPS is a highly sensitive technique, providing quantitative analysis of the elemental composition and chemical states present on the sample surface and has a sampling depth of ~10nm (Urquhart and Alexander, 2007). Operating under ultra high vacuum, X-rays are generated by bombarding an anode, typically aluminium or magnesium, with high energy electrons (Briggs, 1998). The resulting X-rays, or photoemissions, are focused onto the sample surface resulting in the ejection of electrons (photoelectrons) from core and valence orbitals of atoms (Briggs, 1998; Urquhart and Alexander, 2007).

Atoms are identified by discrete binding energies of the core orbitals, with binding energy (BE) determined using the following equation:

\[ \text{BE} = \frac{h\nu}{\text{KE}} - \phi \]

Where KE is the kinetic energy of the emitted photoelectron and \( h\nu \) is the energy of the exciting radiation. \( \phi \) represents the work function and depends on the sample and spectrometer used (Briggs, 1998; Urquhart and Alexander, 2007).

As the number of electrons with a particular BE equates to the amount of a particular atom, XPS can quantify the atomic composition of a sample by comparing relative intensities of each element identified (Briggs, 1998; Urquhart and Alexander, 2007). As well as varying between atoms, the BE of an electron is also altered by the chemical environment in which the parent atom resides causing small chemical shifts (maximum of ~10eV) in binding energy (Briggs, 1998; Urquhart and Alexander, 2007). The shifts provide information of the chemical states present at the surface. However, the shifts combine to form a broader intensity peak and individual chemical states are often difficult to refine. Core level peaks are therefore fitted with smaller peaks corresponding to the different chemical states present at the surface of the sample. Typically, a combination of Gaussian and Lorentzian peak shapes are used and by restricting the FWHM and consulting the literature on
the positioning of particular chemical states it is possible to determine which chemical states are present and in what relative amounts.

Although the X-rays can penetrate micrometers into the sample, the photoelectrons deeper in the sample are scattered by the solid with only photoelectron at the surface escaping. However, the solid scattered photoelectrons do cause a background beneath the photoemission peak which has to be taken into consideration when processing data (Briggs, 1998; Urquhart and Alexander, 2007).

Charging of the sample can occur as electrons are depleted from the surface, forming a positive charge that causes an increase in binding energies. However, this can be corrected by use of an electron flood gun and by charge correcting/calibrating data against the known C-H BE of 285.0 eV (Briggs, 1998; Urquhart and Alexander, 2007).
2.2 ES Cell Culture

All media was filtered using bottle top filters (0.2µm, Nalgene). Bottles for culture medias were purchased from Nalgene. All tissue culture plastic was purchased from Greiner Bio-One. Sterile 50 ml and 15 ml tubes were purchased from StarLab. All pipette tips (Anachem) were sterilised by autoclaving. A Universal 320 Hettich centrifuge was used to pellet cell suspensions. Cells were counted using disposable haemocytometers (BM Browne Ltd.) All H₂O was provided by a Milli-Q filter system unless specified otherwise.

2.2.1 Maintenance of Mouse ES Cell Lines

Five types of mouse ES cells were used in the present study. The Oct4 reporter cell line (Oct4-GFP), were a kind gift from Austin Smith, The University of Cambridge and express green fluorescent protein (GFP) under the control of the Oct4 promoter (Ying et al., 2002; Ying et al., 2003b). E14 ES cells were also donated by Austin smith and derived by Martin L. Hooper (Fisher et al., 1989). E-cadherin-/- mouse ES cells were obtained from the laboratory of Dr Chris Ward and are characterised by Larue et al. (1996) and Spencer et al. (2007). EXT1-/- mouse ES cells were generously donated by Jeffrey Esko and were generated and characterised by Lin et al. (2000). Wildtype 1 (WT1) ES cells were derived in house from an l-129 mouse background according to the protocol detailed by Bryja et al. (2006).

All mouse ES cells were maintained on gelatinised tissue culture treated 6 well plates. When expansion was necessary, cells were cultured on gelatinised tissue culture treated 25cm², 75cm² and 175cm² tissue culture flasks. Plates/flasks were gelatinised by addition of 0.1% gelatin (Sigma) in H₂O to each well, ensuring the whole surface area was covered with the gelatin solution. Plates/flasks were gelatinised overnight at 4°C or for one hour at 37°C. Upon cell seeding, excess gelatin solution was removed and the surfaces allowed to dry in the tissue culture hood.

Cells were maintained in standard ES media consisting of KnockOUT DMEM (Invitrogen) supplemented with 10% FCS (batch tested, BioSera), 1x non-essential amino acids (Gibco), 2mM L-Glutamine (PAA), 50µm 2-mercaptoethanol (Gibco) and 1000U/ml LIF (ESGRO, Chemicon International). When cultured on scaffolds, 5units/ml penicillin and 5µg/ml streptomycin (Gibco) was added to the culture media.

To detach cells, media was removed and cells were washed twice with PBS. For 6 well plates, 1.5ml of 1x trypsin/EDTA (PAA) was added for 10 seconds and then removed, followed by incubation at 37°C for 2 minutes. Trypsin was neutralised with 600µl of culture media and the wells typically split 1:6 or 1:10 onto dry gelatinised wells. Volumes of the above reagents were increased with culture in larger flasks with the essential elements of the protocol remaining the same.

All cells were routinely frozen. Trypsinised cells were detached as above, before being resuspended in 600µl standard mouse ES media supplemented with 10% DMSO (Sigma), transferred to 1.8ml cryovials (Nunc) and slow frozen at -80°C. After a minimum of two days, vials were transferred to liquid nitrogen for long term storage.
2.2.2 Maintenance of Human ES Cell Lines

The human ES cell line HUES7 (Cowan et al., 2004) was obtained from Harvard University (Human ES cell facility, Melton Laboratory) and was donated to the lab by Dr Chris Ward. Cells were maintained on mitotically inactivated MEFs in standard human ES media consisting of Knockout DMEM, 20% KSR (Gibco), 1x non-essential amino acids, 2mM L-Glutamine, 50µm 2-mercaptoethanol, 10units/ml penicillin and 10µg/ml streptomycin and 10ng/ml FGF2 (Invitrogen). Thawed MEFs previously stored in liquid nitrogen were pelleted at 700rpm for three minutes and resuspended in standard human ES media minus FGF2. MEFs were seeded onto 6 well plates at a density of $7 \times 10^4$ cells/cm$^2$ and cultured for 24 hours in 2ml standard human ES media minus FGF2. Upon seeding of HUES7 cells, media was removed and thawed HUES7 cells in standard human ES media were seeded at approximately $4 \times 10^5$ cells/cm$^2$. Media was replaced every day except for the first day after seeding to allow cells to settle and attach to the MEF monolayer.

To passage HUES7 cells, media was removed and wells were washed twice with PBS. Cells were then incubated with 300µl trypsin/EDTA for 3 minutes at 37°C. Trypsin was neutralised with 700µl standard human ES media and seeded onto MEFs or gelatinised TCP at typically a 1:3 split. Before seeding onto ppAm microfibre scaffolds, cells were cultured for at least two passages on gelatinised TCP in MEF conditioned media supplemented with 10ng/ml FGF2.

MEF conditioned media was created by seeding mitotically inactivated MEFs on 6 well plates at a cell density of $7 \times 10^4$ cells/cm$^2$. Cells were defrosted and seeded as described above and cultured in 2ml standard human ES media minus FGF2. Media was collected every day for 10 days and stored at 4°C used within 14 days. 10ng/ml FGF2 was added to media prior to addition to HUES7 cultures.

2.3 Electrospun PLGA Scaffolds

2.3.1 Scaffold Preparation

2.3.1.1 Microfibre meshes

PLGA with a PDLA: PGA molar ratio of 85:15, a molecular weight of 50,000-75,000 g/mol and inherent viscosity of 0.55-0.75dL/g, was purchased from Sigma. 1g of PLGA pellets was fully dissolved in 5ml hexafluoroisopropanol (HFIP, 99.9%, Apollo Scientific) to create a 20% (w/v) solution.

For electrospinning microfibre meshes, the solution was drawn into a 10ml syringe capped with a blunted 21G needle (both BD Bioscience). The loaded syringe was then mounted onto a syringe pump (WPI-Europe) and polymer solution pumped through the blunted needle at 20ml/hr until regular dripping occurred. Feed rate was then slowed to 0.5ml/hr and 25kV applied to the needle tip by a high voltage unit (Glassman High Voltage Inc.). The solution was spun onto a cylindrical rotating mandrel (9.2cm in length, 6.3cm diameter) grounded by an earthed wire. The mandrel was covered in aluminium foil and rotated at 100rpm. Working distance was set to approximately 20cm. Dry polymer meshes covering the mandrel were collected and removed after approximately two hours of spinning. Samples were then placed in a vacuum oven at room temperature (RT) for 48 hours at 1000 mbar to remove any residual solvent.
2.3.1.2 Spin Coated Films

To create spin coated films, 200µl of the 20% (w/v) PLGA solution described above was spin coated at 2000rpm for 20 seconds onto a glass slide using a WA-400B 6NPP/LITE spin coater (Laurell Technologies). Glass slides were cut from microscope slides to fragments 2cm x 2.5cm and washed with 100% ethanol before use. Spin coated films were left for 24 hours in a fume hood before 48 hours treatment in a vacuum oven.

2.3.1.3 Macrofibre Scaffolds

Electrospinning apparatus was as described above, including needle gauge and syringe size. To create larger fibre diameters from the 20% (w/v) PLGA solution described above, flow rate was increased to 1, 2.5, 5, 7.5 and 10ml/hr. Polymer concentration was also increased to 30% with flow rate set at 1 and 5ml/hr.

To gain large enough meshes for cell culture, 30% (w/v) PLGA with a 5ml/hr flow rate was electrospun onto a flat grounded metal plate 10cm by 10.3cm and 1.1cm thick covered in aluminium foil. Working distance remained at approximately 20cm and fibres were spun at 20kV and 25kV applied voltage.

PLGA (85:15 PLLA: PGA molar ratio) with a viscosity of 2.3 dL/g and a molecular weight of approximately 363,000 g/mol was supplied by PURAC Biomaterials. Electrospinning apparatus was as described in section 2.3.1 with the exception that all fibres were electrospun onto the flat collector plate described above. PURAC PLGA was dissolved in HFIP creating 10% and 12.5% (w/v) spinning solutions. 10% PLGA solutions were electrospun at 1, 2.5, 5 and 7.5ml/hr flow rates at 20kV with a 20cm working distance. 12.5% PLGA solutions were electrospun at 2.5 and 3ml/hr with applied voltages of 20 and 25kV and a 20cm working distance.

The fibre diameters of selected meshes were compared using two sample t-tests assuming equal variance. At least 100 fibres were measured from SEM images of electrospun meshes (one mesh created per set spinning parameters) using ImageTool 3.0. A one-way ANOVA was used to compare varying flow rate on average fibre diameter of meshes created from a 10% PURAC PLGA solution electrospun at 20kV and a 10cm working distance.

2.3.2 SEM Analysis

For SEM analysis, segments of scaffolds were mounted on an aluminium SEM stub via a carbon tab (Agar Scientific). Samples were gold sputter coated and analysed on either a Zeiss EVO60 VPSEM or a Topcon SM 300 SEM. Accelerating voltage was set at 5kV and working distance was approximately 10cm. Fibre diameter was measured using ImageTool 3.0.

2.3.3 TGA

PLGA pellets, microfibre meshes and spin coated films were analysed for any residual HFIP using a 2950 HiRes modulated TGA (TA Instruments). Temperature was ramped at 5°C min⁻¹ to 110°C under nitrogen. Data were analysed using Universal Analysis 2000. The total weight loss from microfibre meshes and spin coated films were compared using a two-sample t-test assuming equal variance (n=2).
2.3.4 FTIR

Attenuated total reflectance FTIR was conducted using a Nicolet 5700 FTIR. Measurements were recorded as % transmission and acquired in the 400-4000cm\(^{-1}\) range at a resolution of 4cm\(^{-1}\). Samples were analysed using a Smart Orbit diamond ATR accessory.

2.3.5 Sterilisation

To assess the effect of ethanol on microfibre meshes, sections of scaffold were soaked in 70% and 100% ethanol for 5, 10 and 15 minutes, one section of mesh per condition. Scaffolds were removed and allowed to air dry before immobilisation on aluminium stubs via adhesive carbon tabs. Scaffolds were visualised by SEM and fibre diameter measured using ImageTool 3.0. 100 fibres from each mesh were measured. Ethanol soaked/treated scaffolds were compared with untreated meshes using two sample t-tests assuming equal variance.

For UV sterilisation, sections of scaffolds were exposed to UV light for 5 and 10 minutes each side (one section of mesh per condition) and then analysed by SEM. The UV light was situated within a Class II tissue culture hood with the UV source approximately one meter away from the scaffolds. Scaffolds were visualised by SEM and fibre diameter measured using ImageTool 3.0. 100 fibres from each mesh were measured. UV treated scaffolds were compared with untreated meshes using two sample t-tests assuming equal variance.

2.3.6 Degradation

For incubation in media, 1.5 by 1.5cm sections of microfibre scaffolds and 2 x 2.5cm spin coated films were UV sterilised for 10 minutes each side as described above and immobilised in 12 well plate CellCrowns (Scaffdex) as described in Figure 10. CellCrowns were sterilised by soaking for 10 minutes in 70% ethanol and then dried on a sterile surface in the laminar flow of the tissue culture cabinet. Scaffolds were placed in 12 well plates and incubated with 1.5ml standard mouse ES media. Empty wells of the 12 well plates were also incubated with 1.5ml standard ES media and served as a negative control. Where possible, three replicates per time point, per condition were measured. Every three days media was replaced and the pH of removed media recorded.

Figure 10. Immobilisation of scaffolds in CellCrowns. Scaffolds were cut to size and anchored in tissue culture wells by folding over the base of the CellCrown insert. Scaffolds were secured in place with a CellCrown ring. Immobilised scaffolds were then placed firmly within the tissue culture well.
For SEM analysis, 1.5cm by 1.5cm sections of microfibre scaffolds were immobilised and incubated in standard ES media as described above. Every five days, scaffolds were removed and washed three times in PBS before drying at RT overnight. Two meshes were incubated per time point where possible. Scaffolds were then immobilised on an aluminium SEM stub, gold coated and analysed as described in 2.2.1. Fibre diameter was analysed using ImageTool 3.0, with at least 100 fibres per scaffold measured. Fibre diameters from each mesh were then compared with day 0 control scaffolds using two sample t-tests assuming equal variance.

For degradation in H$_2$O, samples were treated as described for incubation in media. However, samples were incubated in 1.5ml H$_2$O plus 0.02% sodium azide (Sigma). Surrounding H$_2$O was not changed throughout the incubation period with pH measured every three days.

### 2.3.7 Wettability

WCA was measured by depositing a 5µl droplet of H$_2$O onto the scaffold surface with a Krüss DSA 100B. Scaffolds were immobilized on glass slides using double sided tape. Images of the droplet on the scaffold surface were recorded over a 15 second period at one second intervals. WCAs were measured using Tangent method 1 (Figure 11).

![Figure 11. WCA measured using Tangent method 1. The droplet shape is fitted assuming an elliptical droplet shape (rather than a circular shape, for instance) and is fitted using a conic section method. The WCA is the angle between the baseline and the tangent at the three phase (surface, liquid and gas) contact point. WCA was determined by averaging left and right contact angles of the droplet.](image)

For analysis of WCA after exposure to mouse ES media, microfibre meshes and spin coated films were sterilised and mounted onto CellCrowns as described in section 2.3.6 and soaked overnight at 37°C in standard mouse ES media, washed twice with H$_2$O and allowed to dry. Once dry, scaffolds were immobilised and WCA measured as described above.

### 2.3.8 Mouse ES Cell Culture on Electrospun Meshes and Spin Coated Films

#### 2.3.8.1 Scaffold Preparation

Electrospun meshes and spin coated films were sterilised and mounted onto CellCrowns as described in section 2.3.6. Scaffolds were soaked (pre-conditioned) in standard ES media
containing 10 units/ml penicillin and 10 µg/ml streptomycin overnight at 37°C prior to cell seeding. Upon cell seeding, the existing media was removed and replaced with 1 ml (24 well plate) or 1.5 ml (12 well plate) fresh ES media. Cells were trypsinised as described above. Detached cells were pooled in 50 ml tubes, counted and centrifuged at 700 rpm for three minutes. Supernatant was discarded and cells re-suspended in an appropriate volume of standard ES media. Cells were then seeded in a 100-200 µl volume onto the centre of the scaffolds to reduce the chances of cells floating to the outskirts of the well and attaching to standard TCP. Plates were then gently agitated to disperse cells over the scaffold surface.

2.3.8.2 Short Term Culture

For short term culture (up to 7 days), Oct4-GFP and E-cadherin-/- ES cells were seeded as described above. Oct4-GFP ES cells were initially seeded onto microfibre and spin coated films at a high concentration of 1.5 x 10⁵ cells/cm² which was later lowered to 5 x 10⁴ cells/cm². E-cadherin-/- ES cells were seeded onto microfibre and macrofibre meshes at 1 x 10⁴ and 5 x 10⁴ cells/cm². Media was changed every other day except at later time points when media was changed daily. Cells were cultured mainly on scaffolds immobilised in 24 well plates, with the exception of the DNA assay where Oct4-GFP cells were cultured on 12 well plates.

2.3.8.3 Long Term Culture

For passaging of Oct4-GFP mouse ES cells, microfibre scaffolds and spin coated films were sterilised, immobilised in 12 well plates and pre-conditioned as described above. Gelatinised scaffolds were also included in the experimental setup. Scaffolds were gelatinised by incubation with 1 ml 0.1% gelatin overnight at 4°C. Excess gelatin was removed before pre-conditioning. Gelatinised wells served as positive controls. Where possible, two replicates per condition were prepared at each passage. Oct4-GFP cells were seeded onto scaffolds at 1 x 10⁴ cells/cm² as described above. Once cells reached approximately 70% confluence cells were passaged and reseeded onto pre-prepared scaffolds and gelatinised wells. Cells growing on gelatinised 12 well plates were detached as described in section 2.2.1. To detach cells on scaffolds, samples were transferred to 6 well plates, washed twice with PBS and incubated with 1 ml trypsin/EDTA for three minutes at 37°C. Cells were then detached in the trypsin to create single cell suspensions from aggregates present on the scaffold surface. Cell suspensions were then transferred to sterile 15 ml tubes containing 2 ml mouse ES media. Cells were counted and then centrifuged at 700 rpm for three minutes. Supernatant was removed and cell pellets re-suspended in an appropriate volume of standard mouse ES media and reseeded onto pre-prepared scaffolds. Excess cells were centrifuged at 700 rpm for three minutes and re-suspended in 300 µl 1% formaldehyde (Sigma) for flow cytometry (see section 2.3.10).
At each passage, detached cells were counted enabling the population doubling time during the culture period to be calculated using the following equation:

\[
\text{doubling time} = \frac{z}{q}
\]

\[
z = \text{number of hours in culture}
\]

\[
q = \frac{\log x - \log y}{0.301}
\]

\[
x = \text{the cell number at a specific passage day}
\]

\[
y = \text{the original seeding density}
\]

Average doubling times on all surfaces were calculated and compared using a one way ANOVA (n=6).

### 2.3.9 DNA Assay

DNA assays were conducted on 12 well plates. Oct4-GFP ES cells were seeded at 5 x 10^4 cells/cm^2 and cultured on microfibre, spin coated films and gelatinised TCP as described in section 2.3.8, with three replicates per condition, per time point. At days one, three and seven culture medium was removed and samples washed twice in PBS before 750µl of H_2O was added to each well. Samples were freeze-thawed three times using a -80°C freezer. 3 x 50µl of each sample was aliquoted to separate wells in a clear flat bottomed 96 well plate (Greiner Bio-One). 50µl of TNE buffer (10mM Tris Base, 2M NaCl, 1mM EDTA, pH 7.4) was then added to each well followed by 100µl Hoescht staining solution (Sigma, 20µg/ml) diluted in TNE buffer. Samples were fluorescence excited at 355nm and emission was read at 460nm using a fluorescent plate reader (FLUOStar Optima BMG, Labtech). Controls consisted of blank wells incubated with media. Data was analysed using a one-way ANOVA at each time point (n=3).

Cell number was calculated by comparing emission at 460nm with a pre-prepared standard curve. The standard curve was prepared by seeding a gradient of cell concentrations from 8,000 – 2,000,000 onto gelatinised 12 well plates. Cells were allowed to attach for two hours before wells were treated as described above. Results were calibrated against blank wells and emission was plotted against cell number (Figure 12).
Figure 12. Hoescht DNA standard curve. Emission was read at 460nm. Readings from three separate wells (n=3) for each cell number were averaged and calibrated against a no cell control. For the line of best fit, the intercept was set at 0. The linear equation was rearranged to produce: 
\[ x = \frac{y}{0.0007} \]
with x= cell number and y=emission.

2.3.10 Flow Cytometry

Oct4-GFP ES cells cultured on the surface of microfibre, spin coated films and gelatinised TCP were detached as described in section 2.3.8.3. Cells were centrifuged at 700rpm for three minutes and re-suspended in 300µl 1% formaldehyde. Fluorescence of Oct4-GFP cells was analysed using a Becton Dickinson FACScalibur. Non-fluorescent E-cadherin\(^-\) ES cells served as a negative control. Forward and side scatter were used to gate viable cells, with fluorescence measured from this population.

2.3.11 SEM Cell Analysis

For standard SEM analysis, scaffolds immobilised in CellCrowns were washed twice with PBS and fixed with 1.5% Gluteraldehyde (TAAB) in 0.1M phosphate buffer, pH 7.3 for 30 minutes at 4°C. Samples were then washed twice with 0.1M phosphate buffer. If it was necessary, samples were stored for 1-3 days in 0.1M phosphate buffer, 0.1M sucrose at 4°C. Before dehydration, samples were washed three times with 0.1M phosphate buffer to ensure complete removal of residual sucrose. Samples were dehydrated with increasing concentrations of ethanol:

- 2 x 3 minutes 50% ethanol
- 2 x 3 minutes 70% ethanol
- 2 x 3 minutes 90% ethanol
- 2 x 5 minutes 100% ethanol

Samples were then treated twice with hexamethyldisilazane (HMDS, Sigma) for 5 minutes and allowed to dry. To obtain a cross-section view of E-cadherin\(^+\) ES cells on macrofibre and microfibre scaffolds, dehydrated samples were cut down the centre into four sections whilst still taut on the CellCrown. All samples were immobilised on aluminium SEM stubs using an adhesive carbon tab.
Samples were then gold splutter coated and visualised using either a Topcon SM 300 SEM or a Zeiss EVO60 VPSEM. Accelerating voltage was set at 5kV and working distance was approximately 10cm. To visualise the cross section of macrofire and microfibre scaffolds cultured with E-cadherin<sup>E</sup>-/- ES cells, the stage within the SEM chamber was tilted 55-75°.

For analysis of hydrated samples, scaffolds were fixed with 1.5% gluteraldehyde as described above. However, samples were not dehydrated and were kept wet in 0.1M phosphate buffer prior to analysis. Samples were analysed using a Zeiss EVO60 VPSEM with a 5kV accelerating voltage and a 10 cm working distance. Pressure within the chamber was altered from 1.3 x 10<sup>-4</sup> mbar for standard SEM to 0.55 mbar and the standard 30 µm aperture was exchanged for a 100µm aperture.

### 2.3.12 Immunocytochemistry

For antibody and actin staining, samples were washed twice with PBS and fixed with 4% paraformaldehyde (Sigma) for 10 minutes at 4°C. Samples were then washed twice with PBS and blocked for 30 minutes at RT in immunocytochemistry (ICC) buffer consisting of 1mg/ml BSA, 0.1% triton and 1% goat serum (all purchased from Sigma) in PBS.

For actin staining of Oct4-GFP cells cultured for seven days on microfibre scaffolds and spin coated films, blocked samples were treated with rhodamine phalloidin (Molecular Probes) in ICC buffer (1µl/ml). Samples were then washed twice with PBS. Scaffolds were removed from the CellCrown holders and mounted using ProLong Gold antifade reagent with DAPI (Molecular Probes). Cover slips were purchased from VWR international and glass slides were supplied by Thermo Scientific. Samples were visualised using a Nikon upright laser confocal microscope with parameters set to detect GFP and the rhodamine phalloidin stained actin cytoskeleton. Unfortunately, the DAPI did not stain properly and the nucleus could not be visualised.

For Nanog staining of Oct4-GFP cells passaged on microfibre scaffolds and spin coated films, blocked samples were incubated for one hour with rabbit anti-Nanog (mouse) polyclonal antibody (Chemicon International) at a 1:500 dilution in ICC buffer. Samples were then washed five times with PBS before incubation with secondary antibody AlexaFluor-488 goat anti-rabbit IgG (Molecular Probes) for one hour in the dark at RT at a 1:1000 dilution in ICC buffer. Samples were washed five times with PBS and incubated with DAPI solution (Molecular Probes, 3µl/ml) in PBS for five minutes in the dark. Scaffolds were washed five times with PBS and mounted with coverslips on microscope slides using ProLong Gold Antifade reagent (Molecular Probes). Cells within 6 well TCP plates were mounted directly within the well using SlowFade Gold Anifade reagent (Molecular Probes). Clear nail varnish was used to seal the coverslip.

Samples were analysed on a Nikon Eclipse 50i fluorescent microscope. As a four second exposure is necessary to detect GFP within Oct4-GFP cells, exposure was kept below 750ms when visualising Nanog expression to ensure no GFP was detected.
2.4 ppAm Microfibre Scaffolds

2.4.1 ppAm Microfibre Meshes

Coating parameters were as described in Mahoney et al. (2004). Briefly, microfibre meshes produced as described in section 2.3.1 were placed in the reactor chamber. The stainless steel plasma chamber was 50cm in diameter and 60cm long with an internal electrode. Using a rotary pump and a liquid N\textsubscript{2} cold trap, pressure within the chamber was evacuated to 1 x 10\textsuperscript{-3} mbar. Allyl amine vapour was flowed through the reactor at 4cm\textsuperscript{3} min\textsuperscript{-1} until pressure within the chamber reached 2 x 10\textsuperscript{-2} mbar. Plasma was excited by a 13.56 MHz radiofrequency with a continuous wave power of 5W for 40 minutes. Both sides of the microfibre meshes were treated in this manner.

2.4.2 XPS Analysis

Both ppAm microfibre and uncoated microfibre scaffolds were analysed with a Kratos Axis Ultra using monochromatic Al K\alpha radiation with an X-ray power of 15kV, 10mA. High-resolution analysis of C 1s, O 1s and N 1s were obtained with a pass energy of 20eV. Elemental composition of the scaffolds was determined using wide scan analysis with a pass energy of 80eV. Sample charging was neutralised using a flood of low energy electrons.

Samples were initially immobilised on metal bars using double sided tape, however the samples were contaminated with silicon from the releasing agent polydimethylsiloxane (PDMS). Also, due to the porous nature of the mesh it was difficult to isolate the signal from the meshes from that of the iron bar. These issues were overcome by immobilising the samples across a gap in the bar using PDMS free tape.

Data was analysed using CasaXPS version 2.3.15dev57. In widescan analysis, elements were identified and quantified using an elemental library and relative sensitivity factors specific to the Kratos Axis Ultra used. All scans were fitted with a shirley background and charge corrected to the C-H environment at 285.0 eV. High-resolution scans were fitted with Gaussian/Lorentzian (G/L) line shapes. For high-resolution analysis, the FWHM was restricted to a maximum of 1.2 for core C 1s peaks and 1.5 for O 1s spectra. Corresponding chemical environments in C 1s and O 1s spectra were linked to facilitate accurate peak fitting. Peak fitting was guided by previous analysis on allyl amine coatings (Barry et al., 2005; Dehili et al., 2006; Shard et al., 2004; Whittle et al., 2000).

2.4.3 RP-HPLC Analysis

To determine the HS loading capacity of the ppAm microfibre meshes, 1cm\textsuperscript{2} sections of ppAm microfibre scaffold were immobilised in 24 well plates using CellCrowns (Scaffdex). Scaffolds were incubated overnight with varying 0.1-5µg of HS (from pig mucosa, Iduron) in 500µl PBS, at RT in the dark. The HS used throughout the study was a heterogenous population of high and low sulphated HS with molecular weight ranging between 9,000 – 35,000 daltons.

To assess the effect of UV treatment, 1cm\textsuperscript{2} sections of ppAm microfibre scaffold were sterilised with UV light (see section 2.3.5) for 10 minutes each side and then immobilised in 24 well plates. Scaffolds were then incubated overnight with 1µg and 5µg HS as described above alongside untreated controls.
To assess HS attachment after scaffold soaking, 1cm² sections of ppAm microfibre scaffold were immobilised in 24 well plates and soaked overnight in 1ml PBS in the dark at RT. Scaffolds were then washed three times with PBS, mounted in fresh CellCrowns and transferred to fresh wells before incubation with HS.

After HS immobilisation, samples were washed three times in PBS before incubation with 4M NaCl for 30 minutes to detach the bound HS. The detached HS was then desalted through a PD-10 column (Amersham). In summary, the 1ml detached HS was applied to a PD-10 column followed by successive application of 7 x 1ml H₂O. The elutant was collected in 1 ml fractions, with fractions 4 and 5 containing the immobilised HS. Fractions 4 and 5 were pooled and frozen at -80°C before freeze drying. Dried samples were then resuspended in 100µl heparinise buffer consisting of 50mM sodium acetate, 5mM calcium acetate, pH 7. 2.5 mIU of heparinases I, II and III (all heparinises purchased from Iduron) were added to the preparations and left to digest for at least three hours at RT. An additional 2.5 mIU of heparinases I, II and III were then added and samples were left to digest at RT overnight. Digested samples were then frozen at -80°C before freeze drying.

Digested samples were resuspended in 0.1M AMAC (2-aminacridone) in 85% DMSO/15% acetic acid (Sigma). Samples were incubated at RT for 10 minutes before the addition of 10µl 1M sodium cyanoborohydride (Sigma). Samples were then left overnight at RT. If not used immediately after preparation, samples were stored -20°C.

2-10µl of the labelled samples were diluted in H₂O to a total volume of 20µl before being applied to a Zorbax Eclipse XDB-D18 RP-HPLC column (2.1mm x 150mm, 3.5µm; Agilent Technologies). The column was equilibrated in 100% 0.1M ammonium acetate. Once the sample had been applied, the column was exposed to increasing concentration gradient of methanol. A sharp 2 minute gradient of increasing methanol (0-10%) was followed by a shallow 50 minute gradient of 10-30% methanol. Flow rate was set at 0.250ml/min and fluorescence (excitation 425nm, emission 520nm) of eluting disaccharides recorded (Deakin and Lyon, 2008). Once the run was completed, the column was restored by washing with 100% methanol. The RP-HPLC column was run on an Agilent 110 Series RP-HPLC system and results recorded and analysed on Agilent Chemstation software. As efficiency of AMAC labelling varies according to specific disaccharides the raw peaks were multiplied by pre-determined correction factors (Deakin and Lyon, 2008).

Statistical analysis of the composition of HS bound to unsoaked ppAm microfibre scaffolds at 1-5µg HS was conducted using two sample t-tests assuming equal variance, comparing HS applied at each concentration with pre-binding HS. Two meshes were analysed for HS applied at 1 and 2µg (n=2). Three replicates were analysed for 5µg and pre-binding HS (n=3).

2.4.4 ³H HS Analysis

Due to the need for specific training in the appropriate and safe handling of radioactive material, the preparation of and experiments involving ³H HS were conducted by Dr Rebecca Holley.

Metabolically labelled ³H HS was produced from immortalised MEFs (Lamanna et al., 2006). At approximately 70% confluence, MEFs were incubated with ³H-glucosamine for 48 hours. Media was then removed and cells were washed twice with PBS. Cells were incubated with 1% triton in PBS for two hours at RT whilst agitated on a table top rocker. 1% triton solutions were removed,
pooled and digested with 100µg/ml pronase (Roche) for four hours at 37°C. Samples were then loaded onto a 1ml DEAE column (Sigma), pre-equilibrated with sequential application of 10ml PBS, 5ml 1.5M NaCl and 20mM sodium phosphate (pH 7) followed by 10ml PBS. Samples were eluted from the DEAE with a gradient running from 0.15M to 0.75M NaCl. Eluted fractions containing HS/DS/CS were pooled and desalted on a PD10 column as described in section 2.3.1. Samples were freeze dried and digested with 5 mIU chondroitinase ABC (Iduron) in 50µl of 50mM Tris-acetate (pH 7.5). Samples were then applied to a Sephadex CL6B size exclusion column (Pharmacia Biotech), purifying the intact HS chains.

For incubation analysis in N2B27 and PBS, 1cm² sections of ppAm microfibre and uncoated microfibre scaffolds were UV sterilised for 10 minutes either side and placed in sterile 1.5ml eppendorf tubes. Scaffolds were incubated overnight with 10,000cpm ³H HS in 1ml PBS at RT in the dark. The ³H HS/PBS solution (unbound ³H HS) was then removed and added to a scintillation tube (PerkinElmer) for analysis. Scaffolds were washed twice in 1 ml PBS with each wash added to separate scintillation tubes for analysis. Day 0 scaffolds were added to scintillation tubes for analysis. Remaining scaffolds were incubated in either 1ml PBS or 1ml N2B27 media (N2B27 was prepared as described in section2.4.8) in the dark at 37°C. Both N2B27 and PBS were supplemented with 5units/ml penicillin and 5µg/ml streptomycin. During the incubation period, media/PBS was changed every two days followed by one wash with corresponding incubation medium (either 1ml PBS or N2B27). Removed media/PBS and washes were added to separate scintillation tubes for analysis. All samples were analysed using a Wallac 1409 Liquid Scintillation Counter, with 2ml Scintillation fluid (Optiphase ‘HiSafe’ 3, PerkinElmer) added to each sample. Due to the rarity of both ppAm scaffolds and ³H HS, only one replicate per sample point was prepared.

To assess the stability of the ppAm coating, a 1cm² section of microfibre mesh was soaked overnight in a scintillation tube with 1ml PBS. The scaffold was then washed once with 1ml PBS before being transferred to a fresh scintillation tube. 7,000cpm of ³H HS was added to both the evacuated tube and to the moved scaffold and incubated overnight at RT in the dark. The unbound ³H HS was removed from both the scaffold and the evacuated tube, both were washed three times with 1ml PBS. PBS from each wash was transferred to fresh scintillation tubes for analysis. Before analysis, the scaffold was moved to a third scintillation tube. The evacuated tube, the tube in which the scaffold was incubated with ³H HS and the scaffold itself were all analysed for the presence of ³H HS. An unsoaked scaffold was treated alongside and after incubation in ³H HS was washed three times with 1ml PBS and transferred to a fresh scintillation tube. Both the tube in which the scaffold was incubated with ³H HS and the scaffold itself were analysed for the presence of ³H HS as described above.
2.4.5 ScFv Binding

All phage display ScFv heparin/HS binding antibodies were kindly provided by Dr Toin van Kuppevelt (Nijmegen Centre for Molecular Life Sciences).

2.4.5.1 HS4C3 and NS4F5

For initial binding of NS4F5 and HS4C3, 0.75cm by 0.75cm sections of ppAm microfibre scaffolds were placed in the wells of a v-form 96 well microplate (Greiner Bio-One). Epranex plates (BD heparin binding plate, BD Bioscience) wells were incubated with 200µl PBS containing 0-5000ng heparin (porcine origin, Iduron, average molecular weight 7,400 daltons) overnight at RT, in the dark. Due to volume constraints, empty 96 plate wells and scaffolds were soaked overnight with 100µl PBS containing 0 – 5µg heparin. After overnight incubation, samples were washed three times with 150µl (96 well plates, scaffolds) and 200µl (Epranex plates) pH 6 standard assay buffer (SAB6). SAB6 was comprised of 50mM sodium acetate, 100mM NaCl, 0.2% Tween20 (all Sigma), pH 6. Scaffolds were then incubated for 90 minutes with 150-200µl 1% BSA in SAB6 at 37°C. Samples were then washed twice with 150-200µl SAB6 and incubated with 100µl ScFv antibody (HS4C3 or NS4F5) at a 1:10 dilution in SAB6 for 1 hour at RT in the dark. Samples were washed twice with SAB6 and incubated with 100µl biotinylated secondary antibody raised against the VSV-G tag of the ScFv (rabbit polyclonal antibody, 1:1000 dilution in SAB6, Abcam) for 90 minutes at RT in the dark. Samples were then washed twice with 150-200µl SAB6 before incubation with ExtrAvidin Alkaline Phosphatase (1:10,000 dilution in SAB6, Sigma) for 30 minutes at RT in the dark. Samples were then washed twice with 150-200µl SAB6 before addition of 200µl 1mg/ml p-nitrophenylphosphatase in 0.2M Tris buffer, 5mM magnesium chloride (Sigma). Cleavage of p-nitrophenylphosphate to p-nitrophenyl by the immobilised alkaline phosphatase led to colorimetric change that was read as absorbance at 405nm using a Multiskan Ascent plate reader (Lab systems). Epranex plates were read directly by the plate reader. As scaffolds would obscure the reading, 85µl of developing sample was transferred to empty wells for analysis. Absorbance was recorded after 10 minutes development.

To assess the ability of increasing BSA concentrations to reduce non specific binding, 0.75cm by 0.75cm sections of ppAm microfibre scaffolds were placed in the wells of a v-form 96 well microplate and incubated with 100µl PBS overnight at RT in the dark. Samples were washed twice with 150µl SAB6 before incubation with 1, 5, 10 and 15% BSA in SAB6 for 90 minutes at 37°C in the dark. Scaffolds were then treated as described above.

For NS4F5 binding at 5% BSA block concentration, 0.75cm by 0.75cm sections of ppAm microfibre scaffolds were placed in a v-form 96 well microplate and incubated with 0-5000ng heparin in PBS overnight at RT in the dark. Scaffolds were washed twice with 150µl SAB6 before incubation with 150µl 5% BSA in SAB6 at 37°C, for 90 minutes in the dark. Scaffolds were then treated as described above with NS4F5 serving as primary ScFv antibody (1:10 dilution in SAB6). As the stability of the ppAm coating had come into question, scaffolds were moved to fresh wells before the addition of 200µl 1mg/ml p-nitrophenylphosphate. 200µl 1mg/ml p-nitrophenylphosphate phosphate was also added to the vacated wells. 85µl aliquots of the developing samples were moved to fresh wells before absorbance was read at 405nm after 10 minutes development.
E14 and Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> ES cells were cultured for at least four passages in standard ES media (see section 2.2.1) supplemented with 10% KSR instead of 10% FCS to ensure extracted HS originated from the cells rather than supplemented serum. Cells were cultured in gelatinised T175 flasks. When cells reached approximately 70% confluence, media was removed and cells were washed twice with 15ml PBS (PAA). 10ml 1% Triton (Sigma) in PBS was added to each flask and cells were incubated at RT for one hour, gently agitated on a table top rocker. The triton solutions were then removed and pooled. In the case of E14 ES cells, removed media was pooled with the cell extracts. Samples were then treated with 100µg/ml pronase for four hours at 37°C to cleave core proteins and release the HS chains. Samples were then loaded onto separate 1ml DEAE columns (Sigma) pre-equilibrated with 10ml PBS followed by 5ml 1.5M NaCl, 20mM sodium phosphate, pH 7 and a further 10ml PBS. Once the sample was added, the column was washed with 50ml 0.25M NaCl, 20mM sodium phosphate, pH 7 to remove loosely bound material such as HA. Sulphated GAGs were eluted and collected with 5 x 1ml applications of 1.5M NaCl, 20M sodium phosphate, pH 7. Collected samples were then desalted on a PD-10 column, freeze dried and re-suspended in 100µl (Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> HS) or 500µl (E14 HS) H<sub>2</sub>O and stored at -20°C. A portion of the samples were then analysed for RP-HPLC analysis as described in section 2.4.3. To estimate the quantity of HS present, total fluorescence was compared to 5µg Celsus HS (Iduron) which was simultaneously desalted, digested and AMAC labelled. Once quantities had been established, HS stock solutions were adjusted to a final concentration of 0.1mg/ml.

For RB4EA12 binding, 0.75cm by 0.75cm sections of ppAm microfibre scaffold were placed in the wells of a v-form 96 well microplate and pre-soaked in 100µl PBS overnight at RT in the dark. Scaffolds were then washed twice with 150µl PBS before being transferred to fresh wells for HS incubation. Scaffolds were incubated with 100µl 0-200ng E14 or Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> HS in PBS overnight at RT in the dark. Epranex plates were also incubated with both HS preparations at 0-200ng in 200µl PBS. Samples were washed twice with 150-200µl SAB6 before blocked with 150-200µl 5% BSA in SAB6 at 37°C for 90 minutes in the dark. Samples were then washed twice with 150-200µl SAB6 and incubated with 100µl RB4EA12 ScFv antibody (1:100 dilution in SAB6) for one hour at RT in the dark. Samples were then treated as described above, with scaffolds transferred to fresh wells before the addition of 200µl p-nitrophenylphosphate.

For EV3C3 binding, Epranex plates were incubated with 0-200ng E14 or Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> HS in PBS overnight at RT in the dark. Wells were treated as described above with EV3C3 as the primary ScFv antibody at a 1:100 dilution in SAB6.

### 2.4.6 bA-Link_TSG-6 Binding

0.75cm x 0.75cm pieces of microfibre and ppAm microfibre scaffolds were placed in the wells of a non tissue culture treated, v-form 96 well microplate. For pre-soaked ppAm microfibre scaffolds, sections were incubated overnight at RT in the dark with 150µl PBS and then transferred to fresh wells for incubation with heparin. Epranex plate wells were incubated with 200µl PBS containing 0-5000ng heparin (Iduron, average molecular weight 7,400) overnight at RT, in the dark. Due to volume restraints, empty 96 plate wells and scaffolds were soaked overnight with 100µl PBS containing 0 – 5µg heparin. After overnight incubation, samples were washed three times with
150μl (96 well plates, scaffolds) or 200μl (Epranex plates) SAB6. Samples were then incubated for 90 minutes with 150-200μl 1% BSA in SAB6 at 37°C for 90 minutes. After blocking with BSA, samples were washed twice with 150-200μl SAB6 and incubated with biotinylated Link_TSG-6 (bA-Link_TSG6, 2pmol/well) in SAB6 for three hours at RT, in the dark. bA-Link_TSG-6 was kindly supplied by Professor Anthony Day, The University of Manchester. Samples were washed twice with 150-200μl SAB6 before incubation with 200μl ExtrAvidin Alkaline Phosphatase (1:10,000 dilution in SAB6) for 30 minutes at RT in the dark. Samples were then washed twice with 150-200μl SAB6 before addition of 200μl 1mg/ml p-nitrophenolphosphate in 0.05M Tris-HCL, 0.1M NaCl. All scaffolds were moved to fresh wells before the addition of p-nitrophenolphosphate. Absorbance was measured as described above. As the scaffolds would obscure the reading, 85μl of developing sample was transferred to empty wells. As the absorbance was being directly compared with Epranex plates, absorbance values were multiplied by 2.35 to compensate for the reduction in volume.

2.4.7 LIF Withdrawal – PCR Analysis

2.4.7.1 Experimental Setup

E14 ES cells were seeded at 1 x 10^4 cells/cm² onto ppAm microfibre scaffolds +/- heparin, uncoated microfibre meshes, spin coated films, ppAm TCP +/- heparin, and standard gelatinised TCP. Electrospun meshes and spin coated films were sterilised and mounted onto CellCrowns as described in section 2.3.6.

For heparin immobilisation, 1.5cm by 1.5cm ppAm microfibre scaffolds were UV treated for 10 minutes each side, immobilised in sterile 12 well CellCrowns and incubated overnight in the dark with 0.5μg/cm² heparin in 1ml PBS. ppAm microfibre scaffolds without heparin were incubated in just PBS. After incubation, ppAm microfibre meshes +/- heparin were washed three times with PBS and all scaffolds were pre-conditioned with standard ES media –LIF. ppAm TCP consisted of 24 well plates coated with ppAm in the same manner as Epranex plates and ppAm microfibre scaffolds. As with ppAm microfibre scaffolds, wells were either incubated in 0.5μg/cm² heparin in 500μl PBS (+heparin) or just with 500μl PBS (-heparin) overnight, at RT, in the dark. Wells were then washed twice with PBS and gelatinised with 0.1% gelatin overnight at 4°C. Excess gelatin was removed before cells were seeded.

Cells were seeded and cultured in standard ES media minus LIF. Media was changed every other day except near the end of the 10 day culture period when media was replaced daily. Cells cultured on gelatinised TCP and ppAm TCP +/- heparin were detached as described in 2.2.1. To detach cells from scaffolds, all scaffolds were transferred to sterile 6 well plates, washed twice with PBS and incubated for 3 minutes at 37°C with 1ml trypsin/EDTA. Trypsin was neutralised with 1ml standard ES media -LIF and the cell suspension transferred to a sterile 15ml tube. Cells were pelleted at 700rpm for 3 minutes and resuspended in 1ml standard ES media minus LIF and then transferred to sterile DNase/RNase free eppendorfs (Greiner Bio-One). Cells were then centrifuged for 1000rpm for 5 minutes and cell pellets stored at -80°C.
2.4.7.2 RNA Extraction

RNA extraction, cDNA synthesis and PCR were all conducted using RNase/DNase free filter tips (Anachem) and RNase/DNase free 1.5ml and 0.2ml (StarLab) tubes. Centrifuge steps were conducted using a table top microcentrifuge (Sigma). All PCR and cDNA synthesises were conducted using a Bio-Rad DNA Engine Dyad Peltier thermo cycler.

RNA was extracted using an RNeasy RNA Mini Kit for total RNA purification from animal cells, animal tissues, bacteria and yeast (Qiagen). Cell pellets were lysed with 350-600µl Buffer RLT containing 10µl/ml β-mercaptoethanol (Sigma), applied to a QIAShredder column (Qiagen) and spun at 13,000 rpm for 2 minutes. An equal volume of 70% ethanol prepared from RNase/DNase free H2O (Sigma) and ethanol (Fluka) was thoroughly mixed with the elutant before being applied to an RNeasy spin column and centrifuged at 10,000 rpm for 30 seconds. Flow through was discarded and the column washed with 350µl Buffer RW1, spun at 10,000rpm for 30 seconds. Flow through was discarded and the column treated with DNase I (Qiagen). Stock solution of DNase I was prepared by dissolving lyophilized DNase I in 550µl RNase free water, giving a final concentration of 2.7 Kunitz units/µl. 10µl of DNase I stock solution was then diluted in 70µl Buffer RDD and applied to the RNeasy spin column membrane and incubated for 15 minutes at RT. The DNase I solution was then removed with 350µl Buffer RW1, spun at 10,000 rpm for 30 seconds.

500µl Buffer RPE was added to the column, which was then spun at 10,000 rpm for 30 seconds. Before application, the supplied concentrated solution of Buffer RPE was diluted with four volumes of RNase/DNase free ethanol. Flow through was discarded and a second dose of 500µl Buffer RPE was applied to the column, which was centrifuged at 10,000rpm for 2 minutes. Columns were then transferred to fresh 2ml collection tubes and spun for a further 2 minutes at 13,000 rpm to removed any residual Buffer RPE.

To elute RNA, columns were transferred to RNase/DNase free 1.5ml eppendorfs and 30µl RNase/DNase free dH2O was applied to the spin column membrane. Columns were then centrifuged for 1 minute at 10,000 rpm. To maximise RNA collection, the eluted solution was reapplied to the column and spun as described above. Eluted RNA was quantified and stored at -80°C.

2.4.7.3 cDNA Synthesis

cDNA was synthesised in a total reaction volume of 50µl. All reagents were supplied by Promega unless specified otherwise. For each sample, 2µg RNA in 20µl dH2O was heated to 65°C for 3 minutes and then transferred to ice. To each solution, a pre-prepared mixture of the following reagents was added –

10µl 5X RT Buffer
11.5µl RNase/DNase free dH2O
5µl dNTP (2.5mM Stock solution)
2.5µl Oligo dT

Total Volume: 29µl
After addition to the RNA preparation, 1µl AMV RT was added and mixed thoroughly with each sample, bringing the total reaction volume to 50µl.

Samples were then incubated at 42°C for 1 hour followed by treatment at 98°C for 5 minutes to terminate AMV RT activity. Samples were stored at -20°C.

2.4.7.4 PCR

All primers were provided by VH Bio and are detailed in Table 1. Primers were pre-established sequences used within the lab. Lyophilised primers were reconstituted in RNase/DNase free dH₂O to a 1µg/µl stock concentration.

Primers were diluted to a working concentration containing both forward and reverse primers each at 100ng/ml in RNase/DNase free dH₂O. All solutions were stored at -20°C. Reaction solutions consisted of:

- 7.5µl 2x Biomix Red (Bioline)
- 1µl Primer working solutions (100ng/ml of forward and reverse primers)
- 1µl cDNA
- 5.5µl RNase/DNase free dH₂O.

Total volume: 15µl

If the band intensity of the house keeper gene GAPDH was not equal across samples, suggesting the total cDNA in each reaction varied despite the concentration of RNA being maintained at 2µg, the volume of cDNA added above was manually adjusted until bands were of relative intensity. The volume of dH₂O was consequently altered to maintain the total reaction volume at 15µl.

The PCR cycle applied to each sample was as follows:

\[
\begin{align*}
95°C & - 5 \text{ minutes} \\
95°C & - 1 \text{ minute} \\
60°C & - 1 \text{ minute} \\
72°C & - 1 \text{ minute} \\
72°C & - 10 \text{ minutes}
\end{align*}
\]

Cycle repeated \( n \) amount of times.

The total number of cycles for each primer set is detailed in Table 1.

1% agarose gels were prepared by diluting agarose (Fisher Scientific) in 1 X TAE Buffer by microwave heating until solutions were clear. TAE buffer consisted of 1mM EDTA tetrasodium salt, 40mM Tris base and 1.14ml/litre acetic acid, pH 8 supplemented with 0.05µg/ml ethidium bromide (Fisher Scientific). Agarose solutions were poured into a gel mould and allowed to set before samples were loaded. Samples were run on the set gel at 100 volts for 45 minutes and visualised using a UV doc.
Table 1. Primer sets and cycle number. Although cycle number varied between each primer set, the annealing temperature was always set at 60°C.

2.4.8 Neural Differentiation on Uncoated and ppAm Microfibre Meshes +/- HS

1.5cm by 1.5cm sections of uncoated and ppAm microfibre scaffolds were UV sterilised for 10 minutes each side and mounted in 24 well plates by CellCrowns sterilised as described in section 2.3.6. For HS immobilisation, samples were incubated overnight in the dark at RT with 2.25µg (1µg/cm²) Celsus HS in 500µl PBS. Scaffolds without HS were incubated with 500µl PBS alone. Scaffolds were then washed three times with PBS before pre-conditioning with 1ml mouse ES media overnight at 37°C. EXT1⁻/⁻ ES cells were cultured and detached from gelatinised TCP as described in section 2.2.1. Detached cells were collected in sterile 15ml tubes, counted and spun at 700rpm for three minutes. Supernatant was removed and the cell pellet resuspended in an appropriate volume of ES media. Pre-conditioning media surrounding the scaffolds was removed.
and replaced with fresh ES media. Cells were seeded into the centre of the CellCrown holder to ensure the maximum amount of cells settled on the scaffold surface at $1 \times 10^4$ cells/cm$^2$ in a 100µl volume. Plates were gently agitated to encourage cell dispersion across the scaffold surface before incubation at 37°C, 5% CO$_2$. After 24 hours, media was removed and all samples were washed three times with 500µl PBS. Samples were then incubated with 1ml N2B27 media composed of a 1:1 ratio of Neurobasal media and DMEM F12 supplemented with 50µg/ml BSA (fraction V), 500µM L-glutamine (PAA), 5ml N2 and 10ml B27 supplements (all supplied by Gibco).

The first day of culture in N2B27 media was considered as day 0 of neural differentiation. Samples were incubated at 37°C, 5% CO$_2$ with media replaced every other day until the end of the 8 day neural differentiation period. At selected time points, samples were fixed and prepared for immunocytochemistry as described in section 2.4.12.

### 2.4.9 Neural Differentiation on ppAm Microfibre Meshe$\pm$/HS$\pm$/FGF4

For unsoaked samples, ppAm microfibre scaffolds were prepared and EXT1$^{-/-}$ ES cells were seeded as described in section 2.4.8. However, standard ES media was replaced with N2B27 with or without 2ng/ml FGF4 (BioSource) creating four conditions; -HS-FGF4, -HS+FGF4, +HS-FGF4 and +HS+FGF4. FGF4 was added to N2B27 immediately prior to culture and media was changed every other day during the 8 day neural differentiation procedure.

To confirm that pre-soaked scaffolds retained the capacity to direct neural differentiation, 1.5cm by 1.5cm sections of ppAm microfibre scaffold were UV sterilised as described previously and then soaked in PBS overnight at RT. Eight pieces of scaffold were soaked in 15ml PBS in a 10cm non tissue culture treated petri dish (Greiner Bio-One). After soaking, scaffolds were transferred to fresh petri dishes containing 20ml PBS where the aqueous solution prevented crumpling of the mesh and enabled immobilisation in sterile 24 well plate CellCrowns. Scaffolds were then treated as described in section 2.4.8 with the exception that scaffolds were transferred to fresh wells within the 24 well plates after HS immobilisation. Both EXT1$^{-/-}$ and WT1 ES cells (wild type ES cells derived from a SV129 mouse strain) were seeded as described in section 2.4.8 and cultured with and without HS and FGF4 as described above.

### 2.4.10 Human ES Cell Culture on ppAm microfibre meshes $\pm$/HS

1.5cm by 1.5cm ppAm microfibre scaffolds were sterilised and incubated with HS as described in section 2.4.8. However, scaffolds were pre-conditioned overnight at 37°C with standard human ES media minus FGF2, rather than mouse ES media.

HUES7 ES cells were cultured and detached as described in section 2.2.2. Cells detached from two 70% confluent 6 well plates were collected in two separate sterile 15ml tubes, counted and spun at 700rpm for three minutes. One pellet was resuspended in 400µl human ES cell media supplemented with 100ng/ml FGF2 whilst the second pellet was resuspended in 400µl MEF conditioned media supplemented with 10ng/ml FGF. The resuspended cells were then split between four scaffolds, two with HS and two without immobilised HS and cultured in either 1ml of human ES cell media supplemented with 100ng/ml FGF2 or MEF conditioned media supplemented with 10ng/ml FGF. Media was changed every day except for the first day to enable cells to settle
and fully attach to the scaffold surface. Scaffolds were sampled for immunocytochemistry at days three and five of the culture period as described in section 2.4.12.

2.4.11 SEM Analysis

After eight days of culture in N2B27, EXT1−/− ES cells cultured with HS immobilised on unsoaked ppAm microfibre scaffolds were washed twice with 1 ml PBS, transferred to fresh 12 well plates (Nunc) and fixed in 1.5ml 1.5% gluteraldehyde. Samples were then washed twice with 2ml 0.1M phosphate buffer, dehydrated and prepared for SEM analysis on a Zeiss EVO60 VPSEM as described in section 2.3.11.

2.4.12 Immunocytochemistry

For βIII tubulin and Map2 staining of neural differentiation samples, scaffolds were removed from the 24 well culture plates and transferred to fresh 24 well plates. Scaffolds were washed twice with 1ml PBS and then fixed with 1ml 4% paraformaldehyde (Sigma) in PBS for 10 minutes at 4°C. Samples were washed twice with PBS before being incubated with 500µl ICC buffer for 30 minutes at RT. For βII tubulin staining, samples were incubated with 300µl of βIII tubulin primary antibody (monoclonal mouse IgG, Millipore) at a 1:250 dilution in ICC buffer. For Map2 staining, samples were incubated with 300µl Map2 primary antibody (mouse polyclonal IgG, Chemicon International) at a 1:200 dilution in ICC buffer. Both primary antibodies were incubated with the samples for one hour at RT. Samples were then washed five times with PBS and incubated with 300µl AlexaFluor-488 goat anti-mouse IgG (Molecular Probes) at a 1:1000 dilution in ICC block for one hour at RT in the dark. Samples were washed five times with PBS and incubated with DAPI solution (Molecular Probes, 3µl/ml) in PBS for five minutes in the dark. Scaffolds were washed five times with PBS and mounted using ProLong Gold Antifade reagent.

For Oct4 and actin staining of human ES cells cultured on ppAm microfibre meshes, scaffolds were moved to fresh 24 well culture plates and washed twice with PBS before fixing with 4% paraformaldehyde for 10 minutes at 4°C. Samples were then washed twice with PBS and blocked with 500µl ICC buffer. Samples were incubated with 300µl Oct4 primary antibody (rabbit polyclonal IgG, Santa Cruz) at a 1:100 dilution in ICC buffer for one hour at RT. Samples were then washed five times with PBS and incubated with 300µl ICC buffer containing secondary antibody (1:1000 dilution, AlexaFluor-546 goat anti-rabbit IgG, Molecular Probes) and FITC-phalloidin (1:200 dilution from a 1mg/ml stock dissolved in methanol, Sigma) for one hour at RT in the dark. Samples were then stained with DAPI and mounted as described above. All samples were visualised on a Nikon Eclipse 50i fluorescent microscope.
3 Electrospun PLGA Scaffolds

The work described in this section aimed to explore the potential of electrospun scaffolds for the 2D and 3D culture of mouse ES cells. Electrospinning is an effective method of creating meshes composed of nanometer size fibres that replicate the fibrous structure of the ECM (Stevens and George, 2005). Fibrous architecture provided by electrospun meshes has influenced cell signalling in mouse ES cells causing Rac upregulation, enhanced Nanog expression and increased proliferation (Nur et al., 2006). Taking this into consideration, the work presented here focused on determining if scaffolds composed of fibres in the nanometer range would assist in maintaining ES cell pluripotency in standard ES cell media.

Although electrospun matrices have shown promise as instructive substrates for ES cell culture, little cell infiltration has been observed. However, several approaches have sought to overcome this issue by creating looser mesh architecture (Tzezana et al., 2008) and by increasing fibre diameter and therefore pore size (Pham et al., 2006). The infiltration of ES cells into electrospun meshes and the formation of a truly 3D culture environment would be desirable for both the propagation and differentiation of mouse ES cells. Therefore, this study also aimed to investigate and encourage cell infiltration into the meshes by increasing average fibre size with a target diameter of >3µm.

PLGA was selected as it has proved a promising material for ES cell culture, with PLGA sponges supporting the differentiation of human ES cells into multilayered tissue constructs including vascularised cardiomyocytes (Caspi et al., 2007). Electrospun PLGA scaffolds have also been successfully applied to the culture and differentiation of MSCs (Xin et al., 2007). PLGA has a number of advantages over other synthetic polymers by possessing an easily tailored degradation rate through adjustment of the PLA: PGA ratio. PLGA has also demonstrated extensive versatility in its capacity to be electrospun with natural polymers, including gelatin and elastin (Li et al., 2006b) and has been post-synthetically modified with ECM peptide motifs (Kim and Park, 2006).

In summary, electrospun PLGA microfibre scaffolds composed of fibres in the nm range were assessed for their ability to support the culture of pluripotent mouse ES cells compared with flat spin coated films. Cell morphology and proliferation on the scaffold surface were assessed with pluripotency monitored using an Oct4-GFP reporter cell line. To investigate the development of 3D meshes for ES cell culture, spinning parameters were adjusted to increase fibre size and the ability to support cell infiltration assessed using E-cadherin<sup>−/−</sup> ES cells.
3.1 Microfibre Scaffold Preparation

The initial aim was to create a reproducible, bead free microfibre mesh with an average fibre diameter of less than 1µm. A solution of 20% (w/v) PLGA (85:15 lactide: glycolide, average Mw 50,000 – 75,000) dissolved in HFIP was prepared. The solution was electrospun using a potential of 25kV, a solution flow rate of 0.05ml/hr and a 20cm working distance. The electrospun fibres were collected onto a rotating mandrel (100rpm) and the resultant mat gold coated and analysed using SEM. The mesh was found to consist predominantly of fibres ranging between 0.1 – 1µm (Figure 13), with an average fibre diameter of 0.570µm and a standard deviation of 0.38µm (± 0.38µm). Fibres were bead free with no detectable surface texture. Meshes were reproducible; with the fibre distribution in Figure 13 calculated from three meshes spun on 3 different days from 3 separately prepared spinning solutions.

Figure 13. Morphology and fibre distribution of microfibre meshes. 20% (w/v) PLGA dissolved in HFIP was electrospun onto a rotating mandrel (100 rpm) at 25 kV, with a 0.5 ml/hr flow rate and a 20 cm working distance. Fibres were visualised by SEM and fibre distribution was calculated by measuring at least 100 fibres from three separate meshes using ImageTool 3.0.

A flat spin coated film was used as a flat control in the cell culture experiments against the fibrous morphology of the microfibre mesh. The flat films were produced by spin coating 200µl of the electrospinning solution onto a 2.5cm x 2cm glass slide for 20 seconds at 200rpm. Spin coated films were flat with no surface texture evident by SEM analysis. Both the microfibre meshes and
the spin coated films were kept in a vacuum oven for 48 hours to remove as much of the residual HFIP as possible.

3.1.1 FTIR Analysis

FTIR spectroscopy was used to characterise the PLGA scaffolds and ascertain whether any residual HFIP was present. The absorbance, or transmittance spectra, is unique to the material as absorption is dependent on the specific frequencies at which molecules in the compound rotate or vibrate (Young and Lovell, 1991). Results can be presented as percent absorption or percent transmittance of the infrared light. In this case, results are presented as percent transmittance. The samples were studied using an attenuated FTIR setup.

![Chemical structure of PLGA (A) and HFIP (B).](image)

Figure 14. Chemical structure of PLGA (A) and HFIP (B).

The molecular structures of PLGA and HFIP are depicted in Figure 14. The HFIP had two strong bands at 3000-2850 cm\(^{-1}\) (Figure 15 A) and 3800–3100 cm\(^{-1}\) (Figure 15 B) representing absorption by carbon-hydrogen bonds and hydroxyl groups respectively. The carbon-hydrogen band was at 2985 and 2881 cm\(^{-1}\). In comparison the PLGA had a carbon-hydrogen band with three strong peaks at 2997, 2947 and 2881 cm\(^{-1}\), which is in agreement with previous findings (Figure 16, B) (Caticker et al., 2000). PLGA was also characterised by a band at 1850-1650 cm\(^{-1}\) from the carbonyl groups present on the polymer chain (Figure 16, C).

The FTIR spectra for the microfibre meshes (Figure 17) and spin coated films (Figure 18) contained the same carbonyl bands (1650-1850 cm\(^{-1}\)), carbon-hydrogen bands (3000-2850 cm\(^{-1}\)), and carbon-hydrogen bond bands (2997-2995, 2947 and 2883 cm\(^{-1}\)) present in the as-received PLGA. Despite the treatment in a vacuum oven for 48 hours, the spin coated films still had a weak band at 3800–3100 cm\(^{-1}\), suggesting residual HFIP was still present within the film (Figure 18, A). In comparison, microfibre meshes display no deficit in this region (Figure 17 A).
Figure 15. FTIR analysis of HFIP. FTIR analysis of HFIP is characterised by the presence of an aliphatic C-H band (A) at 3000-2850 cm\(^{-1}\) and a hydroxyl band (B) at 3800–3100 cm\(^{-1}\).

Figure 16. FTIR analysis of PLGA. FTIR analysis of a PLGA pellet is characterised by the presence of an aliphatic C-H stretching band (B) at 3000-2850 cm\(^{-1}\) and a carbonyl band (C) at 1850-1560 cm\(^{-1}\).
Figure 17. FTIR analysis of Microfibre Meshes. Microfibre meshes are characterised by an aliphatic C-H band (B) at 300-2850 cm\(^{-1}\) and a band at 1850-1650 cm\(^{-1}\) representing the C=O stretching mode (C). No reduction in transmittance was observed at the hydroxyl group band at 3800–3100 cm\(^{-1}\) (A).

Figure 18. FTIR analysis of Spin Coated Films. The spin coated films are characterised by an aliphatic C-H stretching band (B) at 300-2850 cm\(^{-1}\) and a band at 1850-1650 cm\(^{-1}\) (C) representing C=O stretching mode. The spin coated films also absorbed slightly at the hydroxyl group band at 3800–3100 cm\(^{-1}\) suggesting some residual HFIP was present (A).
3.1.2 TGA

TGA was used to quantify the residual HFIP present in the PLGA scaffolds. The samples were heated from 0 – 100°C (The boiling temperature of HFIP is 60°C) at a heating rate of 5 °C min\(^{-1}\) in a nitrogen atmosphere.

![TGA profile of PLGA pellets](image)

**Figure 19. TGA analysis of PLGA pellets.** A typical TGA profile of PLGA pellets heated from 0-100°C at a heating rate of 5 °C min\(^{-1}\) in an air atmosphere.

An average loss of 0.3% was observed in the raw PLGA pellets (Figure 19). This loss was due to water from the atmosphere entering the polymer during its time in storage. The weight loss from the meshes that had been held for 48 hours in a vacuum oven was 4.39 wt% (SD: 0.27, n=2) with maximum weight loss occurring at 60.2 °C (SD: 0.54) (Figure 20). This temperature corresponds to the boiling point of HFIP and suggests that the microfiber mesh contained 4.3 wt % HFIP. An increase in weight was consistently observed between 40 – 60°C for the electrospun fibres and none of the other samples. This temperature range corresponds to the Tg of the polymer used and thus it is possible that the weight increase actually relates to the movement of the polymer as it softened in the pan. To avoid underestimating the residual HFIP, total weight loss was calculated from the maximum weight recorded rather than the initial weight at the beginning of the procedure.

![TGA profile of microfibre meshes](image)

**Figure 20. TGA analysis of microfibre meshes.** A typical TGA profile of microfibre meshes after 48 hours vacuum treatment heated from 0-100°C at a heating rate of 5 °C min\(^{-1}\) in an air atmosphere.

Spin coated films had an average weight loss of 10.9 wt%, significantly larger than microfibre meshes (samples compared using a two sample t-test. \(P=0.01\), n=2). Maximum weight loss
occurred at 74.2°C (SD: 1.57), 14.2°C higher than the boiling point of HFIP (Figure 21). This higher temperature could be due to the lower surface area of spin coated films compared to microfibre scaffolds. Therefore more time and higher temperatures may be necessary to facilitate solvent diffusion out of the films as the solvent has a longer distance to travel.

![TGA analysis of spin coated films.](image)

**Figure 21. TGA analysis of spin coated films.** A typical TGA profile of spin coated films after 48 hours vacuum treatment heated from 0-100°C at a heating rate of 5 °C min⁻¹ in an air atmosphere.

### 3.1.3 Wettability

To assess the hydrophobicity of PLGA scaffolds, 5µl of dH₂O was deposited onto the scaffold surface and the WCA measured over a 15 second period at one second intervals (Figure 22)

![Surface contact angle measurements of microfibre meshes and spin coated films.](image)

**Figure 22. Surface contact angle measurements of microfibre meshes and spin coated films.** 5µl of dH₂O was deposited on the scaffold surface and WCA measured over 15 seconds at one second intervals.

Both scaffolds had high contact angles of 133.6° for microfibre meshes and 75.5° for the spin coated films. In both cases, the droplets remained unchanged on the scaffold surface over the 15 second measurement period.

### 3.1.4 Degradation in Culture

In the presence of water, PLGA degrades by hydrolysis into lactic acid and glycolic acid. Accumulation of degradation products can lead to an increase in acidity which is detrimental to the
cell culture environment. To assess possible adverse effects of PLGA degradation during culture, microfibre and spin coated scaffolds were incubated in standard ES media (supplemented with 10% FCS) for 30 days and the pH of the surrounding media monitored. In order to replicate culture conditions as closely as possible, scaffolds were incubated at 37°C and media was changed every three days (Figure 23).

![pH Analysis - PLGA Scaffolds in Standard ES Media](image)

**Figure 23. pH analysis of PLGA scaffolds incubated in ES cell media.** Microfibre meshes, spin coated films and standard TCP wells were incubated in standard ES media at 37°C over 30 days. The surrounding media was replaced every 3 days and the pH of the removed media recorded. 3 replicates per condition were analysed.

Throughout the 30 day incubation period pH did not drop below pH 7.8 and pH of media surrounding the PLGA scaffolds did not deviate from the TCP controls.

To monitor possible changes in fibre morphology of the microfibre meshes during culture, microfibre scaffolds were incubated in ES media for 30 days at 37°C and sampled for SEM analysis every fifth day (Figure 24). Where possible, two separate meshes for each time point were analysed. At day 0, microfibre meshes are composed of discrete fibres with average fibre diameter 0.470µm ±0.2. At days 5-10, fibres appear to be joining together and thinner fibres show signs of breakage. By day 15, melding of the fibres has progressed with junctions between fibres becoming less defined. Degradation of larger fibres is apparent at days 20 and 25 with fibre breakage accompanied by erosion leaving small “stumps”. At the end of the incubation period, individual fibres are difficult to identify and smaller fibres appear to have completely degraded.
Figure 24. Morphology of microfibre meshes incubated in ES cell media. Microfibre meshes were incubated as described in Figure 23. Meshes were sampled for SEM analysis at 5 day intervals. Two meshes per time point were analysed where possible.
Figure 25. Fibre diameter distribution of microfibre meshes incubated in ES cell media. 100 fibres from two separate meshes incubated for 20 (B) and 25 (C) days as described in Figure 23 were measured from SEM images using ImageTool 3.0. Statistical analysis compared measurements with day 0 (A) samples using a two sample t test assuming equal variance.
The loss of thinner fibres from the mesh is evident at day 20, with a significant increase in fibre diameter from 0.470\(\mu\)m ±0.2\(\mu\)m to 0.639\(\mu\)m ±0.3\(\mu\)m (mesh 1, \(P=0.05\)) and 0.639\(\mu\)m ±0.3\(\mu\)m (mesh 2, \(P=0.001\)). A significant increase was also observed after 25 days, with average fibre diameter of mesh 1 increasing to 0.896\(\mu\)m ±0.4\(\mu\)m (\(P=0.001\)). No significant increase in fibre diameter was observed for the second mesh (mesh 2) after 25 days, which may be due to the large degree of variation in fibre diameter present within the microfibre meshes. However, the increase in fibre diameter at day 20 and 25 is shown in Figure 25, with fibre distribution shifting towards larger fibre diameters. The increase in fibre diameter may also be due to fibre swelling, as fibres as large as 2.5\(\mu\)m are present in the meshes after 25 days.

As degradation of the mesh is apparent in Figure 24 an accompanying increase in acidity of the surrounding media would be expected. To determine whether the ES media acted as a buffer and prevented any pH change, PLGA scaffolds were incubated for 30 days in dH2O with 0.02% sodium azide (Figure 26). Apart from an increase in pH at day 20 in the TCP control, the pH of water surrounding the PLGA scaffolds did not deviate from TCP readings and pH at the end of the culture period was pH 7.33 for both scaffolds, an increase from day 0 (pH 6.87). After 30 days incubation microfibre meshes displayed a similar morphology to scaffolds incubated in media with merging of fibres and a loss of structural definition.

**Figure 26. Morphology and pH analysis of PLGA scaffolds incubated in H2O for 30 days.**

PLGA scaffolds were incubated in H2O for 30 days at 37\(^\circ\)C. H2O was not replaced throughout the incubation period and pH was measured every three days. Incubated microfibre meshes were visualised by SEM at the end of the incubation period.
3.2 ES Cell Culture on Microfibre Mesh and Spin Coated Films

3.2.1 Scaffold Preparation

Before PLGA scaffolds could be applied to cell culture it was necessary to find a sterilisation method that did not alter the architecture of the microfibre mesh. Soaking in 100% ethanol caused fibre swelling, significantly increasing the average fibre diameter from 0.475\( \mu \text{m} \) to 0.678\( \mu \text{m} \) (5 minutes), 0.702\( \mu \text{m} \) (15 minutes) and 0.794\( \mu \text{m} \) (30 minutes). The increase in fibre diameter distribution and the effect on the morphology of the microfibre mesh is shown in Figure 27. 70% ethanol (Figure 28) reduced fibre swelling compared with 100% ethanol treatment, with average fibre diameter 0.570\( \mu \text{m} \) at 5 minutes, 0.529\( \mu \text{m} \) at 15 minutes and 0.612\( \mu \text{m} \) at 30 minutes. Nevertheless, fibre swelling did occur with significant increases in fibre diameter observed at 5 and 30 minutes, distorting the mesh and creating a denser network of fibres.

![Figure 27. 100% Ethanol treatment of microfibre meshes. SEM images and fibre diameter distribution of microfibre meshes soaked in 100% ethanol for 5, 15 and 30 minutes. Fibre diameter distribution was assessed by measuring at least 100 fibres from SEM images of each condition. Statistical analysis was conducted comparing ethanol soaked samples with untreated controls using a two sample t-test assuming equal variance. All scaffolds treated with ethanol had a significantly larger average fibre diameter with \( P = 0.001 \).](image)
Figure 28. 70% Ethanol treatment of microfibre meshes. SEM images and fibre diameter distribution of microfibre meshes soaked in 70% ethanol for 5, 15 and 30 minutes. Fibre diameter distribution was assessed by measuring at least 100 fibres from SEM images of each condition. Statistical analysis was conducted comparing ethanol soaked samples with untreated controls using a two sample t-test assuming equal variance. Significantly larger fibre diameters were recorder for scaffolds treated with ethanol for 5 ($P=0.05$) and 30 ($P=0.01$) minutes.

UV treatment of the microfibre mesh for 5 and 10 minutes each side does not appear to significantly affect fibre diameter or fibre morphology/integrity (Figure 29). Therefore UV treatment for 10 minutes each side was used to sterilise both microfibre meshes and spin coated films.

Figure 29. UV treatment of microfibre meshes. SEM images of microfibre meshes UV treated for 5 and 10 minutes each side. There was no significant difference in fibre diameter between meshes treated with UV and untreated controls. Statistical analysis was conducted comparing UV treated samples with untreated controls using a two sample t test assuming equal variance. No significant difference in fibre diameter was observed after UV treatment.

In order to make the scaffolds more compatible for cell culture, meshes were pre-incubated overnight in ES media (supplemented with 10% FCS) to allow the adsorption of serum proteins to the scaffold surface. This dramatically lowered initial WCA from $133.3^\circ \pm 4.87$ to $46.6^\circ$ on microfibre
meshes and from 75.5° ±1.71° to 61.3° ±11.2° on spin coated films. After 15 seconds contact, WCA had further decreased to 32.6° and 55.4° ±3.25° on microfibre meshes and spin coated films respectively.

**Figure 30. WCA measurements for pre-conditioned microfibre meshes and spin coated films.** Microfibre meshes and spin coated films were soaked overnight in standard ES media (plus 10% FCS), washed twice with H₂O and allowed to dry. Once dry, 5ul of dH₂O was deposited on the scaffold surface and contact angle measured over 15 seconds at 1 second intervals.

### 3.2.2 Oct4-GFP ES Cells

Oct4-GFP ES cells express green fluorescent protein (GFP) under the control of the Oct4 promoter and were a kind gift from Professor Austin Smith, The University of Cambridge. The Oct4-GFP reporter cell line allows the expression of Oct4 within the population to be assessed by flow cytometry and serves as an excellent tool to assess the ability of the scaffolds to maintain pluripotency (Ying et al., 2002; Ying et al., 2003b). Oct4-GFP cell phenotype is typical of normal mouse ES cells. They can be passaged on standard gelatinised TCP and grow in tight colonies surrounded by a small population of differentiated cells (Figure 31 A).

Flow cytometry is a highly sensitive and versatile method of assessing cell phenotype. Detached cells are passed single file through a laser beam, scattering the light according to their size and granularity. The laser beam excites the GFP within the cells (or labelled antibodies in antibody staining studies) causing the GFP to emit light at 488nm. This is recorded for each cell passing through the beam, creating profiles depicted in Figure 31 B. As with all mouse ES cell cultures, Oct4-GFP cultures are often comprised of pluripotent ES cells and a small population of differentiated cells. Flow cytometry highlights these two populations, with a GFP positive peak and a smaller GFP negative peak.
Figure 31. Oct4-GFP ES cells. A) Light microscope image of ES cell colonies growing on gelatinised TCP. B) A typical flow cytometry profile of Oct4-GFP cells in standard ES cell culture (green). Non fluorescent cells (E-cadherin^- ES cells) were used as a negative control (purple). Cells were 88.7% GFP positive. C) Oct4-GFP cells visualised using Fluorescent Microscopy. A 4 second exposure was necessary to visualise the GFP within the cells (nucleus: blue, GFP: green). D) Laser confocal microscope imaging of Oct4-GFP cells stained for cytoskeletal protein actin (nucleus: blue, GFP: green, actin: red).

As GFP is not attached to the Oct4 protein it is diffuse within the cell and not localised within the nucleus. It is very difficult to visualise GFP within the cells using standard fluorescent microscopy. Exposure time has to be increased from a standard level of 100 – 500ms to 4.0 seconds in order to visualise GFP within the culture (Figure 31 C). Image D within Figure 31 is a confocal laser scanning microscope image of Oct4-GFP ES cells stained for actin. Confocal laser scanning microscopy enables the GFP to be visualised more easily. As with all ES cells, Oct4-GFP cells have a cortical actin arrangement within the pluripotent colonies. Upon differentiation and cell spreading, the cytoskeleton elongates and becomes polarised. This arrangement is largely restricted to the small population of differentiated cells outside the ES cell colonies.

3.2.3 Short Term Culture on Microfibre Meshes and Spin Coated Films

To assess the attachment, viability and growth of ES cells on microfibre meshes, Oct4-GFP cells were initially seeded at a high density (1.5 x 10^5 cells/cm^2) and cultured in standard ES media over 7 days.
Figure 32. 7 day culture of Oct4-GFP cells on microfibre meshes (seeding density 1.5 x 10^5 cells/cm^2). Oct4-GFP cells were seeded onto microfibre meshes in standard ES media and cultured for 7 days. Cells were monitored using light microscopy and SEM.

Although the cells on the microfibre mesh are difficult to visualize, light microscopy shows the cells attaching to the matrix in round aggregates after 24 hours (Figure 32). These aggregates grew in size with no evidence of cell spreading across the surface. The increased density at the centre at day 7 suggests cell death was occurring, reminiscent of cavitation during EB development. SEM supported the observations made by light microscopy. At day 1, cells were attached to the cell surface in aggregates which grew in size by day 3, with little or no spreading across the mesh. By day 7 the aggregates had formed compact balls of cells covered with ECM, structures closely resembling the tight cell clusters observed when EBs are formed.
Figure 33. Oct4-GFP cells seeded onto microfibre meshes at different cell densities. SEM images of Oct4-GFP cells seeded at $5 \times 10^2$, $5 \times 10^3$ and $5 \times 10^4$ cells/cm$^2$ onto microfibre meshes and cultured in standard ES media for 7 days.

The tight, spherical cell structures may have occurred due to the large number of cells initially seeded on the microfibre meshes. To encourage cell growth and interaction with the scaffold, cells were seeded at $5 \times 10^2$, $5 \times 10^3$ and $5 \times 10^4$ cells/cm$^2$ on both microfibre meshes and spin coated films. At all concentrations tested, cells on the microfibre meshes formed aggregates on the scaffold surface (Figure 33). These aggregates became more flattened over the culture period and were surrounded by spread cells suggesting greater interaction with the meshes, rather than tight clustering into ‘EB-like’ structures.
Figure 34. Matrix deposition and morphology of Oct4-GFP cells on microfibre meshes. SEM images selected to demonstrate the morphology and progressive matrix deposition of Oct4-GFP cells cultured on microfibre meshes. A-D were seeded at 5 \times 10^4 cells/cm^2, E-L were seeded a 5 \times 10^3 cells/cm^2.

Over the culture period progressive ECM deposition was observed (Figure 34). At day 3 a light fibrous mesh covered the cells, which formed a dense plaque covering the colonies by day 7. Cells beneath the ECM plaque appeared to have varying morphologies indicating a mixed population of cell types. Cells in the centre of the aggregates possessed a rounded morphology reminiscent of ES cells (Figure 34, G-J) whilst cells on the outside appeared polarised and were simultaneously attached to both the microfibre mesh and the overlying ECM (Figure 34, K-L).

Similar aggregates were observed on the spin coated films (Figure 35). However a monolayer of spread cells appeared to form by day 7. The cells are difficult to visualise on the flat surface and the monolayer had undergone extensive cracking. Such distortion of the samples if also present on the microfibre meshes and is due to the dehydration necessary for standard SEM imaging.
Figure 35. Oct4-GFP cells seeded onto spin coated films at different cell densities. SEM images of Oct4-GFP ES cells seeded at 5 x 10^2, 5 x 10^3 and 5 x 10^4 cells/cm^2 onto spin coated films and cultured in standard ES media for 7 days. Unfortunately, there were no cells present to visualise when seeded at 5 x 10^2 cells/cm^2.

To visualise cells and the surrounding ECM in a hydrated state, E-SEM was used instead of standard SEM imaging (Figure 36). By lowering the vacuum chamber pressure from a minimum of 1.3 x 10^-4 mbar to 0.55mbar it was possible to visualise the cells in a fully hydrated state. Although E-SEM images lack the surface detail seen in Figure 34, the images give a more realistic view of the cell environment, particularly the size of the fully hydrated cell compared to the fibres of the microfibre mesh. As seen in standard SEM analysis, cells on the microfibre mesh grew primarily in tight aggregates with some surrounding spread cells. Although cells on the spin coated films formed aggregates, there appeared to be a greater amount of cell spreading across the scaffold surface.
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<th>Day 1</th>
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<td>Spin Coated</td>
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Figure 36. E-SEM images of Oct4-GFP cells on PLGA Scaffolds (seeding density: $5 \times 10^4$ cells/cm$^2$). Oct4-GFP cells were cultured on microfibre meshes and spin coated films in standard ES media for 7 days. After fixing in 1.5% gluteraldehyde, cells were not dehydrated as for standard SEM analysis. Instead cells were stored in PBS and visualised in a hydrated state using VP-SEM.
To further assess cell morphology on the scaffold surface, cells were stained for actin (Figure 37). Samples were analysed using confocal microscopy enabling the GFP within the cells to be visualised. Cells on the microfibre mesh grew in tight cell aggregates with less spreading compared to the spin coated films. By day 7, Oct4-GFP is primarily expressed by cells within the aggregates with little expression observed in the surrounding spread cells. On the spin coated films, aggregates were observed. However, there appeared to be a greater proportion of spread cells at day 3 which then grew to form a cell monolayer by day 7. Oct-GFP expression was present in the monolayer, with some areas of greater intensity suggesting a mixed population of cell phenotypes.

Figure 37. Actin staining of Oct4-GFP cells cultured on PLGA Scaffolds (seeding density: 5 x 10⁴ cells/cm²). Oct4-GFP cells cultured on microfibre meshes and spin coated films in standard ES media over 7 days were fixed with 4% paraformaldehyde and stained for actin (red). Samples were visualised using laser confocal microscopy with scan settings optimised to detect GFP expression (green) within the Oct4-GFP reporter cell line.

Hoechst DNA assay was used to assess proliferation on the PLGA scaffolds compared to growth on gelatin (Figure 38). Cell numbers were calculated using a standard curve created by conducting the assay on known cell concentrations (see section 2.3.9). Cell numbers on all surfaces increased over the 7 day period with cells grown on gelatin reaching a maximum cell number of 14.5 x 10⁶ after 7 days. In comparison, cells reached a maximum number of 2.6 x 10⁶ and 2 x 10⁶ on microfibre and spin coated scaffolds respectively. Proliferation on PLGA scaffolds was significantly less than standard gelatinised TCP at all time points. However, there was no significant difference between microfibre meshes and spin coated films.
Figure 38. Proliferation on PLGA scaffolds (seeding density: 5 x 10^4 cells/cm^2). Proliferation of Oct4-GFP cells on microfibre meshes and spin coated films over 7 days culture in standard ES media was assessed using Hoechst 33258 DNA assay. Statistical analysis was conducted using a one way ANOVA at each time point. No significant difference in proliferation between microfibre meshes and spin coated films was observed during the culture period. Significantly lower cell numbers were recorded on microfibre meshes (**) and spin coated films (*) compared to gelatin at all time points (n=3, P=0.001).

According to the DNA assay, only 4.5 x 10^4 and 2.1 x 10^4 cells were present on microfibre and spin coated films, respectively, after one day of culture. This is compared to 5.3 x 10^5 cells present on gelatinised TCP and despite a seeding density of 5 x 10^4 cells/cm^2 (a total of 2 x 10^5 cells/scaffold). The data suggest a much lower level of ES cell attachment on microfibre meshes and spin coated films compared to gelatinised TCP.

3.2.4 Passaging ES cells on Microfibre Meshes and Spin Coated Films

In order to determine whether the PLGA scaffolds were candidate surfaces for long term ES cell culture, Oct4-GFP cells were seeded at 5 x 10^4 cells/cm^2 and passaged on microfibre meshes and spin coated films over a 21 day period. Each time the cells were passaged and reseeded, ‘spare’ cells were analysed for Oct4-GFP expression by flow cytometry and compared with a parallel culture maintained on standard gelatinised TCP. However, GFP expression was lost on all surfaces, including gelatinised TCP (results not shown). In order to address the loss of Oct4 expression, cells were cultured at a lower seeding density (1 x 10^4 cells/cm^2) over 17 days. Also, microfibre meshes and spin coated films were coated with gelatin to assess if the presence of gelatin would enhance the ability of the scaffolds to maintain Oct4-GFP expression (Figure 39).

At the lower seeding density, Oct4-GFP expression was maintained at 76-87% on all surfaces tested, with all scaffolds maintaining pluripotency at a level equivalent to standard culture conditions.
Figure 39. Oct4-GFP expression of cells passaged on PLGA scaffolds (seeding density: $1 \times 10^4$ cells/cm$^2$). Flow cytometry was used to assess Oct4-GFP expression of cells passaged on microfibre meshes and spin coated films over 17 days compared with a parallel culture on standard gelatinised TCP. PLGA scaffolds pre-coated with gelatin were also assessed for their ability to maintain Oct4-GFP expression. Cells were split when approximately 70% confluent and reseeded onto fresh scaffolds/gelatinised wells at $1 \times 10^4$ cells/cm$^2$. Remaining cells were fixed in 1% formaldehyde for flow cytometry with non-fluorescent E-cadherin$^-/$- ES cells used as a negative control.

ES cell pluripotency on the surfaces was confirmed by Nanog staining. Cells grown on gelatinised TCP were re-plated in a 6 well plate to allow them to be stained and visualised by fluorescent microscopy (Figure 40). Cells passaged on gelatin were positive for Nanog expression, which was localised to the nucleus.
Figure 40. Nanog staining of Oct4-GFP cells passaged on gelatin for 17 days (seeding density: $1 \times 10^4$ cells/cm$^2$). Oct4-GFP cells were cultured on gelatinised TCP as described in Figure 39. Cells were fixed with 4% paraformaldehyde and stained for Nanog (green) at passage day 17. Fluorescent microscope settings were optimised to ensure that no Oct4-GFP expression was detected alongside Nanog staining.

Cells cultured on microfibre scaffolds grew in rounded colonies that were positive for Nanog expression after 17 days (Figure 41). Gelatinised microfibre scaffolds also supported cell growth in rounded aggregates with Nanog expression localised to the cell clusters (Figure 42).

Figure 41. Nanog staining of Oct4-GFP cells passaged on microfibre scaffolds for 17 days (seeding density: $1 \times 10^4$ cells/cm$^2$). Oct4-GFP cells were cultured on microfibre scaffolds as described in Figure 39. Cells were fixed with 4% paraformaldehyde and stained for Nanog (green) at passage day 17. Fluorescent microscope settings were optimised to ensure that no Oct4-GFP expression was detected alongside Nanog staining.
Figure 42. Nanog staining of Oct4-GFP cells passaged on gelatinised microfibre scaffolds for 17 days (seeding density: $1 \times 10^4$ cells/cm$^2$). Oct4-GFP cells were cultured on gelatinised microfibre scaffolds as described in Figure 39. Cells were fixed with 4% paraformaldehyde and stained for Nanog (green) at passage day 17. Fluorescent microscope settings were optimised to ensure that no Oct4-GFP expression was detected alongside Nanog staining.

ES cells cultured on spin coated films were also positive for Nanog expression (Figure 43), with Nanog positive aggregates present on the scaffold surface. At lower magnification, it is evident that cells express Nanog on gelatinised spin coated films (Figure 44). However, higher magnification images reveal poor intensity of staining which could be due to poor lens contact with applied oil or the variability inherent in mounting rigid samples such as spin coated films.

Figure 43. Nanog staining of Oct4-GFP cells passaged on spin coated films for 17 days (seeding density: $1 \times 10^4$ cells/cm$^2$). Oct4-GFP cells were cultured on spin coated films as described in Figure 39. Cells were fixed with 4% paraformaldehyde and stained for Nanog (green) at passage day 17. Fluorescent microscope settings were optimised to ensure that no Oct4-GFP expression was detected alongside Nanog staining.
Figure 44. Nanog staining of Oct4-GFP cells passaged on gelatinised spin coated films for 17 days (seeding density: 1 x 104 cells/cm2). Oct4-GFP cells were cultured on gelatinised spin coated films as described in Figure 39. Cells were fixed with 4% paraformaldehyde and stained for Nanog (green) at passage day 17. Fluorescent microscope settings were optimised to ensure that no Oct4-GFP expression was detected alongside Nanog staining.

Average doubling times on all surfaces were calculated and compared using a one way ANOVA (Figure 45), revealing no significant difference between the conditions tested. Average doubling times on spin coated films and microfibre meshes were 23.9 and 21.3 hours, respectively. Coating scaffolds with gelatin did not affect proliferation, with the average doubling time on gelatin coated microfibre meshes calculated as 21.2 hours and doubling time on spin coated films averaging at 23.2 hours. The lack of a significant difference between the scaffolds and gelatinised TCP is surprising considering the comparatively low doubling time on gelatinised TCP (14.8 hours). This could be attributed to the large amount of variability in doubling time on the PLGA scaffolds, which may be in part due to variable cell recovery from the scaffolds mounted in Scaffdex CellCrowns.
Figure 45. Proliferation of Oct4-GFP cells passaged on PLGA scaffolds. Seeding density: $1 \times 10^4$ cells/cm$^2$. Oct4-GFP Cells were passaged on microfibre meshes and spin coated films as described in Figure 39. At each split, cells were counted and the doubling time calculated using the equation detailed in 2.3.8.3. Statistical analysis was conducted using a one way ANOVA, comparing averaged population doublings over 17 days culture on the different surfaces (n=6).
3.3 Macrofibre Scaffolds

The microfibre scaffolds supported the growth and maintained the pluripotency of mouse ES cells. However, the meshes created an essentially 2D culture environment with no infiltration into the scaffold observed. A number of studies have sought to overcome this issue by increasing fibre diameter, which in turn increases pore size and encourages cell infiltration (Ekaputra et al., 2008; Pham et al., 2006; Tzezana et al., 2008). To investigate this further, spinning parameters were altered to increase average fibre diameter. The ability of the scaffolds to support cell infiltration was assessed using pro-migratory E-cadherin^−/− ES cells.

3.3.1 E-cadherin^−/− ES Cells

Engineered via homologous recombination, E-cadherin^−/− ES cells lack the cell: cell contact protein E-cadherin normally expressed by pluripotent ES cells (Larue 1996, Spencer 2006). As a result, E-cadherin^−/− ES cells grow as single cells in culture and not in compact colonies (Cavallaro and Christofori, 2004). Despite the lack of cell: cell contact, E-cadherin^−/− ES cells remain pluripotent and, when E-cadherin is reintroduced, can form all three germ layers (Soncin et al., 2009). They are also more migratory than wildtype ES cells (Eastham et al., 2007; Spencer et al., 2007) making them an excellent tool to assess the ability of the macrofibre meshes to support cell infiltration.

3.3.2 Sigma Macrofibre Scaffolds

The initial aim was to create scaffolds comprised of fibres with an average diameter over 3µm. 20% (w/v) PLGA (Sigma) dissolved in HFIP was electrospun onto a rotating mandrel (100rpm) at 25kV with a 20 cm working distance. Polymer flow rate was increased from 0.5ml/hr to 1, 2.5, 5, 7.5 and 10ml/hr (Figure 46). Increasing flow rate showed a slight increase in average fibre diameter, reaching a maximum of 1.24µm ± 0.62µm at 10ml/hr.
Figure 46. Effect of increased flow rate on fibre distribution: PLGA, Sigma. 20% (w/v) PLGA dissolved in HFIP was electrospun onto a rotating mandrel (100 rpm) at 25 kV, with a 20 cm working distance. Polymer flow rate was tested at 0.5, 1, 2.5, 5, 7.5 and 10 ml/hr. Fibre diameter distribution was calculated by measuring at least 100 fibres from SEM images of the electrospun meshes using ImageTool 3.0, with one mesh per spinning parameters analysed.

Increasing polymer concentration to 30% significantly increased fibre diameter compared to 20% solutions spun at the same settings. Average fibre diameter reached 1.86\(\mu\)m ± 0.46\(\mu\)m at 1ml/hr flow rate (Figure 47) and significantly increased to 2.7\(\mu\)m ± 0.8\(\mu\)m at 5ml/hr (\(P=0.001\)) (Figure 48).

Figure 47. Effect of increased polymer concentration on fibre distribution at 1ml/hr flow rate: PLGA, Sigma. 30% and 20% (w/v) PLGA dissolved in HFIP was electrospun onto a rotating mandrel (100 rpm) at 25 kV with a 20 cm working distance. Fibre diameter was measured as described in Figure 46. Fibre diameters were compared using a two sample t-test assuming equal variance (\(P=0.001\)).
Figure 48. Effect of increased polymer concentration on fibre distribution at 5ml/hr flow rate: PLGA, Sigma. 30% and 20% (w/v) PLGA dissolved in HFIP was electrospun onto a rotating mandrel (100 rpm) at 25 kV with a 20 cm working distance. Fibre diameter was measured as described in Figure 46. Fibre diameters were compared using a two sample t-test assuming equal variance (P=0.001).

Electrospinning onto a rotating mandrel only produced a thin strip of electrospun mesh that was not large enough for cell culture. Therefore, meshes were electrospun onto a grounded metal plate (10.3 cm by 10 cm). Electrospinning at standard voltage and working distance of 25kV and 20cm with a 5ml/hr flow rate produced a mesh with an average fibre diameter of 2.1µm ±0.4µm. However, lowering the voltage to 20kV significantly increased average fibre diameter to 2.43µm ±0.53µm (Figure 49).

Figure 49. Effect of lower applied voltage on fibre distribution: PLGA, Sigma. 30% (w/v) PLGA dissolved in HFIP was electrospun onto a flat collector plate with a 20 cm working distance and a 5ml/hr flow rate. To test the effect of altering applied voltage, the polymer solution was electrospun at 20kV and 25kV. Fibre diameters were compared using a two sample t-test assuming equal variance (P=0.001).

Increasing flow rate over 5ml/hr and lowering working distance was also attempted to increase fibre diameter, however no fibre meshes formed on the grounded collector plate. Therefore, the meshes
with the largest average fibre diameter that could be collected in a form applicable to ES cell culture were the meshes electrospun at 20kV in Figure 49.

E-cadherin⁻/⁻ ES cells were seeded on microfibre meshes and the Sigma macrofibre mesh electrospun at 20kV as described in Figure 49. Despite the advantages of using E-SEM to visualise cells in a hydrated state, standard SEM was employed to analyse cell attachment and infiltration into the meshes. E-SEM demonstrated a high degree of variability in image quality and resolution. Considering it was necessary to analyse how the cells interacted with and moved through the fibres, standard SEM was used instead. Cells were seeded at 1 x 10⁴ cells/cm² and cultured for 5 days in standard ES media (Figure 50). By day 3 cells were attached to the fibre meshes with a number of cells observed below the top layer of fibres. At day 5, cells had proliferated, adopting a spread morphology with some growing below the surface of the Sigma macrofibre mesh. In comparison, cells seeded on microfibre scaffolds appear more rounded, with no cells infiltrating into the mesh.

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<td>Day 5</td>
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![Microfibre Mesh](image1)

![Sigma Macrofibre Mesh](image2)

Figure 50. E-cadherin⁻/⁻ ES cells cultured on microfibre and Sigma macrofibre meshes (seeding density: 1 x 10⁴ cells/cm²). E-cadherin⁻/⁻ ES cells were seeded on microfibre meshes and Sigma macrofibre meshes (average fibre diameter 2.43µm ±0.53µm). Cells were cultured in standard ES media for 5 days. Sigma macrofibre meshes were prepared as in Figure 49. Samples were prepared for SEM at days 3 and 5.

To ascertain how far the cells had migrated into the mesh, day 5 samples were fixed for SEM and then cut down the centre giving a cross section view of the scaffolds. Sigma macrofibre meshes were 64.71µm thick whilst microfibre meshes were 7.99µm (Figure 51). Although the cells had not infiltrated through the whole of the macrofibre scaffold, cells can be observed below the top layer of fibres with some in the scaffold centre. No cells were observed within the microfibre mesh and it is highly unlikely the thin scaffold could support 3D culture of ES cells.
Figure 51. Cross section view of E-cadherin^- ES cells cultured on microfibre and Sigma macrofibre meshes (seeding density: 1 x 10^4 cells/cm^2). E-cadherin^- ES cells were seeded on microfibre and Sigma macrofibre meshes (average fibre diameter 2.43µm ±0.53µm). Cells were cultured in standard ES media for 5 days. Sigma macrofibre meshes were prepared as in Figure 49. At day 5 scaffolds were prepared for SEM analysis and then sliced through the centre whilst still mounted tightly on the scaffold holder. Samples were mounted flat onto SEM stubs. The stage of the SEM was tilted approximately 55° – 75° allowing a cross section view of the mesh. All scaffolds are orientated with the surface facing upwards and the base of the scaffold at the bottom. Yellow arrows indicate cells within the macrofibre mesh.

3.3.3 PURAC Macrofibre Scaffolds

Solutions of Sigma PLGA above 30% (w/v) could not be electrospun. In order to create meshes with larger fibre diameters, a PLGA polymer with a higher inherent viscosity was used. It has been reported that polymer solutions with high viscosity create larger fibre diameters (Fong et al., 1999; Huang et al., 2003). By using a polymer with a higher inherent viscosity, it was hoped that highly viscous solutions could be formed from a low polymer concentration. Sigma PDLG (inherent viscosity: 0.55-0.75 dLg) was replaced with PURAC PDLGA (85:15 PDLA: PGA), inherent viscosity: 2.3dLg.

10% PURAC PLGA (w/v) dissolved in HFIP was electrospun onto a flat collector plate at 25kV with a 20 cm working distance. Average fibre diameter increased with faster flow rates, reaching a maximum of 3.27µm at 7.5ml/hr (Figure 52).
Figure 52. Effect of increased flow rate on fibre distribution: PLGA, PURAC. 10% (w/v) PLGA dissolved in HFIP was electrospun onto a flat collector plate at 25 kV, with a 20 cm working distance. Polymer flow rate was tested at 1, 2.5, 5 and 7.5ml/hr. Fibre distribution was calculated by measuring at least 100 fibres from SEM images of the electrospun meshes. Fibre diameter was analysed using a one-way ANOVA, with significant differences observed between all flow rates (P=0.001).

Increasing polymer concentration to 12.5% (w/v) restricted the maximum flow rate to 3ml/hr. Electrospinning at 2.5ml/hr at 15kV and 20kV created meshes with significantly different average fibre diameters of 3.12µm and 2.63µm respectively (Figure 53). A similar significant increase of fibre diameter at lower applied voltage was also observed at a 3ml/hr flow rate, producing average fibre diameters of 4.25µm at 15kV and 2.92µm at 20kV (Figure 54).
**Figure 53.** Effect of lower applied voltage on fibre distribution at 2.5ml/hr flow rate: PLGA, PURAC. 12.5% (w/v) PLGA (PURAC) dissolved in HFIP was electrospun onto a flat collector plate with a 20 cm working distance. To test the effect of altering applied voltage, the polymer solution was electrospun at 15kV and 20 kV. Fibre diameters were measured as described in Figure 52. Statistical analysis was conducted using a two sample t test assuming equal variance (P=0.001).

Despite reaching a maximum average fibre diameter of 4.25 µm, the fibres appeared swollen as though still wet with solvent. Due to this swollen morphology, the pore size appeared to be compromised. Therefore, the second largest average fibre diameter mesh, electrospun at 7.5ml/hr flow rate described in Figure 52, was applied to E-cadherin⁻/⁻ ES cell culture. This mesh will be referred to as PURAC macrofibre mesh.

**Figure 54.** Effect of lower applied voltage on fibre distribution at 3 ml/hr flow rate: PLGA, PURAC. 12.5% (w/v) PLGA (PURAC) dissolved in HFIP was electrospun onto a flat collector plate with a 20 cm working distance. To test the effect of altering applied voltage, the polymer solution was electrospun at 15kV and 20 kV. Fibre diameters were measured as described in Figure 52. Statistical analysis was conducted using a two sample t test assuming equal variance (P=0.001).
**Figure 55. SEM images of E-cadherin−/− ES cells cultured on PURAC macrofibre meshes (seeding density: 5 x 10⁴ cells/cm²).** E-cadherin−/− ES cells were cultured on PURAC macrofibre meshes (average fibre diameter 3.27μm ± 0.4μm) in standard ES media for 5 days. PURAC macrofibres were prepared by electrospinning a 10% PLGA (PURAC) dissolved in HFIP onto a flat collector plate at 25kV with a 7.5ml/hr flow rate and a 20cm working distance. Samples were prepared for SEM analysis at days 1, 3 and 5.

To encourage cell infiltration, cells were seeded at a higher density of 5 x 10⁴ cells/cm² and cultured for 7 days in standard ES media. After 24 hours, cells were attached to the PURAC macrofibre mesh with some cells below the top fibre layer (Figure 55). At 3 days, cells had spread with some present below the surface of the mesh. This is more evident at day 7, where cells have attached to fibres within the mesh and spread under cells on the scaffold surface.
Figure 56. SEM images of E-cadherin<sup>−/−</sup> ES cells seeded on microfibre meshes (seeding density: 5 x 10<sup>4</sup> cells/cm<sup>2</sup>). E-cadherin<sup>−/−</sup> ES cells were cultured in standard ES media on microfibre meshes and prepared for SEM analysis at day 1, 3 and day 7.

E-cadherin<sup>−/−</sup> ES cells seeded on microfibre meshes adopted a similar spread morphology at day 7 (Figure 56). However, there were areas where cells appeared to grow on top of one another rather than on the mesh itself. No cell infiltration into the mesh was observed throughout the 7 day culture period.
Cross section views of the PURAC macrofibre meshes show no cell infiltration after 24 hours (Figure 57). After 3 days, cells had migrated into the mesh. However, despite migration by day 3 there was not a dramatic increase in the number of cells within the mesh by day 7.

Microfibre meshes were very thin with a thickness of 3.05µm compared with 106.25µm for PURAC macrofibre scaffolds. No infiltration was observed and it is unlikely that the microfibre mesh could create a 3D culture environment for ES cells (Figure 58).
Figure 58. Cross section view of E-cadherin<sup>-/-</sup> ES cells cultured on microfibre meshes (seeding density: 5 x 10<sup>4</sup> cells/cm<sup>2</sup>). E-cadherin<sup>-/-</sup> ES cells were cultured on microfibre meshes in standard ES media for 5 days. Scaffolds were prepared for SEM analysis as described in Figure 51. All scaffolds are orientated with the surface facing upwards and the base of the scaffold at the bottom. Yellow arrows indicate cells within the macrofibre mesh.

3.4 Discussion

Initial electrospinning parameters produced a microfibre mesh comprised of fibres ranging between 0.1µm and 1 µm in diameter. These dimensions are in agreement with PLGA meshes previously electrospun by Li et al. (2002b) (500-800nm) and Xin et al. (2007) (550-970nm).

FTIR and TGA analysis revealed the presence of residual HFIP within the scaffolds, with 10.9 wt% present within the spin coated film despite 48 hours treatment in a vacuum oven. The lower amount present within the microfibre scaffold (4.3 wt%) can be attributed to the electrospinning process, where solvent evaporates as the polymer jet progresses towards the grounded collector. The post spun mesh also has a higher surface area to volume ratio compared to spin coated films allowing for enhanced solvent evaporation during vacuum treatment.

PLGA degrades in aqueous media by hydrolysis of ester bonds. To limit the implications of degradation in the current study, a ratio of 85:15 lactic acid: glycolic acid was used. The results presented here are by no means an exhaustive study of scaffold degradation in culture but are rather a preliminary assessment to pre-empt any issues which may adversely affect mouse ES cell culture. To replicate culture conditions, scaffolds were initially incubated at 37°C in standard ES media with media changed every 3 days. SEM images reveal fibre breakage occurring at day 5 and
day 10 which is followed by further degradation leaving small “stumps”. This appears to begin with smaller fibres, progressing to larger fibres by day 20. The degradation of electrospun fibres may predominantly occur through surface erosion, without autocatalysis within the fibres. As autocatalysis occurs due to the retention of degradation products, the nanometer scale of the fibres would facilitate diffusion of degraded oligomer species and remove the by-products necessary for the process to occur (Dong et al., 2009; Shin et al., 2006). A significant increase in fibre diameter was observed at later stages of degradation, possibly due to degradation of smaller fibres and fibre swelling. Fibre swelling of PLGA electrospun meshes has been reported previously and attributed to water penetration into the fibres (Cui, 2008). The loss of smaller fibres from the meshes may be due to the increased surface area resulting in higher exposure to the surrounding media and as a consequence, increased surface erosion (Dong et al., 2009; Shin et al., 2006). This would lead to fibre breakage, with the end of the stumps subsequently exposed to the aqueous phase and shortened due to progressive erosion at the exposed tip (Dong et al., 2009). A lack of structural definition is also apparent from day 15 onwards with individual fibres difficult to identify by day 30. The change in morphology observed is supported by Cui et al. (2008), where incubation of PDLA electrospun meshes in PBS led to conglutination of degraded fibres and fibre swelling due to water entering the polymer mesh. A similar loss of structural definition was also observed in electrospun scaffolds formed from 50:50 PLGA polymer blends (You et al., 2004). The ‘melted’ appearance could be attributed to a reduction in Tg, causing increased chain mobility and fibre ‘melting’ to reduce surface tension (Dong et al., 2009).

Despite the morphological changes occurring, no change in pH was observed in media surrounding both spin coated films and microfibre meshes. The absence of any dramatic change in pH is in agreement with Shin et al. (2006) who reported little change in pH during 10 week incubation of electrospun PLGA (75:25) scaffolds in PBS. The possibility of the media buffering any pH change was rejected when no change in pH was observed when scaffolds were incubated in H2O for 30 days. It would appear that the degradation occurring is sufficient to alter fibre morphology but does have an adverse effect on the pH of the culture environment. In order to determine what is occurring during the degradation of the PLGA scaffolds, extensive analysis using gel permeation chromatography, differential scanning calorimetry and weight loss analysis would be needed over an extended time period.

Considering that limited fibre degradation occurs by day 15, a time point beyond most culture periods and differentiation protocols for ES cells, and that no pH change was observed over a 30 day incubation period, it was concluded that the scaffolds were potential surfaces for either ES cell propagation and/or differentiation.

Cell density is an important parameter for ES cell propagation and differentiation and is highlighted during short term and long term culture of mouse ES cells on microfibre and spin coated scaffolds. During short term culture, the initial high seeding density of 1.5 x 10^5 cells/cm^2 led to the formation of large cell aggregates similar in morphology to EBs on the scaffolds surface, characterised by a necrotic/apoptotic centre and extensive ECM deposition. The observed matrix deposition was reminiscent of that observed by Sachlos et al. (2008) during EB formation. As with results presented here, a light fibrous coating formed by day 3 that subsequently developed into a smooth plaque by day 7. In the study, the outer ECM shell contained collagen I and collagen IV. However,
the composition of the plaque observed at both high and lower seeding densities remains to be determined.

Despite lowering cell density, cells on microfibre meshes formed discrete aggregates, displaying a preference for cell: cell contact rather than cell: surface adhesion. In comparison, ES cells seeded on spin coated films formed aggregates but spread across the scaffolds surface after 7 days of culture. Bashur et al. (2006) reported a lack of cell spreading of fibroblasts on electrospun PLGA scaffolds compared to solvent cast films. MSCs cultured on electrospun scaffolds also adopted a rounded morphology after initial seeding but eventually spread after 7 days of culture (Xin et al., 2007). Bashur et al. hypothesised that the lack of cell spreading could be due to the scale of the fibres limiting the size of focal adhesion complexes. This is supported by the increased spreading of E-cadherin\(^{-}\) ES cells on both sigma and PURAC macrofibre meshes (average fibre diameter > 2 µm) compared to microfibre scaffolds.

The reduced cell spreading could also be due to the hydrophobicity of the microfibre meshes. According to water contact angle analysis, microfibre meshes are significantly more hydrophobic than spin coated films. This difference between electrospun meshes and flat films was also observed by Cui et al. (2008) using PDLA and was attributed to the water droplet sitting on top of air pockets on the rough surface as in the Cassie-Baxter model (Whyman et al., 2008). PLGA is a naturally hydrophobic polymer due in part to the methyl groups present in PLA. XPS analysis by Cui et al. revealed an increase in the methyl groups within the XPS analysis layer (~10nm depth), suggesting the electrospinning process orientates these groups to the surface of the fibres. This could contribute to the increased hydrophobicity observed on the electrospun mesh and is supported by XPS data presented in section 4.1.2.

Focal adhesions and networks of actin stress fibres are not characteristic of mouse ES cells, which normally adopt a rounded morphology and cortical actin arrangement. Therefore, it could be argued that the heaped aggregates present on microfibre meshes are more reminiscent of pluripotent ES cells. This is supported by studies which reported a preference for the formation of membrane ruffles in fibroblasts, accompanied by an upregulation of Rac, on electrospun polyamide (Nur et al., 2005). Culture of mouse ES cells on the same scaffolds also induced Rac activation that was associated with an increase in proliferation and Nanog expression (Nur et al., 2006). However, there was no difference in the ability of the surfaces to maintain Oct4-GFP expression during long term culture. Positive Nanog expression staining was observed within the cell aggregates on the microfibre meshes and within the nucleus of more spread cells on the spin coated films, indicating the scaffolds maintain ES cell pluripotency to the same degree after long term culture period despite the differences in morphology.

According to the results presented here, doubling times on gelatinised TCP and PLGA scaffolds (+/- gelatin) were comparable, with no significant difference observed between the surfaces. This is despite an average doubling time of 14.8 hours on gelatinised TCP compared to 21.2-23.9 hours on PLGA scaffolds (+/- gelatin). The absence of any significant difference could be due to the large degree of variability observed, possibly introduced by unreliable levels of cell recovery from the PLGA scaffolds. In addition to contributing to a high degree of variability, the cell recovery could have led to an underestimation of doubling time. The doubling time could also be lower due to the
reduced cell attachment suggested by the DNA assay. The doubling times were calculated using
the initial seeding density, essentially assuming all cells that were seeded attached to culture
surfaces. As the DNA assay suggests this may not be the case, the average doubling time of cells
on the scaffolds could potentially be lower than that presented here. Further analysis will be
necessary to accurately calculate the proliferation of mouse ES cells on the scaffold surface in
relation to attachment efficiency. The reduced attachment could be attributed to the hydrophobicity
of the electrospun meshes and spin coated films. Wells et al. (2009) used plasma deposition to
form carboxylic acid gradients which in turn created a range of water contact angles. In serum free
media, mouse ES cell attachment increased with increasing carboxylic acid density and decreasing
WCA, with no attachment observed at the highest WCA of 88°. Therefore, the inherent
hydrophobicity of PLGA may have contributed to reduced cell attachment (which translated into
reduced proliferation) on both spin coated films and microfibre meshes.

As hydrophobic surfaces are not favourable for cell attachment (Webb et al., 1998; Wells et al.,
2009), the scaffolds were conditioned overnight in standard ES media to allow the adsorption of
serum proteins before cell seeding. Although conditioning introduces an unknown composition of
proteins onto the surface, it was effective in decreasing WCA on both spin coated films and
microfibre meshes. Based on a previous study by Chastain et al. (2006), proteins possibly
adsorbing to the scaffolds are collagen I, fibronectin and vitronectin. However, addition of
supplements to the culture media reduced fibronectin binding suggesting protein adsorption may
vary with media composition. In addition, proteins may not be adsorbed onto the surface in a
conformation favourable for cell attachment. Keselowsky et al. (2003) revealed surfaces
functionalised with methyl groups (CH₃), carboxyl groups (COOH) and amine groups (NH₂) bound
similar levels of fibronectin. However, surfaces functionalised with methyl groups had reduced
affinity for fibronectin antibodies and reduced integrin binding, indicating fibronectin was not
adsorbed in an optimal form for cell attachment (Keselowsky et al., 2003). A possible lack of ECM
molecules, combined with the hydrophobicity of the PLGA, may have contributed to the reduced
attachment and proliferation on the PLGA scaffolds.

High levels of attachment, proliferation and maintenance of pluripotent ES cells are a necessary
pre-requisite if a surface is to be applied to ES cell propagation. Ascertaining whether ES cells are
truly maintained in a pluripotent state would require more extensive assessments such as testing
whether cultured cells can generate all three germlayers (*in vivo* and *in vitro*).

As discussed in section 1.3.8, one of the limiting factors of electrospun meshes is the lack of cell
infiltration. As with microfibre meshes, cells grow on the surface of the mesh creating a fibrous, 2D
culture system. Infiltration into the network of fibres will be necessary in order to create a truly 3D
culture environment for propagation or differentiation of ES cells. Although the electrospinning
fibres in the nm range creates meshes that mimic the architecture of the natural ECM (Stevens and
George, 2005), it has been proposed that smaller fibre diameters increase the number of fibre
crossings and decrease pore size creating a denser fibre mesh that limits cell infiltration into
electrospun scaffolds (Eichhorn and Sampson, 2005). To explore this further, fibre diameter was
increased with an initial aim of reaching an average fibre diameter > 3µm. The parameters altered
were flow rate, polymer concentration/solution viscosity and applied voltage. Increasing flow rate
and polymer concentration increased fibre diameter, as supported by previous studies (Deitzel et
al., 2001; Fong et al., 1999; Huang et al., 2003; Kumbar et al., 2008; Pham et al., 2006; Son et al., 2004). Decreasing applied voltage appeared to increase fibre diameter, supporting the theory that elongation forces are enhanced by higher voltages resulting in a lower fibre diameters (Deitzel et al., 2001; Son et al., 2004). By increasing both concentration and flow rate, the amount of polymer within the ejected fibre is increased. Together with increased viscosity of the polymer solution restricting bending stability, a maximum average fibre diameter of 3.27µm was achieved (Deitzel et al., 2001; Ekaputra et al., 2008).

Increasing average fibre diameter to 3.27 µm permitted the migration of E-cadherin^{+/−} ES cells below the top layer of fibres by day 3. If cells were migrating readily into the mesh and were able to proliferate an increase in cell number within the mesh would be expected. However, little difference is seen after 7 days of culture with cells forming a monolayer on the surface.

Cross-section views of the scaffold reveal its potential as a 3D construct for tissue engineering. The microfibre meshes ranged between 3.05 - 7.99µm thick despite a two hour collecting time on a rotating mandrel. In comparison, PURAC macrofibre scaffolds had an average thickness of 106.25µm after 15 minutes collection on a collector plate. However, the difference in thickness could be in part due to the different collection methods. Microfibre meshes are collected onto a rotating mandrel creating meshes approximately 8 cm x 16 cm. Macrofibre meshes did not cover the entire plate, collecting instead in a concentrated circle approximately 7 cm in diameter. Therefore, a thicker microfibre mesh could be produced if the collections method was modified. However, no infiltration under the top layer of fibres was observed on the microfibre scaffolds. Even if a thicker mesh was produced, it is unlikely any infiltration would occur. Infiltration into the electrospun mesh could be improved by further increasing fibre diameter. Pham et al. (2006) developed electrospun scaffolds comprised of fibres 5µm in diameter with an average pore size of 25 µm. Rat mesenchymal stem cells were able to infiltrate into the macrofibre mesh. However, migration into the scaffold was inhibited by the inclusion of a nanofibre layer with an average fibre diameter of 600nm.

During electrospinning, fibres are stacked on top of one another creating a dense network. There is little evidence supporting the theory that cells are able to move fibres in order to accommodate migration or proliferation. Therefore, migration and proliferation in electrospun scaffolds could be improved by creating a looser network of fibres. Ekaputra et al. (2008) achieved this by electrospinning PCL/Collagen fibres (average fibre diameter 1.61µm) whilst simultaneously electrospraying a degradable hydrogel composed of heparin and HA. The two substances were spun using complimentary positive and negative applied voltages to ensure that no charge repulsion occurred. The degradable heparasil reduced volume density of the fibre mesh allowing infiltration of human fetal osteoblasts throughout the full thickness of the scaffold (210µm).

A layered hydrospinning method was introduced by Tzezana et al. (2008). Microfibrous (0.8µm) and macrofibrous (8.3µm) hydrospun meshes were produced by electrospinning PCL into a PBS bath. Electrospun meshes were collected at intervals and layered on top of one another. The layered constructs were comprised of 100 separate meshes which were then placed into a vacuum oven. The evacuation of the trapped water stretched the fibrous mesh, increasing the width of the
scaffold 10 fold and creating a looser network of fibres. Myoblasts and EBs formed from human ES cells were able to infiltrate into both microfibrous and macrofibrous hydrospun scaffolds.

Electrospinning of PLGA proved an effective method of producing electrospun matrices with nanometer size fibres. Degradation was observed in culture and dramatic changes in fibre morphology occurred from day 15 onwards with no adverse effects on pH of the surrounding media. Electrospun meshes and spin coated PLGA films were cytotocompatible with mouse ES cell culture. Despite a difference in cell morphology, both scaffolds supported the culture of Oct4-GFP ES cells to the same degree, with cells proliferating and maintaining pluripotency at levels comparable to gelatinised TCP. The development of macrofibre scaffolds demonstrated the ability to dramatically alter scaffold architecture by altering spinning parameters. Increasing fibre diameter >3µm encouraged infiltration of E-cadherin−/− ES cells below the top layer of fibres and produced a thick mesh with potential for 3D culture. The ability and potential to manipulate scaffold architecture demonstrates the flexibility of electrospinning and the advantages over 2D flat films. Further infiltration may be facilitated by further increasing fibre diameter (Pham et al., 2006); or creating a looser mesh work (Ekaputra et al., 2008; Tzezana et al., 2008). The results presented here suggest reduced attachment on the surface of PLGA scaffolds compared to standard TCP. However, it would appear that once attached, the cells may have a comparable doubling time to cells cultured on gelatinised TCP. Although the architecture of the microfibre mesh mimics that of the native ECM, modification of the scaffold surface chemistry and functionalisation with components of the ES cell microenvironment may enhance attachment and proliferation on the scaffold surface.
A secondary aim of this study was to functionalise the electrospun scaffolds with HS/heparin molecules, which could subsequently function to influence ES cell behaviour. Incorporation of heparin within electrospun scaffolds has led to leaching of the heparin from the fibre mesh and into the media (Casper et al., 2005; Luong-Van et al., 2006). This is advantageous in some systems but immobilisation of heparin/HS on the fibre surface may serve to enhance growth factor signalling, as reported for covalently bound growth factors (Fan et al., 2007; Nur et al., 2008). The immobilisation of HS on fibres also better replicates the way in which HS chains are presented in vivo, attached to and branching out from core proteins. Therefore, the project aimed to immobilise HS/heparin on the fibre surface, rather than to incorporate them into the fibre structure.

However, it was important that the chosen method did not alter the microfibre scaffold architecture. Plasma polymerisation has proved an effective method of dramatically altering the surface chemistry of a material without affecting bulk properties or scaffold structure. For instance, plasma polymerisation of electrospun PLGA meshes with acrylic acid enhanced the attachment and growth of fibroblasts without adversely affecting scaffold dimensions (Park et al., 2007). In addition, plasma polymerised surfaces also provide functional groups for covalent immobilisation of ECM molecules that otherwise would not be present on the naked material. However, the functional groups have also been utilised to mediate non-covalent interactions with molecules such as fibronectin (Bible et al., 2009) and GAGs (Mahoney et al., 2004; Marson et al., 2009). ppAm microtitre plates have successfully immobilised a wide array of GAGs, including heparin, HS, HA and CS in forms that are free to participate in protein interactions that occur naturally in vivo (Mahoney et al., 2004; Marson et al., 2009). Indeed, the microtitre plate system assisted in determining the heparin binding domain within the Link module of TSG-6 (Mahoney et al., 2005). The non-covalent attachment of GAGs to ppAm surfaces is a very attractive and elegant approach to functionalise biomaterial scaffolds, as the heparin/HS chains remain completely unmodified and are immobilised in a form that is free to participate in protein interactions. Therefore, in the current study, microfibre scaffolds characterised in the previous chapter were treated with ppAm and surface chemistry analysed using WCA and XPS.

HS plays a fundamental role in regulating ES cell behaviour. Mouse ES cells express an unusually low sulphated form of HS, with differentiation accompanied by and dependent on the generation of specific sulphation motifs within the HS chains (Baldwin et al., 2008; Johnson et al., 2007). Taking this into consideration the current study aimed to ensure that immobilised HS could bind proteins such as the link module of TSG-6 and presented the sulphation epitopes implicated in mediating their biological activity. This aspect was investigated using binding assays incorporating the link module of TSG-6 and anti-HS/heparin antibodies with high affinity for specific sulphation motifs within the heparin/HS chains. It was also essential to confirm that HS retained its biological activity whilst bound to the scaffold and could influence ES cell behaviour. This was assessed by testing the ability of the ppAm scaffolds functionalised with HS to rescue the neural differentiation capacity of HS deficient EXT1−/− ES cells, with and without the addition of exogenous FGF4.
The culture of human ES cells requires high levels of FGF2 in unconditioned media, with MEF secreted HSPGs stabilising and facilitating the binding of FGF2 in culture (Levenstein et al., 2008). It was hypothesised that the immobilised HS could facilitate the binding of FGF2 and together with the fibrous morphology of the scaffolds sustain human ES cell pluripotency. Therefore, scaffolds with and without immobilised HS were assessed for their ability to support human ES cells in both conditioned and unconditioned media.
4.1 Surface Analysis

4.1.1 Wettability

Allyl amine plasma polymerisation did not alter average fibre diameter, with no significant difference between coated (0.63µm ±0.38) and uncoated (0.67µm ±0.51) microfibre meshes (Figure 59). The morphology of the fibres remained unchanged with no surface texture visible by SEM.

Microfibre scaffolds coated with plasma polymerised allyl amine (ppAm microfibre scaffolds) were more hydrophilic than uncoated microfibre meshes (Figure 60). ppAm microfibre meshes had a significantly lower initial WCA of 117° compared to 131° on uncoated microfibre scaffolds. Over the twenty second contact period, the water droplet on the surface of microfibre scaffolds remained at 130-132°. In comparison, the WCA of ppAm microfibre scaffolds decreased rapidly at 0-4 seconds as the droplet spread across and possibly into the porous mesh, before levelling out and reaching 41° after 15 seconds. The WCA of microfibre meshes are hydrophobic due to the lotus leaf effect of the water drop sitting on fine polymeric structures and air pockets. It is plausible that as the water wetted the ppAm microfibre scaffold, it displaced the air, loosing the lotus-leaf effect and adopting the Wenzel state.
Figure 60. Water contact angle of ppAm microfibre and uncoated microfibre scaffolds. 5µl drops of dH₂O were placed on the surface of the meshes and recorded over 15 seconds with WCA measured at one second intervals. Statistical analysis of microfibre (n=10) versus ppAm microfibre meshes (n=6) was conducted using a two sample t-test at 0 and 15 seconds, revealing a significant difference (P=0.001) at both time points.

4.1.2 XPS Analysis

XPS analysis was conducted on uncoated microfibre meshes and ppAm microfibre meshes 5 days after coating. Initial analysis revealed a high degree of contamination with silicon (3.85 at.%). As the scaffolds are porous the contamination most likely originated from PDMS present in the tape used to immobilise the meshes on the sample bar. An alternative method of immobilising the meshes across a gap in the sample bar removed the contamination. The results presented are derived from the uncontaminated samples and are representative of peaks present in both contaminated and uncontaminated scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>C 1s [%]</th>
<th>O 1s [%]</th>
<th>N 1s [%]</th>
<th>F 1s [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfibre</td>
<td>63.19</td>
<td>36.81</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>ppAm Microfibre</td>
<td>77.1</td>
<td>5.28</td>
<td>16.24</td>
<td>1.38</td>
</tr>
</tbody>
</table>

Table 2. Elemental composition of microfibre and ppAm microfibre meshes. Wide scan analysis of microfibre and ppAm microfibre meshes revealed element composition of the scaffolds. Scans were charge corrected to the C-C/H environment at 285.0 eV. Elements were identified and quantified using an element library and relative sensitivity factors specific to the Kratos Axis Ultra used.

Wide scan analysis of microfibre meshes reveals a surface elemental composition of carbon (63.19%) and oxygen (36.81%) (Table 2). Plasma polymerisation of allyl amine onto the microfibre meshes introduces nitrogen (16.24%) and lowers total oxygen content to 5.28%. The ppAm
microfibre meshes also have contamination with fluorine (1.38%). As it is not present in the uncoated microfibre scaffolds, the fluorine must have been introduced during the plasma polymerisation process.

![Figure 61. Unlinked High resolution C 1s spectra: Microfibre meshes.](image)

<table>
<thead>
<tr>
<th>C 1s Microfibre</th>
<th>C-C/CH 285.0 eV</th>
<th>C-O 287.0 eV</th>
<th>C(=O)-O 289.1 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1s %</td>
<td>30.9</td>
<td>34.4</td>
<td>34.7</td>
</tr>
</tbody>
</table>

A high-resolution C 1s spectrum of uncoated microfibre scaffolds is shown in Figure 61. Uncoated microfibre meshes are characterised by three peaks. The hydrogen carbon environment at 285.0 eV is representative of the CH₃ groups present in PLA. The C-O and C(=O)-O environments from both PLA and PGA are present at shifts of 2.0 and 4.0 eV (±0.1eV) relative to C-H binding energy. The molar fraction of PLA present in the sampling depth can be estimated by dividing the intensity of the C-H component by the intensity of the C(=O)-O environment (Ogaki et al., 2008). Within the sampling depth of the microfibre scaffolds, the molar fraction is 89%. This is greater than the 85% within the original pellet and could be attributed to the adsorption of adventitious carbon from the atmosphere or the configuration of the co-polymer within the XPS sampling depth (Cui, 2008; Ogaki et al., 2008).
A high-resolution O 1s spectrum (Figure 62) of uncoated microfibre scaffolds reveals the presence of two peaks representing the two oxygen environments present in PLGA: C-OR (532.3 eV) and C=O (533 eV).

<table>
<thead>
<tr>
<th>O 1s Microfibre</th>
<th>C(=O)-O 532.3 eV</th>
<th>C-O 533.8 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1s %</td>
<td>48</td>
<td>52</td>
</tr>
</tbody>
</table>

**Figure 62. Unlinked High resolution O 1s spectra: Microfibre meshes.** Curves were fitted using a G-L line shape and a shirley background. FWHM was restricted to 1.5. Scans were charge corrected to the C-C/C-H environment at 285.0 eV and peaks were linked with corresponding environments in high-resolution C 1s spectra. Blue line: C(=O)-O, black line: C-O.

Peak fitting of distinct chemical environments can be assisted by ensuring the corresponding chemical environments in separate core spectra are of equal intensity. However, when the shared chemical environments of the C 1s and O 1s spectra (C(=O)-O and C-O) were linked, ensuring they were of equal proportion, a drop in intensity of the C(=O)-O peak in the C 1s spectra was observed Figure 63. This in turn increased the estimated molar fraction of PLA to 92%. However, the obvious deficit present within the C(=O)-O environment suggests that, in this instance, linking the peaks of the C 1s and O 1s spectra was not effective in assisting peak fitting.
**Figure 63. Linked High resolution C 1s spectra: Microfibre meshes.** Curves were fitted with a restricted FWHM of 1.2 using a G/L line shape and a shirley background. Scans were charge corrected to the C-C/C-H environment at 285.0 eV and peaks were linked with corresponding environments in high-resolution O 1s spectra. Purple line: C-H, red line: C(=O)-O, blue line: C-O.

Plasma polymerisation of allylamine dramatically altered the peak shape of both the C 1s and O 1s core spectra. Instead of being distinguished by three distinct peaks, the C 1s spectra was characterised by a large broad peak composed of overlapping chemical environments. Considering the variability of the plasma polymerisation process and the lack of distinct peaks, corresponding chemical environments in the C 1s and O 1s spectra were linked in this instance to aid peak fitting.

Consistent with the decrease in oxygen content, high-resolution C 1s spectra of ppAm microfibre meshes showed a dramatic drop in intensity of the C-O and C(=O)-O environment compared to uncoated microfibre meshes (Figure 64). Peaks at shifts of 1 eV (± 0.1eV), 1.5 eV (± 0.1 eV) and 3.0 eV eV (± 0.1eV) relative to the C-H/C-C binding energy were necessary to fit the spectra. The shift at 1.0 eV represents the C-N environment present in primary, secondary and tertiary amines, whilst the peak at +1.5 corresponds to imines. The peak at +3.0 eV indicates the presence of amides (N-C=O) within the deposited film.
Figure 64. Linked High resolution C 1s spectra: ppAm microfibre meshes. Curves were fitted as described in Figure 63. Purple line: C-H/C-C, light blue line: C-N, red line: C=N, dark blue line: C-O, black line N-C=O and brown line: C(=O)-O.

Compared to microfibre scaffolds, the intensity of the core level O 1s peak and its components has decreased dramatically with a greater reduction in the C-OR component. Also present is a third peak at 531.2 eV which has been allocated to the O=C-NR₂ environment representative of amides.
Figure 65. Linked High resolution O 1s spectra: ppAm microfibre meshes. Curves were fitted using a G-L line shape and a shirley background. FWHM was restricted to 1.5. Scans were charge corrected to the C-C/C-H environment at 285.0 eV and peaks were linked with corresponding environments in high-resolution C 1s spectra. Green line: O=C-NR$_2$, yellow line: C=O, purple line: C-O.

High-resolution core level N 1s peak was well fitted by a symmetrical single peak at 399.44 eV (Figure 66) encompassing amine, amide and imine functionalities. No N 1s signal was detected in the uncoated microfibre meshes.

<table>
<thead>
<tr>
<th>O 1s ppAm Microfibre</th>
<th>O=C-NR$_2$ 531.2 eV</th>
<th>C(=O)-O 532.5 eV</th>
<th>C-O 533.8 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1s %</td>
<td>33.6</td>
<td>38.4</td>
<td>28.0</td>
</tr>
</tbody>
</table>
Figure 66. High resolution N 1s spectra: ppAm microfibre meshes. The signal was fitted with a signal peak using a G-L line shape and a Shirley background. Scans were charge corrected to the C-C/C-H environment at 285.0 eV.

The results above illustrate the difference in surface chemistry of the ppAm microfibre mesh compared to uncoated scaffolds. Plasma polymerisation of allyl amine had introduced amine groups onto the scaffold surface and resulted in a marked increase in hydrophilicity. The peaks described above are in agreement with previous research investigating ppAm coatings (Barry et al., 2005; Dehili et al., 2006; Harsch et al., 2000; Mahoney et al., 2004; Shard et al., 2004).

4.2 HS Immobilisation

4.2.1 Disaccharide Composition Analysis

To initially assess whether ppAm microfibre scaffolds bound HS, 1 cm² sections of ppAm microfibre scaffold and uncoated microfibre scaffold were incubated with 10µg Celsus HS in PBS overnight. The Celsus HS used for the following RP-HPLC analysis is a heterogeneous preparation, containing both low sulphated and highly sulphated HS chains. A typical disaccharide analysis of Celsus HS is depicted in Figure 67.
Figure 67. Disaccharide analysis of Iduron HS. The peak areas from the raw HPLC profile (A) are quantified and values adjusted using known correction values (Deakin and Lyon, 2008) to give percentage composition for each disaccharide unit from which the total sulphation and N-acetylation within the preparation can be calculated (B). A minor AMAC related peak occurs at 24.5-25 minutes elution. At moderate - high concentrations of HS, it is readily discernable from the neighbouring UA-GlcNAc6S peak (Deakin and Lyon, 2008).

After incubation in 10µg HS, scaffolds were washed twice in PBS before the bound HS was competed off with 4M NaCl and processed for RP-HPLC as described in section 2.4.3 and briefly in Figure 68. The amount of bound HS was estimated by comparing the total fluorescence of the detached HS with a 10µg HS control (referred to as pre-binding HS) that simultaneously underwent the same desalting, digesting and AMAC labelling process. The amount of HS bound to the ppAm microfibre scaffold was approximately 1.29µg, whilst the microfibre scaffold only bound 0.0025µg. The disaccharide composition and total sulphation of the HS bound to ppAm microfibre scaffolds is shown in Figure 68. The amount of HS bound to microfibre scaffolds was too small for reliable compositional analysis.
Figure 68. Disaccharide analysis of bound HS: ppAm microfibre meshes. 1cm² sections of scaffold were immobilised in a 24 well plate by Scaffdex CellCrowns and soaked overnight with 10µg HS in PBS. Incubated samples were washed three times with PBS and then soaked for 30 minutes in 4M NaCl to detach any HS bound to the scaffolds. The detached HS was subsequently desalted in a PD-10 column and freeze dried. To prepare disaccharides for HPLC analysis, the HS samples were digested by heparinise I, II and III for approximately 18 hours and then freeze dried. Digested samples were then fluorescently labelled with AMAC, diluted in dH₂O to a total volume of 20µl and applied to a C18 RP-HPLC column. The disaccharide composition (A) and total sulphation (B) of HS bound to ppAm microfibre scaffolds was characterised and compared with pre-binding HS.

Disaccharide analysis of bound HS reveals an increase in percent composition of sulphated disaccharide species compared to pre-binding HS, particularly in the 6 sulphated species UA-GlcNAc 6S (+8.2%) and UA-GlcNS 6S (+2.7%) and the tri-sulphated units UA2S-GlcNS 6S (+12.0%). There was also a corresponding reduction in contribution of the non-sulphated disaccharide unit UA-GlcNAc to 18.6% from a pre-binding value of 35.5%. This increased composition of sulphated species is also evident in the total sulphation analysis with total 6 sulphation increasing by 23.0%. Total N sulphation and total 2-sulphation increased by 8.8% and 11.0% respectively, with a corresponding reduction in total N-acetylation by 8.8%.
In order to determine the maximum amount of HS a 1cm$^2$ piece of ppAm microfibre scaffold could bind, scaffolds were introduced to increasing amounts of HS ranging from 0.1 – 5µg. The amount of bound HS was estimated by comparing the total fluorescence of the detached HS with a 5µg HS control (referred to as pre-binding HS) that simultaneously underwent the same processing procedure. Quantification reveals a dose dependent increase in bound HS with increased applied HS until levels begin to plateau at 1.4µg (2µg applied HS), reaching a maximum of 1.7µg bound at 5µg applied HS (Figure 69). The large amount of variation observed in the amount of bound HS at 2 and 5µg may be due to variability in scaffold structure and volume and also variability in the ppAm coating. The number of processing steps involved in the RP-HPLC analysis, such as desalting, heparinase digestion and AMAC labelling could also have contributed to the large amount of variation observed.

![Figure 69. Loading capacity of ppAm microfibre meshes.](image)

At 1 and 2µg applied HS the disaccharide composition is not significantly different from pre-binding HS (Figure 70). The slight increase in UA-GlcNAc6S is influenced by the neighbouring AMAC related peak that elutes at 24.5-25 minutes. At lower HS concentrations, the separation of these two peaks is less defined. 5µg applied HS resulted in a significant increase in the tri-sulphated disaccharide UA2S-GlcNS6S composition (+4.7%) and a significant decrease in the non-sulphated disaccharide UA-GlcNAC (-7.1%) compared to pre-binding HS. This is accompanied by increased UA-GlcNS6S (+2.5%) and UA-GlcNAC6S (+2.3%) creating a profile similar to HS applied at 10µg (Figure 68). This increase in percentage composition of sulphated disaccharides is also reflected in a total sulphation analysis where increased total N-sulphation (+4.7%) and total 2-sulphation (+4.0%) is observed with a significant increase in total 6-sulphation (+9.5%) compared with pre-binding HS. This was accompanied by a corresponding loss in total N-acetylation (-4.7%). It appears that at the saturation level of ~1-2µg/cm$^2$ the ppAm microfibre scaffolds bind all forms of HS. However, under competitive binding conditions well above the saturation level (5µg/well) the scaffolds preferentially bind more sulphated species from within the heterogeneous preparation.
Figure 70. Loading capacity of ppAm microfibre meshes: Disaccharide analysis and total sulphation of bound HS. The disaccharide composition (A) and total sulphation (B) of bound HS applied at 1, 2 and 5µg/scaffold was characterised and compared with pre-binding HS. Bound HS was removed and prepared for HPLC as described in Figure 68. Statistical analysis was conducted using a two sample t-test assuming equal variance comparing HS applied at 1-5µg with pre-binding HS (n=2 for 1 and 2µg, n=3 for 5µg and pre-binding HS). 5µg HS had significantly higher UA2S-GlcNS6S (**) and total 6S (***) and significantly lower UA-GlcNAc (*) compared to pre-binding HS (P=0.05).

To determine whether UV treatment necessary for ES cell culture alters HS binding, 1 cm² sections ppAm microfibre scaffolds were treated with UV for ten minutes each side and incubated in 1 and 5µg HS alongside untreated controls. Bound HS was removed and prepared for RP-HPLC as described in Figure 68. UV treated scaffolds bound 2.7µg (5µg applied HS) and 0.7µg (1µg applied HS) compared to 2.2µg (5µg applied HS) and 0.6µg (1µg applied HS) bound to non-treated controls Figure 71. The increased binding to UV treated scaffolds could be attributed to variability between scaffolds, between quality of ppAm coating and also variation introduced as the samples are being processed. This variation could also be responsible for the amount binding at 5µg applied HS being greater than the previously determined 1-2µg saturation level.
Figure 71. UV treatment of ppAm microfibre meshes: Quantification. 1cm² sections of ppAm microfibre scaffolds were treated with UV light for 10 minutes each side and then incubated overnight with 1 and 5µg HS in 500µl PBS. Bound HS was removed and prepared for HPLC as described in Figure 68. The total amount of bound HS was estimated by comparing the total fluorescence of the detached HS with a 5µg control (referred to as pre-binding HS) that simultaneously underwent the same preparation procedure.

Composition of bound HS to UV treated microfibre scaffolds did not appear to greatly differ from non-treated controls (Figure 72). The results differed from previous findings, as the preferential binding of more sulphated species at 5µg applied HS observed in Figure 70 was not as prominent. However, there was still the characteristic increase of total 6S on UV treated (51.5%) and non-treated (55.3%) scaffolds at 5µg applied HS. The absence of UA-GlcNAc6S at 1µg applied HS is due to the inability to discern between the UA-GlcNAc6S peak and the AMAC related peak at 24.5-25 minutes. This in turn contributes to the lower total 6S observed at 1µg applied HS. As there was no marked difference between UV and non-treated scaffolds, it would appear that UV sterilisation has minimal/no affect on the capacity for ppAm microfibre scaffolds to bind HS.
Figure 72. **UV treatment of ppAm microfibre meshes: Disaccharide analysis and total sulphation.** The disaccharide composition (A) and total sulphation (B) of HS bound to UV treated scaffolds was calculated and compared with pre binding HS. Bound HS was removed and prepared for HPLC as described in Figure 68.

### 4.2.2 Retention of Bound HS

\(^3\text{H}\) radiolabelled HS (\(^3\text{H}\) HS) was used to test the longevity of HS binding to ppAm microfibre scaffolds in N2B27 neural differentiation media and PBS. 1cm\(^2\) pieces of ppAm and uncoated microfibre scaffolds were UV sterilised and incubated overnight with \(^3\text{H}\) HS in 1.5ml eppendorf tubes. After \(^3\text{H}\) HS immobilisation, samples were washed twice with PBS before incubation in either PBS or N2B27 for 10 days at 37°C. The amount of \(^3\text{H}\) HS present on ppAm microfibre scaffolds was analysed after 0, 1, 5 and 10 days incubation. At each time point, scaffolds were washed and the amount of \(^3\text{H}\) HS assessed before scaffolds were discarded. As materials were limited, only one scaffold per time point, per condition was analysed. In order to replicate culture conditions, the surrounding media was replaced every other day, with scaffolds washed once at each media change. Removed media and all washes were analysed for the presence of \(^3\text{H}\) HS in order to account for any \(^3\text{H}\) HS lost from the scaffolds.
After HS incubation (day 0) ppAm microfibre mesh radioactivity levels were 1370cpm compared to 9cpm on the uncoated microfibre scaffold, supporting RP-HPLC analysis revealing a lack of HS immobilisation on uncoated microfibre scaffolds. During incubation in PBS, no consistent loss of $^3$H HS from the scaffolds was observed with $^3$H HS maintained between 1240cpm (day 5) and 1590cpm (Day 10) (Figure 73).

**Figure 73. Retention of $^3$H HS ppAm microfibre meshes during 10 day incubation in PBS.**

ppAm microfibre meshes were incubated with $^3$H HS overnight. After washing twice with PBS, scaffolds were incubated in PBS for 10 days at 37°C. Surrounding PBS was also removed every two days, with scaffolds washed once at each media change. At selected time points, scaffolds were washed once with PBS and the amount of $^3$H HS present on the scaffolds recorded (red bars). The amount of $^3$H HS within surrounding PBS and all washes were also recorded (blue bars).

As in PBS, no consistent loss of counts was observed from ppAm microfibre scaffolds incubated in N2B27 over ten days, with $^3$H HS maintained above 1140cpm (Figure 74). These results indicate that $^3$H HS was retained by ppAm microfibre scaffolds throughout the ten day culture period in PBS or N2B27.
Figure 74. Retention of $^3$H HS ppAm microfibre meshes during 10 day incubation in N2B27. ppAm microfibre meshes were incubated with $^3$H HS overnight. After washing twice with PBS, scaffolds were incubated in N2B27 for 10 days at 37°C. Surrounding N2B27 was also removed every two days, with scaffolds washed once at each media change. At selected time points, scaffolds were washed once with N2B27 and the amount of $^3$H HS present on the scaffolds recorded (red bars). The amount of $^3$H HS within surrounding N2B27 and all washes were also recorded (blue bars).

However, $^3$H HS was detected in media and washes from ppAm microfibre scaffolds incubated in both PBS and N2B27. The total $^3$H HS lost from PBS incubated samples to media changes and washes after 10 days was 420cpm. In contrast, the total $^3$H HS lost from meshes incubated in N2B27 was 1360cpm, a value comparable to the 1370cpm recorded on day 0 scaffolds. However, despite this accumulative loss of $^3$H HS, the amount on the scaffolds remained high throughout the incubation period, suggesting the $^3$H HS was released from another source.

In addition, not all $^3$H HS applied to ppAm microfibre meshes was accounted for. Despite approximately 10,000cpm of $^3$H HS being added to each scaffold, average total counts recovered from ppAm microfibre samples was 3480cpm compared to 9380cpm recovered from uncoated microfibre meshes (Figure 75).

As all fluid which came into contact with the scaffolds was measured for $^3$H HS, it was hypothesised that the ppAm coating may have detached from the scaffold and adhered to the eppendorf wall, resulting in $^3$H HS attaching to the inside of the eppendorf tube. As the eppendorf tube could not be read by the scintillation counter the $^3$H HS immobilised on the detached coating would not be recorded, resulting in a reduced total count compared to uncoated microfibre meshes. If this were the case, then the $^3$H HS detected in surrounding media and washes could have originated from this unstable, detached ppAm rather than from the ppAm microfibre scaffolds.
Figure 75. Total HS counts recorded on microfibre and ppAm microfibre scaffolds. Total counts are an average of all readings recorded for each individual scaffold including counts present on the scaffold, in unbound $^3$HHS and in the surrounding N2B27/PBS and PBS washes.
4.2.3 Coating Stability

In order to investigate the possible detachment of the ppAm layer, a 1cm² piece of ppAm microfibre scaffold was pre-soaked in PBS in a scintillation tube overnight as depicted in Figure 76. It was then washed twice with PBS before moving to a second tube. Both the vacated tube and the scaffold were then incubated with $^3$H HS. An unsoaked ppAm microfibre scaffold was incubated alongside. Both soaked and pre-soaked scaffolds were moved to fresh scintillation tubes before the amount of $^3$H HS was read.

![Figure 76. Method for assessing $^3$H HS binding to pre-soaked ppAm microfibre scaffolds.](image)

A 1cm² piece of ppAm microfibre scaffold was pre-soaked in PBS overnight in a scintillation tube (Tube A). The scaffold was then washed twice with PBS before being moved to a fresh tube (Tube B). Tube A and the scaffold in Tube B were both incubated in $^3$H HS overnight. The pre-soaked ppAm microfibre scaffold was then moved to third tube (Tube C). Scintillation fluid was added to tubes A, B and tube C containing the ppAm microfibre mesh. Results were compared with an unsoaked control ppAm microfibre scaffold which was soaked overnight in $^3$H HS in tube D and then moved to a second tube (Tube E). Scintillation fluid was then added to tube D and tube E containing the unsoaked ppAm microfibre scaffolds. All fluid which came into contact with the scaffolds was kept and analysed for $^3$H HS.

The tube in which the ppAm microfibre scaffold was soaked (tube A) bound 1890cppm $^3$H HS (Figure 77, $\Upsilon$). Tube B in which the pre-soaked scaffold was incubated with $^3$H HS contained 120cppm (Figure 77, ¶). This difference suggests that during the soak, some of the ppAm coating detached from the scaffold and stuck to the wall of tube A, enabling the binding of $^3$H HS. Once this unstable layer of ppAm coating had detached, very little was removed during HS incubation resulting in the low counts present in tube B. This is supported by the 1680cppm present in tube D (Figure 77, φ), in which the unsoaked scaffold was incubated with $^3$H HS. Removal of the unstable ppAm by pre-soaking in PBS did not reduce binding of $^3$H HS to the ppAm microfibre mesh, in fact a slight increase was observed with 2070cppm present on the soaked scaffold (Figure 77, ¥) and 1650cppm recorded on the unsoaked control (Figure 77, ¥).
Figure 77. Radiolabelled HS binding to pre-soaked ppAm microfibre scaffolds. Assessment of $^3$H HS binding to pre-soaked ppAm microfibre scaffolds and unsoaked ppAm microfibre scaffolds. Samples were treated as depicted in Figure 76.

To test whether pre-soaking ppAm microfibre meshes affected the amount and type of HS that bound to the scaffold, a 1 cm$^2$ piece of ppAm microfibre mesh was soaked overnight in 500 µl PBS, before incubation with 5 µg HS. An unsoaked ppAm microfibre mesh was incubated with 5 µg alongside the soaked scaffold. Both pre-soaked and unsoaked samples were washed in PBS and moved to fresh wells before bound HS was competed off with 1 ml 4M NaCl. Removed HS was treated as described in Figure 68.

Disaccharide composition of HS bound to soaked scaffolds was largely similar to HS bound to untreated meshes, except for an increase in UA2S GlcNS6S (+4.3%) and a decrease in UA2S GlcNS (-2.7%, Figure 78). The composition of bound HS was similar to previous results, with a prominent increase in tri-sulphated disaccharide UAS GlcNS6S (+11.0% pre-soak, +6.7% unsoaked) and a decrease in the non-sulphated unit UA-GlcNAc (-8.2% pre-soak, -7.5 unsoaked).

The total sulphation of HS bound to soaked and unsoaked scaffolds revealed the characteristic increase in total N-sulphation (+10.7% pre-soak, +9.6% unsoaked), 2-sulphation (+8.0% pre-soak, +6.4% unsoaked), and 6-sulphation (+11.6% pre-soak, +7.3% unsoaked), accompanied by a concomitant decrease in total N-acetylation (-10.7% pre-soak, -9.6% unsoaked) observed previously at 5 µg applied HS.
Figure 78. HPLC analysis of HS bound to pre-soaked scaffolds. A 1cm$^2$ piece of ppAm microfibre scaffold was immobilised in a 24 well plate with a scalfex and soaked overnight in 1 ml PBS. The scaffold was then immobilised in a fresh scalfex and moved to a neighbouring well where it was soaked in 5µg HS in 500µl PBS overnight, alongside an unsoaked scaffold. The scaffolds were then washed three times in PBS before being moved to a fresh well for incubation in 1 ml 4M NaCl for 30 minutes. The detached HS was then desalted and prepared for HPLC as described in Figure 68. The disaccharide composition (A) and total sulphation (B) of HS bound to both soaked and unsoaked scaffolds was characterised and compared with pre-binding HS (5µg HS which simultaneously underwent the same processing procedures as the HS bound to the scaffolds).

Pre-soaked scaffolds bound 3.7µg of HS and untreated scaffolds bound 2.5µg, higher levels than previously observed. To assess the saturation level of pre-soaked ppAm microfibre mesh, 1cm$^2$ pieces of ppAm microfibre mesh were incubated overnight in 500µl PBS and moved to fresh wells for incubation in 1, 2 and 5 µg HS. Scaffolds were washed twice in PBS before being moved to fresh wells for incubation in 1ml 4M NaCl. Samples were processed as described in Figure 68. Soaked ppAm microfibre scaffolds bound a maximum of 1.1µg at 5µg applied HS (Figure 79), indicating that the saturation level remains at the 1-2µg/cm$^2$ level previously estimated in Figure 69.
Figure 79. **Loading capacity of pre-soaked ppAm microfibre scaffolds.** 1cm² pieces of ppAm microfibre scaffold were pre-soaked as described in Figure 78 and incubated with 1, 2 and 5 µg HS. HS bound to both pre-soaked scaffolds was removed and prepared for HPLC analysis as described in Figure 68. The total amount of bound HS was estimated by comparing the total fluorescence of the detached HS with a 5ug control that simultaneously underwent the same preparation procedure.

Disaccharide analysis in Figure 80 reveals a profile similar to that of unsoaked scaffolds in Figure 68, with an increase percent composition of the tri-sulphated disaccharide (+14.6%) and a decrease in the non-sulphated disaccharide (-12.6%) at 5µg applied HS (Figure 80). However, the increased percent composition of UA2S-GlcNS6S is also present at 1µg (+6.14%) and 2µg (+4.7%) HS, as is the decrease in UA-GlcNAc (-4.6% 1µg, -3.8% 2µg). The increased percent composition of more sulphated species of HS bound at 1 and 2µg HS is also evident in total sulphation analysis, with increases in total N-sulphation (+5% 1µg, +3.8% 2µg), total 6-sulphation (+5.9% 1µg, +6.3% 2µg) and total 2-sulphation (+6.2% 1µg, +4.2% 2µg) accompanied by a decrease in total N-acetylation (-5.0% 1µg, -3.8% 2µg). This suggests that soaking ppAm microfibre meshes and removing the unstable ppAm layer may enhance the selective binding of more sulphated species from the heterogeneous HS preparation.
Figure 80. Loading capacity of pre-soaked ppAm microfibre scaffolds: Disaccharide analysis and total sulphation of bound HS. 1cm² pieces of ppAm microfibre scaffold were pre-soaked as described in Figure 78 and incubated with 1, 2 and 5 µg HS. HS bound to both pre-soaked scaffolds was prepared for HPLC analysis as described in Figure 68. The disaccharide composition (A) and total sulphation (B) of HS bound to both soaked and unsoaked scaffolds was characterised and compared with pre-binding HS.

4.3 Ligand Binding

4.3.1 ScFv Binding

The ability of phage display derived ScFv antibodies to bind specific sulphation motifs within the HS/heparin chain makes them an excellent tool to assess whether these motifs are accessible and unchanged when the GAGs are immobilised on the scaffold surface. The ScFv antibodies were created by biopanning phage display libraries (expressing human antibodies) against various HS preparations and have been subsequently used to identify sub-populations of HS e.g. within skeletal muscle (Jenniskens et al., 2000), human lung tissue (Smits et al., 2004) and the rat kidney basement membrane (van Kuppevelt et al., 1998). The binding of ScFv antibodies is dependent on the presence of N- and O- sulphation within heparin and HS chains (Jenniskens et al., 2000; Smits et al., 2004; van Kuppevelt et al., 1998). The antibodies used here demonstrate strict specificity
towards heparin and HS with no reactivity observed with other GAGs (e.g. DS and CS) (Jenniskens et al., 2000; Smits et al., 2004; van Kuppevelt et al., 1998). By using a secondary biotinylated antibody, the ScFv antibodies were used in the assay depicted in Figure 81.

Figure 81. ScFv binding assay to immobilised heparin/HS. Step 1: Heparin was immobilised on ppAm coated surfaces by overnight incubation with a gradient of heparin concentrations dissolved in PBS. Step 2: After overnight incubation, samples were blocked for 90 minutes in 1% BSA at 37°C and incubated with 100µl ScFv antibody (1:10 dilution) for 1 hour. Step 3: Samples were incubated with 100µl biotinylated secondary antibody raised against the vesicular stomatitis virus G protein (VSV-G) tag of the ScFv (1:1000 dilution) for 1 hour. Step 4: Samples were incubated with ExtrAvidin Alkaline Phosphatase for 30 minutes enabling binding of the avidin component to the biotin covalently linked to the secondary antibody. Step 5: Development substrate (1mg/ml p-nitrophenylphosphate) was added to the samples. Enzymatic cleavage of the p-nitrophenylphosphate to p-nitrophenyl by the immobilised alkaline phosphatase created a colorimetric change that was read as absorbance at 405nm.

As the results presented here are preliminary, and the changes in absorbance with increasing heparin/HS has not been fully assessed, the recorded absorbance values are connected by dotted/broken lines.

4.3.1.1 HS4C3 and NS4F5 Binding to Immobilised Heparin

To ascertain whether the modified assay was applicable, Epranex plates were incubated in 0-5000ng heparin and two ScFv antibodies, HS4C3 and NS4F5, were tested for their ability to bind the immobilised heparin (Figure 82). The HS4C3 binding site resembles the antithrombin binding pentasaccharide sequence and has a specific requirement for 3-O-sulphation (Smits et al., 2006; Ten Dam et al., 2006). NSF45 binding requires N-sulphation, epimerisation of glucuronic acid to iduronic acid and high 6-O and 2-O sulphation; all of which is present within the heparin chain.
(Wijnhoven et al., 2008). Plates were read at 10 minutes with absorbance set at 405nm. Results from the wells were averaged and calibrated against wells containing no heparin. The absorbance values for HS4C3 and NS4F5 binding are shown in Table 3 and Table 4 respectively.

![HS4C3 and NS4F5 Binding - Epranex Plate](image)

**Figure 82. HS4C3 and NS5F4 binding to heparin immobilised on Epranex plates.** Epranex 96 well plates were treated as described in Figure 81. Calibrated readings for HS4C3 (Table 3) and NS5F4 (Table 4) binding are plotted against applied heparin concentration at 10 minutes development.

<table>
<thead>
<tr>
<th>HSC4C3 Binding - Epranex Plate - 10 minutes</th>
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<tr>
<td>Applied Heparin (ng)</td>
</tr>
<tr>
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</tr>
<tr>
<td>Well 2</td>
</tr>
<tr>
<td>Average</td>
</tr>
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**Table 3. HS4C3 binding to heparin immobilised on Epranex plates.** Epranex plates were treated as described in Figure 81 with HS4C3 as the primary ScFv antibody (1:10 dilution). Plates were read at 10 minutes with absorbance set at 405nm. Results from the wells were averaged and calibrated against wells containing no heparin.
Table 4. **NS5F4 binding to heparin immobilised on Epranex plates.** Epranex plates were treated as described in Figure 81 with NS5F4 as the primary ScFv antibody (1:10 dilution in SAB6). Results from the wells were averaged and calibrated against wells containing no heparin. Plates were read at 10 minutes with absorbance set at 405nm.

An increase in absorbance was observed with heparin concentrations above 100ng. Absorbance formed a plateau at 500ng, with maximum absorbance values recorded at maximum heparin concentrations for both HS4C3 and NS4F5 (Figure 82).

0.75 cm by 0.75 cm pieces of ppAm microfibre scaffolds were placed in non tissue culture treated 96 well plates and incubated in 0-5000ng heparin. HS4C3 binding was assayed as described in Figure 81. As the scaffolds would interfere with the absorbance measurement, 85µl of the developing sample was removed and added to a fresh 96 well plate at 5 and 10 minutes development. Results from the wells were averaged and corrected against wells containing no heparin (Table 5).

<table>
<thead>
<tr>
<th>Applied Heparin (ng)</th>
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<th>500</th>
<th>1000</th>
<th>5000</th>
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<td>0.1305</td>
<td>0.5730</td>
<td>0.5830</td>
<td>0.6285</td>
</tr>
<tr>
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<td>-0.0210</td>
<td>0.4215</td>
<td>0.4315</td>
<td>0.4770</td>
</tr>
</tbody>
</table>

**Table 5. HS4C3 binding to heparin immobilised on ppAm microfibre meshes.** 75cm x 0.75cm pieces of ppAm microfibre scaffolds were placed in the wells of a 96 well plate and soaked overnight in 200µl PBS containing 0 – 5000ng heparin at RT. Samples were treated as described in Figure 81, with HS4C3 serving as primary ScFv antibody (1:10 dilution)

A no primary antibody control was also included to assess non specific binding of HS4C3. Samples minus HS4C3 contained low levels of background absorbance (0.183 – 0.242) at all heparin concentrations. However, uncorrected absorbance levels of samples incubated with just PBS reached an average absorbance of 0.674 at 10 minutes development. This high absorbance was not present in no primary controls indicating non-specific binding of HS4C3 to the scaffold.
When readings were corrected against samples with no heparin, an increase in absorbance with increasing heparin concentration was observed, reaching a maximum of 0.524 at 5000ng applied heparin (Figure 83).

![HS4C3 Binding - ppAm Microfibre Meshes](image)

*Figure 83. HS4C3 binding to heparin immobilised on ppAm microfibre meshes.* Calibrated readings for HS4C3 binding (Table 5) plotted against applied heparin concentration at 10 minutes development.

NS4F5 binding to ppAm microfibre scaffolds revealed similar results, with wells without any heparin producing absorbance values of 0.5580 after 10 minutes development (Table 6). Omission of NS4F5 reduced absorbance to 0.081 - 0.108 suggesting that, as with HS4C3, non-specific binding of NS4F5 was occurring.

<table>
<thead>
<tr>
<th>Applied Heparin (ng)</th>
<th>0</th>
<th>500</th>
<th>5000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesh 1</td>
<td>0.595</td>
<td>0.984</td>
<td>0.930</td>
</tr>
<tr>
<td>Mesh 2</td>
<td>0.521</td>
<td>0.848</td>
<td>0.897</td>
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<tr>
<td>Mesh 3 - no 1º</td>
<td>0.082</td>
<td>0.108</td>
<td>0.108</td>
</tr>
<tr>
<td>Average</td>
<td>0.6530</td>
<td>0.9160</td>
<td>0.9135</td>
</tr>
<tr>
<td>Calibrated</td>
<td>0</td>
<td>0.3580</td>
<td>0.3550</td>
</tr>
</tbody>
</table>

*Table 6. NS4F5 binding to heparin immobilised on ppAm microfibre meshes.* 75cm x 0.75cm pieces of ppAm microfibre scaffolds were placed in the wells of a 96 well plate and soaked overnight in 200µl PBS containing 0 – 5000ng heparin at RT. Samples were treated as described in Figure 81, with NS4F5 serving as primary ScFv antibody (1:10 dilution)

Calibrated readings showed an increase in absorbance at 500ng and 5000ng heparin (Figure 84). However, unlike HS4C3 binding appeared to level off at 500ng, reaching a maximum of 0.3580.
Figure 84. NS4F5 binding to heparin immobilised on ppAm microfibre meshes. Calibrated readings for NS4F5 (Table 6) binding plotted against applied heparin concentration at 10 minutes development.

To try and reduce non-specific binding of ScFv antibodies, increasing concentrations of BSA block were tested on ppAm microfibre scaffolds incubated overnight in PBS minus heparin. Increasing block concentration to 5% almost halved absorbance after 10 minutes from 0.364 (1%) to 0.189 (Figure 85). Increasing block concentration to 10% and 15% reduced absorbance after 10 minutes to 0.155 and 0.13 respectively.

Figure 85. Increased BSA block concentration. 0.75 cm x 0.75 cm sections of ppAm microfibre meshes were immobilised in 96 well plates and incubated in 100µl PBS overnight at RT. Samples were then incubated in 200µl 1, 5, 10 and 15% BSA block in SAB6. Scaffolds were then treated as described in Table 5 with NS4F5 serving as the primary ScFv antibody at a 1:10 dilution.

Binding of NS4F5 was repeated with a 5% BSA block. 5% concentration was chosen as it halved non-specific absorbance and higher concentrations may have interfered with ScFv binding to
HS/heparin motifs. Due to the coating stability issues raised in 4.2.3, scaffolds were moved to fresh wells before the development substrate was added to ensure that any absorbance recorded was emitted from the scaffolds and not detached coating. Development substrate was also added to the vacated wells to assess binding of ScFv to any detached ppAm coating within the well.

Scaffolds moved to fresh wells produced low absorbance values of 0.075 and 0.09 when scaffolds were incubated in just PBS (Table 7). This is in comparison to the corresponding vacated wells, with recorded values of 0.173 and 0.397 at 5 and 10 minutes development. This suggests that the observed non specific binding of ScFv antibodies may have been to detached ppAm adsorbed to the inside of the well, rather than non-specific binding to the scaffolds.

<table>
<thead>
<tr>
<th>NS4F5 Binding - 1:10 dilution – ppAm Microfibre Meshes – Moved Scaffolds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Applied Heparin (ng)</td>
</tr>
<tr>
<td>Vacated Wells</td>
</tr>
<tr>
<td>Scaffolds</td>
</tr>
<tr>
<td>Scaffolds Calibrated</td>
</tr>
<tr>
<td>5 minutes</td>
</tr>
<tr>
<td>Applied Heparin (ng)</td>
</tr>
<tr>
<td>Vacated Wells</td>
</tr>
<tr>
<td>Scaffolds</td>
</tr>
<tr>
<td>Scaffolds Calibrated</td>
</tr>
<tr>
<td>10 minutes</td>
</tr>
</tbody>
</table>

Table 7. NS4F5 binding to heparin immobilised on ppAm microfibre meshes – Scaffold Move. 75cm x 0.75cm pieces of ppAm microfibre scaffolds were placed in the wells of a 96 well plate and soaked overnight in 200µl PBS containing 0 – 5000ng heparin at RT. Samples were treated as described in Figure 81, with NS4F5 serving as primary ScFv antibody (1:10 dilution). Scaffolds were moved to fresh wells before development substrate p-nitrophenylphosphate was added.

Scaffolds transferred to fresh wells produced absorbance values that increased with increasing applied heparin. Calibrated values reached a maximum value of 0.048 (5 minutes development) and 0.217 (10 minutes) at 5000ng applied heparin (Figure 86). These results indicate that the heparin was bound to the ppAm microfibre scaffolds in a form that exposed the necessary sulphation epitopes to ligand interaction.
Figure 86. NS4F5 binding to heparin immobilised on ppAm microfibre meshes – Scaffold Move. Calibrated readings for NS4F5 binding to moved scaffolds (Table 7) plotted against applied heparin concentration at 5 and 10 minutes development.

4.3.1.2 RB4EA12 Binding to E14 and Sulf1/2−/− ES cell HS

Sulf1 and Sulf2 are 6-O-endosulfatases that post-translationally modify HS chains by selective removal of 6-O-sulphate groups. Primarily acting on di-sulphated (UA-GlcNS6S) and tri-sulphated (UA2S-GlcNS6S) disaccharides within highly sulphated S domains (Lamanna et al., 2006; Lamanna et al., 2008), Sulf1 and Sulf2 influence the binding of growth factors such as FGF2 and VEGF (Narita et al., 2006; Uchimura et al., 2006).

Loss of both enzymes in MEFs resulted in significant increases in all 6-O-sulphated disaccharides (UA-Glc6S, UA-GlcNS6S and UA2S-GlcNS6S) (Lamanna et al., 2006; Lamanna et al., 2008). RB4EA12 ScFv antibody preferentially binds HS rich in IdoA-GlcNS(6S) (Dennissen et al., 2002) and has a specific requirement for 6-O-sulphation of the glucosamine residue (Lamanna et al., 2006). To investigate the specificity of ScFv binding, RB4EA12 binding to HS derived from E14 and Sulf1−/−/Sulf2−/− ES cells was assessed on both Epranex plates and ppAm microfibre scaffolds.

HS was derived from E14 and Sulf1−/−/Sulf2−/− cells as described in section 2.4.5. Briefly, both cell types were cultured on gelatin in ES media supplemented with 10% KSR instead of FCS for at least 4 passages. Cells were washed twice with PBS and rocked for 2 hours with 1% Triton in PBS. Cell extract (and pooled media for E14 cells) was pronase treated for 4 hours at 37°C before being run through a pre-equilibrated DEAE column. After washing with 0.25M NaCl to remove any loosely bound material, sulphated GAGs were eluted in 5 ml 1.5M NaCl. Eluted GAGs were desalted on a PD10 column and freeze dried before being prepared for RP-HPLC analysis as described in section 4.2.1.

The loss of Sulf1 and Sulf2 enzymes resulted in 3.9% increase in tri-sulphated disaccharide UA2S-GlcNS6S compared to E14 HS (Figure 87). This contributed to a total 6 sulphation of 13.4% compared to 11.6% within E14 HS. The increase in tri-sulphated disaccharides is in agreement
with previous findings. This in turn contributed to an increase in total 2-sulphation (+3.15%), total N-sulphation (+3.3%) and a complimentary decrease in N-acetylation (-3.3%) Contrary to previous findings, there was no observed increase in di-sulphated (UA-GlcNS6S) or (UA-GlcNAc6S) monosulphated disaccharides. This could be attributed to the differences in the repertoire of biosynthetic enzymes and HS composition between mouse ES cells and MEFs. However, the increase in tri-sulphated disaccharides confirmed that Sulf1^{-/-}/Sulf2^{-/-} HS would be effective in determining the differential binding of ScFv antibodies to sulphation motifs on the immobilised HS.

**Figure 87. Disaccharide composition and total sulphation analysis of E14 and Sulf1^{-/-}/Sulf2^{-/-} HS.** Extracted HS was prepared for HPLC analysis as described in 4.2.1 and disaccharide composition (A) and total sulphation (B) for both HS preparations calculated and compared.

The quantity of total HS extracted from each preparation was estimated by comparing the total fluorescence of the detached HS with a 5μg HS control that simultaneously underwent the same processing procedure. Both HS preparations were initially immobilised on Epranex plates at 0-200ng/well (Figure 88). Absorbance values calibrated against wells containing no HS revealed an increase in absorbance levels with increasing Sulf1^{-/-}/Sulf2^{-/-} HS concentrations. Maximum values of 1.533 were recorded for Sulf1^{-/-}/Sulf2^{-/-} at 200ng applied HS. These values are particularly high when compared to E14 HS which produced a maximum absorbance value of 0.743 at 100ng applied HS.
Figure 88. RB4EA12 binding to E14 and Sulf1<sup>+/−</sup>/Sulf2<sup>+/−</sup> HS immobilised on Epranex plates. Epranex plates were incubated overnight in 200μl PBS containing 0 – 200ng E14 or Sulf1<sup>+/−</sup>/Sulf2<sup>+/−</sup> HS. After overnight incubation, samples were blocked with 5% BSA at 37°C for 90 minutes. Blocked wells were incubated for 1 hour in 100μl RB4EA12 ScFv antibody (1:100 dilution in SAB6). Wells were then treated as described in Figure 81.

To investigate whether a similar increased binding of RB4EA12 to Sulf1<sup>+/−</sup>/Sulf2<sup>+/−</sup> HS was observed on ppAm microfibre scaffolds, scaffolds were immobilised in 96 well plates and incubated overnight with E14 and Sulf1<sup>+/−</sup>/Sulf2<sup>+/−</sup> HS (0-200ng/scaffold). Scaffolds were pre-soaked in 100μl PBS to remove any unstable coating before being moved to fresh wells for HS immobilisation. As in Figure 86, scaffolds were moved to fresh wells before the development reagent was added to ensure readings originated from the scaffolds themselves (Figure 89).
Figure 89. RB4EA12 binding to E14 and Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS immobilised on ppAm microfibre scaffolds. 0.75cm x 0.75cm sections of ppAm microfibre meshes were pre-soaked overnight in 100ul PBS. Scaffolds were moved to fresh wells before incubation overnight in 0 – 200ng E14 or Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS. After overnight incubation, samples were blocked with 5% BSA at 37°C for 90 minutes. Blocked wells were incubated for 1 hour in 100µl RB4EA12 ScFv antibody (1:100 dilution in SAB6). Wells were then treated as described in Figure 81. Scaffolds were moved to fresh wells before the development substrate was added. 85µl of the developing sample was removed and added to a fresh 96 well plate with absorbance read at 405nm at 20 minutes. Results from the wells were averaged and calibrated against wells containing no heparin.

As with Epranex plates, increased absorbance values were observed for ppAm microfibre scaffolds incubated with Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS. Absorbance values increased with applied Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS concentration, reaching a maximum of 0.811 at 200ng applied HS after 20 minutes development. This was compared to a maximum of 0.202 at 200ng applied E14 HS.

To investigate whether binding was in response to the different sulphation patterns on the HS chain, EV3C3 was used as the primary antibody to detect E14 and Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS bound to Epranex plates. Binding of EV3C3 is inhibited by 6-O-Sulpation (Dennissen et al., 2002) and reduced binding was observed on Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) MEFs compared to wildtype controls. However, equal binding of EV3C3 binding to E14 and Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) mouse ES cells has been observed (Dr. Rebecca Holley, personal communication). Therefore, equal or lower binding of EV3C3 to Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) would be expected compared to E14 HS.
Figure 90. EV3C3 binding to E14 and Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> HS immobilised on Epranex plates.

Epranex plates were incubated overnight in 200ul PBS containing 0 – 200ng E14 or Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> HS. After overnight incubation, samples were blocked with 5% BSA at 37°C for 90 minutes. Blocked wells were incubated for 1 hour in 100µl EV3C3 ScFv antibody (1:100 dilution in SAB6). Wells were then treated as described in (Figure 81).

Increased absorbance values were recorded with increasing Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> HS concentrations, reaching a maximum of 0.849 at 200ng after 20 minutes development (Figure 90). This is in comparison to a maximum absorbance value of 1.533 observed with RB4EA12 binding to Epranex plates after 20 minutes development. A marked reduction in absorbance was also observed with E14 HS, reaching a maximum of 0.057 at 200ng after 20 minutes development.

Therefore, it appears that differential binding of ScFv antibodies to immobilised HS is occurring as indicated by the reduced binding of RB4EA12 to E14 HS compared to Sulf1<sup>-/-</sup>/Sulf2<sup>-/-</sup> HS and the reduced binding of EV3C3. However, the dramatic drop in EV3C3 binding to E14 HS indicates that binding is occurring in a manner not easily predicted by their specified epitopes.

4.3.2 bLink_TSG-6 Binding

The link module of tumour necrosis factor stimulated gene-6 (TSG-6) has the ability to bind heparin, HS (Mahoney et al., 2005) and HA (Mahoney et al., 2001). The Link module has been successfully used to assess heparin immobilisation on commercially available ppAm coated 96 well plates (Epranex plates) (Mahoney et al., 2004) and is depicted in Figure 91.
The assay was applied to 0.75 cm by 0.75 cm pieces of scaffold placed in wells of an uncoated, non-tissue culture-treated 96-well plate. Epranex plates were used as positive controls, while empty wells of the uncoated 96-well plate served as negative controls. ppAm microfibre scaffolds soaked in 200 μl PBS overnight were also included in the assay. The soaked scaffolds were moved to fresh wells before being incubated with heparin.

**Figure 91. bA-Link_TSG-6 Binding Assay.** Step 1: 0.75 cm x 0.75 cm pieces of microfibre and ppAm microfibre scaffolds were placed in the wells of a 96-well plate. Scaffolds, empty 96-well wells and Epranex plate wells were soaked overnight with PBS containing 0 – 5000 ng heparin at RT. Step 2: After overnight incubation, wells were incubated for 90 minutes with 1% BSA at 37°C. After blocking with BSA, samples were incubated with biotinylated Link_TSG-6 (bA-Link_TSG6) for 3 hours (2 pmol/well). Step 3: Samples are incubated with ExtrAvidin Alkaline Phosphatase (1:10,000 dilution in SAB6) for 30 minutes enabling binding of the avidin component to the biotin covalently linked to the Link_TSG-6. Step 4: Developmental substrate (1 mg/ml p-nitrophenylphosphate) was added to the samples. Enzymatic cleavage of the p-nitrophenylphosphate to p-nitrophenyl by the immobilised alkaline phosphatase created a colorimetric change that was read as absorbance at 405 nm. Samples were washed twice in 200 μl SAB6 between each step.
Figure 92. bA-Link_TSG-6 binding to heparin immobilised on ppAm microfibre scaffolds.

Samples were prepared and treated as described in Figure 91. Epranex plates served as positive controls, whilst empty wells of standard non tissue culture treated 96 well plates in which scaffolds were immobilised served as negative controls. As the presence of the scaffold would interfere with the reading, 85 µl of the developing sample was removed and added to a fresh 96 well plate. As absorbance values for scaffolds were plotted alongside Epranex plates, the reduction in volume was compensated for by multiplying the absorbance by 2.35. Scaffolds were moved to fresh wells before the development substrate was added. Absorbance was read at 405nm after 20 minutes development time.

Uncoated microfibre scaffolds exhibited very low absorbance values, reaching a maximum OD of 0.08 at 1000ng heparin (Figure 92). No dose dependent increase in absorbance was observed indicating very little/no heparin binding to the uncoated scaffold, as supported by RP-HPLC and radioactivity data. No heparin bound in a form accessible to bA-Link_TSG-6 binding on 96 well plates with no scaffold present. Epranex plates displayed dose dependent increases in absorbance with increasing heparin concentration, forming a plateau at 1000ng and reaching a maximum absorbance of 0.63 at the maximum heparin concentration of 5000ng. ppAm microfibre scaffolds had a similar fluorescence profile as Epranex plates. Absorbance increased with heparin concentration, forming a plateau at 1000ng and reaching a maximum absorbance of 0.62 at 5000ng heparin, indicating heparin was bound in a form accessible to ligand binding. ppAm microfibre scaffold pre-soaked in PBS demonstrated less absorbance and therefore less bA-Link_TSG-6 binding compared with unsoaked scaffolds. An increase in absorbance was observed, forming a plateau at 500ng and reaching a maximum of 0.29 at 5000ng, suggesting a lower proportion of immobilised heparin was bound in a ligand accessible conformation.
4.4 ES Cell Culture

4.4.1 ES Cell Culture

To assess the ability of ppAm microfibre scaffolds to support ES cell culture, 1.5 cm by 1.5 cm ppAm microfibre and uncoated microfibre scaffolds were immobilised in 24 well plates and soaked in either PBS or HS (1µg/cm² in PBS). Scaffolds were then incubated in standard ES media overnight before Oct4-GFP cells (5 x 10⁴ cells/cm²) were seeded on the scaffold surface. Cells were cultured in standard ES media for 7 days. Viability was assessed using a LIVE/DEAD assay (Figure 93). Very little cell death occurred on both ppAm microfibre and uncoated microfibre scaffolds with and without HS. Cells formed aggregates on all surfaces by day 3, which covered the scaffold surface by day 7.

Figure 93. Viability of Oct4-GFP cells on ppAm microfibre meshes. Oct4-GFP ES cells were seeded at 5 x 10⁴ cells/cm² and cultured over 7 days in standard ES media. LIVE/DEAD staining was used to differentiate between live (green) and dead (red) cells. Live cells fluoresce green due to esterase activity within the cell cleaving calcein AM. The intact membrane of living cells is able to retain the resulting fluorescence. Dead cells allow the entrance of ethidium homodimer 1 which binds to nucleic acid, resulting in a 40 fold increase in red fluorescence. Ethidium homodimer 1 is excluded by the intact membrane of live cells. Stained cells were visualised by fluorescent microscopy.
To further assess cell spreading on the scaffolds, E14 cells were seeded at $1 \times 10^4$ cells/cm$^2$ on ppAm microfibre scaffolds (±/-HS), uncoated microfibre scaffolds and spin coated films. Cells were cultured for 7 days in standard ES media and processed for SEM analysis at days 3, 5 and 7 (Figure 94). Cells seeded on spin coated films could not be visualised using SEM, presumably because the dehydration procedure distorted the samples. On microfibre scaffolds the cells formed rounded aggregates in agreement with the morphology of Oct-GFP cells cultured on the same surface. Very little cell attachment was evident, with minimal spreading observed around the aggregates at day 7. In comparison, E14 cells cultured on ppAm microfibre scaffolds with and without immobilised HS showed a greater degree of cell spreading at all time points. Although the cells formed aggregates, anchorage structures were present on the outskirts of the clusters presumably facilitating attachment to the scaffold surface. The enhanced cell spreading is more prominent at day 7, when cells formed a flattened monolayer across the mesh. No difference was discernable between ppAm microfibre scaffolds with and without immobilised HS.

<table>
<thead>
<tr>
<th>Day3</th>
<th>Day 5</th>
<th>Day 7</th>
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</thead>
<tbody>
<tr>
<td>Microfibres</td>
<td>ppAm Microfibres</td>
<td>ppAm Microfibres +HS</td>
</tr>
<tr>
<td><img src="image" alt="Microfibres" /></td>
<td><img src="image" alt="ppAm Microfibres" /></td>
<td><img src="image" alt="ppAm Microfibres +HS" /></td>
</tr>
</tbody>
</table>

**Figure 94. SEM imaging of E14 cells on ppAm microfibre meshes.** E14 ES cells were seeded at $1 \times 10^4$ cells/cm$^2$ onto microfibre meshes and ppAm microfibres with and without immobilised HS. Cells were prepared for SEM at days 3, 5 and 7. Cells were also seeded onto spin coated films. However, spin coated samples did not reveal any cells attached to the surface indicating the samples were too distorted by the SEM preparation procedure.

To investigate further, cells were seeded as described for SEM analysis and stained for actin at days 3, 5 and 7 (Figure 95). In agreement with previous results, cells on microfibre scaffolds grew in tight aggregates on the scaffold surface with limited areas of cell spreading localised around the cell masses. On spin coated films, although tight aggregates were observed, cells spread across the film forming a monolayer by day 7. There was little difference between ppAm microfibre scaffolds with and without immobilised HS. Both had cells growing in tight aggregates at day 3 with
areas of cell spreading observed at day 5. By day 7 aggregates had grown and cells had spread forming a monolayer punctuated by large masses of cells.

![Figure 95. Actin staining of E14 cells seeded on ppAm microfibre meshes. E14 ES cells were seeded at 1 x 10^4 cells/cm^2 and cultured over 7 days in standard ES media. Cells were fixed with 4% paraformaldehyde at days 3, 5 and 7 and stained for actin (green).](image)

To determine whether growth on a particular substrate promoted the differentiation of mouse ES cells into a specific cell phenotype E14 cells were cultured on an array of surfaces in standard ES media minus LIF. Cells were seeded on standard gelatinised TCP, microfibre meshes, spin coated films, ppAm microfibres without heparin and ppAm microfibres with immobilised heparin. Also included in the experiment were ppAm TCP with and without immobilised heparin. Surfaces with heparin were incubated overnight in 0.5 µg heparin/cm^2 in PBS (+heparin), whilst ppAm surfaces without heparin were incubated in PBS (-heparin). ppAm TCP was gelatinised after heparin immobilisation. Cells were seeded at 1 x 10^4 cells/cm^2 and cultured for 10 days in standard ES media minus LIF. At days 0, 2, 4, 6, 8 and 10 cells were trypsinised, counted and pelleted for RNA extraction.

At each time point the total number of cells was counted, enabling proliferation on the scaffold surface to be assessed. Cell number increased until day 6 on all surfaces except for microfibre meshes and ppAm TCP +heparin where a decrease in cell number was observed between day 4
and day 6 (Figure 96). The immobilisation of heparin on ppAm coated surfaces appeared to improve cell proliferation. ppAm TCP +heparin reached a maximum cell number of $5.3 \times 10^6$ at day 6 compared to a maximum of $2.7 \times 10^5$ at day 4 on ppAm TCP –heparin. At day 6 ppAm microfibre meshes +heparin reached a maximum of $1.9 \times 10^6$ cells compared to $9.6 \times 10^5$ on ppAm microfibre meshes –heparin. The number of cells recovered from ppAm TCP +/- heparin and from gelatinised TCP was consistently greater compared to the PLGA scaffolds. This could be due to lower proliferation. However, scaffolds were mounted in Scaffdex CellCrowns making cell recovery more difficult.

![Figure 96. LIF withdrawal: Proliferation.](image)

**Figure 96. LIF withdrawal: Proliferation.** E14 cells were seeded onto gelatinised TCP, spin coated films, microfibres, ppAm microfibres, ppAm microfibres +heparin, ppAm TCP and ppAm TCP +heparin in standard ES media minus LIF and cultured for 10 days.

For each surface, trypsinised cells were pelleted at 700rpm for 3 minutes. The supernatant was removed and the pellet stored at -80°C. RNA was extracted and cDNA synthesised as described in section 2.4.7. Band intensity of the housekeeper gene GAPDH had to be refined by altering the amount of cDNA added to the PCR reaction until all bands were of equal intensity (Figure 97).
Oct 4 expression was assessed to monitor the loss of pluripotency during the culture period. To assess whether the surfaces promoted differentiation down a particular lineage different primers were selected for the three germ layers. NF68kD and FGF5 were selected to assess ectoderm formation, AFP and TTR were selected for endoderm differentiation whilst T-Bra was used as a marker for mesoderm. PCR parameters for each primer set are detailed in section 2.4.7.

Oct4 expression was gradually lost from all surfaces from day 2. Minimal expression was detected at day 10, except on spin coated films and ppAm TCP where slight increases in band intensity were observed (Figure 98).

At day 4, mesoderm marker T-Brachyury (T-Bra) is detected on all surfaces, with slightly weaker band intensity on gelatinised TCP and ppAm TCP –heparin. Weaker expression is present at day 6, with complete loss from day 8 onwards.

The endoderm marker TTR is detected by day 8 on gelatin, ppAm TCP +/-heparin and ppAm microfibres +/-heparin, with a drop in expression on ppAm microfibres +heparin at day 10. On microfibres and spin coated films, TTR expression is detected at day 6. High expression is maintained on the spin coated films until day 10, whilst expression on micro fibre scaffolds peaks at day 8 and decreases by day 10.

On gelatin and ppAm TCP +/-heparin, strong AFP expression was present at day 10. In comparison, AFP expression was present at day 8 and maintained until day 10 on spin coated films and ppAm micro fibre scaffolds. In comparison, virtually no AFP expression was present on ppAm microfibres +heparin and uncoated micro fibre scaffolds with very low band intensity at days 8 and 10.

NF68kD band intensity reached a peak at day 4 on gelatin and ppAm TCP +/-heparin, with low levels of expression present at day 2 and day 6 onwards. A similar expression profile was also observed on spin coated films. On micro fibre scaffolds, NF68kD expression peaked at day 2 and remained very low from day 4 onwards. In contrast, NF68kD expression on ppAm microfibres +/-
heparin remained low throughout the culture period with greatest band intensity detected at day 2 (-heparin) and day 4 (+heparin).

FGF5 expression was observed at day 4, 6 and 10 in cells cultured on gelatin. On ppAm TCP +/-heparin expression began at day 2 and was detected throughout the culture period on ppAm TCP +heparin. On ppAm microfibre scaffolds +/- heparin, expression is detected at day 4 with little or no expression present at later time points. Slight FGF5 expression is present on spin coated films and microfibre scaffolds at day 2. However, band intensity is very low and, with the exception of day 4 on the spin coated film, no further FGF5 expression is detected.

It appears that compared to gelatinised TCP and ppAm TCP +/- heparin, expression profiles on PLGA scaffolds are somewhat altered. Differentiation of mesoderm is largely unaffected, with expression present at day 4 and down regulated by day 8 on all surfaces. Both endoderm markers TTR and AFP have altered expression profiles on PLGA scaffolds. Most notably, high intensity bands of TTR from day 6 onwards were observed on spin coated films with reduced band intensity present at day 10 on ppAm microfibres +heparin and uncoated microfibre scaffolds. Very low expression of AFP was present on ppAm microfibres +heparin and uncoated microfibre scaffolds with early expression detected on spin coated films and ppAm microfibres –heparin. A similar trend of altered expression was also observed in ectoderm markers FGF5 and NF68kD. Reduced expression of both markers was observed on all PLGA scaffolds, with the exception of spin coated and microfibre scaffolds which had high band intensity of NF68kD at days 2 and 4 respectively.
Figure 98. LIF withdrawal: PCR for Oct4 and lineage specific markers. PCR parameters are detailed in section 2.3.7 along with primer sequences and annealing temperatures.
4.4.2 Neural Differentiation

4.4.2.1 EXT1\(^{-/-}\) mouse ES Cells

EXT1\(^{-/-}\) mouse ES cells lack the glycosyltransferase enzyme Ext1 that works together with Ext2 to catalyse the attachment of glucuronic acid and glucosamine residues to growing HS chains (Lin et al., 2000; Stickens et al., 2005). As a consequence, EXT1\(^{-/-}\) ES cells completely lack endogenous HS (Lin et al., 2000). EXT1\(^{-/-}\) ES cells are morphologically similar to normal mouse ES cells, growing in tight colonies that are maintained in standard culture conditions (Lin et al., 2000). However, they cannot form cavitated EBs due to impaired mesoderm and endoderm formation (Lin et al., 2000) and cannot differentiate down a neuronal lineage (Johnson et al., 2007). This inability to undergo neuronal differentiation can be partially rescued by the addition of exogenous heparin or HS to the culture media (Johnson et al., 2007).

4.4.2.2 EXT1\(^{-/-}\) Neural Differentiation

To assess whether the HS immobilised on ppAm microfibre scaffolds was bound in a biologically accessible and functional form, scaffolds were tested for their ability to rescue the neural differentiation capacity of EXT1\(^{-/-}\) cells. Scaffolds were prepared as described in section 2.4.8 and briefly in Figure 99. Uncoated microfibre meshes and ppAm microfibre meshes were incubated overnight in either PBS or HS (1\(\mu\)g/cm\(^2\)). Scaffolds were incubated in standard ES media overnight before 1 \(\times\) 10\(^4\) cells/cm\(^2\) were seeded onto the scaffold surface. As these cells are particularly sensitive to loss of serum, cells were seeded onto the scaffolds in standard ES media. After 24 hours, standard ES media was replaced with N2B27; this was recorded as day 0 of neural differentiation. Cells seeded onto scaffolds in N2B27 did not survive (results not shown).

After 8 days of culture in N2B27, cells on ppAm microfibre scaffolds with and without HS formed spherical aggregates on the scaffold surface. ppAm microfibre scaffolds +HS stained positive for neural differentiation marker \(\beta\)III tubulin (Figure 99). \(\beta\)III tubulin was present within the aggregates but also in neural processes that extended out from the cell clusters, often interacting with adjacent aggregates. In comparison, no \(\beta\)III tubulin staining was present on ppAm microfibre scaffolds without immobilised HS.
Figure 99. Day 8 neural differentiation of EXT1<sup>−/−</sup> cells on ppAm microfibre scaffolds +/- HS – βIII tubulin staining. 1.5 cm by 1.5 cm ppAm microfibre scaffolds were immobilised in 24 well plates using Scaffdex CellCrowns and incubated overnight in either 500µl PBS (-HS) or 1µg HS/cm<sup>2</sup> in 500µl PBS (+HS). Scaffolds were incubated overnight in 1ml standard ES media before EXT1<sup>−/−</sup> ES cells were seeded at 1 x 10<sup>4</sup> cells/cm<sup>2</sup> in standard ES media and cultured for 24 hours. Cells were then washed three times with PBS and media replaced with N2B27. Media was replaced every two days and cells were fixed with 4% paraformaldehyde after 8 days culture in N2B27. Cells were stained for neural differentiation marker, βIII tubulin.
Cell aggregates on ppAm microfibre scaffolds + HS were positively stained for Map 2 (Figure 100). Map2 was primarily localised to the cell aggregates but was also present in the neural processes extending across the scaffold, supporting βIII tubulin staining in Figure 99. ppAm microfibre scaffold without HS did not have extensive Map2 expression, with positive staining limited to irregular structures within the cell aggregate.

Figure 100. Day 8 neural differentiation of EXT1−/− cells on ppAm microfibre scaffolds +/- HS – Map2 staining. Scaffolds were prepared and cells were seeded as described in Figure 99. Cells fixed at day 8 were stained for a second neural differentiation marker, Map2.

In contrast, no βIII tubulin staining was observed on uncoated microfibre scaffolds with and without HS (Figure 101). Cells seeded on microfibre scaffolds did not form large cell aggregates as observed on ppAm microfibre scaffolds. Cell clusters were present, but the nuclei were small and granular indicating cell death had occurred on the scaffold surface.
Figure 101. Day 8 neural differentiation of EXT1<sup>+/−</sup> cells on microfibre scaffolds +/- HS – βIII tubulin staining. Scaffold preparation and cell culture was as described in Figure 99. Cells cultured on microfibre scaffolds +/- HS were fixed with 4% paraformaldehyde at day 8 in N2B27 media and stained for βIII tubulin.

ppAm microfibre scaffolds +HS were prepared for SEM analysis at day 8 (Figure 102). Standard SEM was used as E-SEM failed to provide enough resolution to visualise the fine neural processes extending from the aggregates. Cells on the surface formed dense aggregates with a rough surface composed of either ECM or cell debris from the immense amount of cell death that occurs during the differentiation process. Neural processes extended out from the aggregates, attaching to the scaffold surface. The extensions did not follow single fibres but extended over the fibres of the scaffold, surrounded by cell debris and ECM.
Figure 102. Day 8 neural differentiation of EXT1−/− cells on ppAm microfibre scaffolds +HS – SEM analysis. Scaffold preparation and cell culture was as described in Figure 99. At day 8 neural differentiation, cells were fixed with 1.5% gluteraldehyde and dehydrated for SEM analysis.

To ascertain whether immobilised HS was rescuing neural differentiation of EXT1−/− cells by assisting the binding and activity of FGF4, 2ng/ml FGF4 was added to N2B27 from day 0 creating 4 different conditions: +HS+FGF4, +HS-FGF4, -HS+FGF4 and –HS-FGF4. Media was changed every 2 days and FGF4 was added at to the appropriate conditions at the same concentration throughout the differentiation period.

By day 6, βIII tubulin was present in small irregular structures within the cell aggregates cultured in conditions +HS-FGF and -FGF+HS. No βIII tubulin was present in –HS-FGF cultures. In comparison, cells cultured in +HS+FGF had more extensive βIII tubulin at day 6, with neural processes beginning to extend out from the aggregates over the scaffold surface.
At day 8, βIII tubulin staining of +HS+FGF samples revealed extensive neural differentiation across the entire scaffold surface (Figure 104). The extensions were more abundant and mature, extending away from the cell aggregates forming a spread network. As in Figure 99, βIII tubulin expression of cells cultured in +HS–FGF conditions revealed neural differentiation occurring with neural processes sprouting out of the cell masses. However the extensions did not form as large a network as cells grown in +HS+FGF and were mainly localised around the cell aggregates. Cells cultured in −HS+FGF conditions were positive for βIII tubulin. However, extensions out of the aggregates were less abundant and were not as organised or as extensive as cells cultured in the presence of HS. Despite no βIII tubulin being previously present in cells cultured on scaffolds without HS, cells grown in −HS-FGF had positive staining in a small number of cell aggregates, the
most developed of which is shown in Figure 104. As with −HS+FGF, the slight positive staining revealed small disorganised neural extensions localised around the cell aggregates.

Figure 104. Day 8 neural differentiation of EXT1−/− cells on microfibre scaffolds +/- HS +/- FGF4 – βIII tubulin staining. Scaffolds were prepared and cells were cultured as described in Figure 99. 2ng/ml FGF4 was added to N2B27 media from day 0 and maintained at the same concentration throughout the differentiation period. Cells were fixed at day 8 of culture in N2B27 and stained for βIII tubulin.

By day 8, Map 2 was expressed by cells grown in all conditions. Map 2 formed a network within the cell aggregates and was present at the origin of the neural extensions (Figure 105), but was not detected in the longer extensions highlighted by βIII tubulin staining in Figure 104.
Figure 105. Day 8 neural differentiation of EXT1⁻/⁻ cells on Microfibre scaffolds +/- HS +/- FGF4 –Map2 staining. Scaffolds were prepared and cells were cultured as described in Figure 99. 2ng/ml FGF4 was added to N2B27 media from day 0 and maintained at the same concentration throughout the differentiation period. Cells were fixed at day 8 of culture in N2B27 and stained for Map2.

As data in section 4.2.3 raised issues concerning coating stability, 1.5 cm by 1.5 cm sections of ppAm microfibre scaffolds were pre-soaked overnight in PBS in a 10cm petri dish. Scaffolds were then immobilised in 24 well plates Scaffdex CellCrowns and incubated in HS (1µg/cm²). As an extra precaution, scaffolds were washed three times with PBS after HS incubation and moved to fresh wells before overnight incubation in standard ES media. EXT1⁻/⁻ ES cells were seeded in standard ES media at 1 x 10⁴ cells/cm² and cultured for 24 hours before media was changed to N2B27 +/- 2ng/ml FGF4.

At day 6, cells cultured in conditions +HS+FGF and +HS-FGF were positive for βIII tubulin with networks present within the cell aggregates (Figure 106), with cells grown in +HS+FGF extending neural processes over the scaffold surface. In comparison, little βIII tubulin was detected in –
HS+FGF and –HS-FGF conditions. Any positive staining was limited to underdeveloped networks within the cell aggregates.

Figure 106. Day 6 neural differentiation of EXT1<sup>+/−</sup> cells on pre-soaked ppAm microfibre scaffolds +/- HS +/-FGF4 – βIII tubulin staining. 1.5 cm by 1.5 cm ppAm microfibre scaffolds were soaked in PBS overnight before immobilisation in Scaffdex CellCrowns. Scaffolds were then incubated overnight in either 500ul PBS (-HS) or 500ul HS (+HS, 1ug/cm2). Before cell seeding, scaffolds were washed three times in PBS before being transferred to fresh wells. Cells were seeded and cultured as described in Figure 99. 2ng/ml FGF4 was added to N2B27 media from day 0 and maintained at the same concentration throughout the differentiation period. Samples were fixed at day 6 of culture in N2B27 and stained for βIII tubulin.

By day 8, cells differentiated in both +HS+FGF4 and +HS-FGF4 conditions developed extensive networks of βIII tubulin positive neural processes (Figure 107). Little difference was observed between the two conditions, with neural processes interweaving between aggregates and across the scaffold surface. Cells cultured in –HS+FGF4 and –HS-FGF4 conditions displayed little βIII tubulin staining after 8 days of culture. Neural processes developed in –HS+FGF4 were disorganised and irregular. As with unsoaked scaffolds, cells cultured in –HS- FGF4 showed some
evidence of neural differentiation with a small amount of βIII tubulin within and extending out of a limited number of cell aggregates.

Figure 107. Day 8 neural differentiation of EXT1+/− cells on pre-soaked ppAm microfibre scaffolds +/- HS +/-FGF4 – βIII tubulin staining. Scaffolds were prepared as described in Figure 106. Before cell seeding, scaffolds were washed three times in PBS before being transferred to fresh wells. Cells were seeded and cultured as described in Figure 99. 2ng/ml FGF4 was added to N2B27 media from day 0 and maintained at the same concentration throughout the differentiation period. Samples were fixed at day 8 of culture in N2B27 and stained for βIII tubulin.

In contrast to previous results using unsoaked scaffolds, there was a clear difference in Map2 staining between cells grown on scaffolds with and without immobilised HS. After 8 days, cells differentiated in +HS+FGF4 and +HS-FGF4 conditions were positive for Map2 expression (Figure 108). Staining highlighted neural networks within the cell aggregates, with intense staining at the origin of neural processes extending out over the scaffold surface. No Map2 was present in cells cultured in −HS+FGF4 and −HS-FGF4 conditions.
Figure 108. Day 8 neural differentiation of EXT<sup>−/−</sup> cells on pre-soaked ppAm microfibre scaffolds +/- HS +/-FGF4 – Map2 staining. Scaffolds were prepared as described in Figure 106. Before cell seeding, scaffolds were washed three times in PBS before being transferred to fresh wells. Cells were seeded and cultured as described in Figure 99. 2ng/ml FGF4 was added to N2B27 media from day 0 and maintained at the same concentration throughout the differentiation period. Samples were fixed at day 8 of culture in N2B27 and stained for Map2.

It should be noted that attempts to differentiate EXT<sup>−/−</sup> ES cells in N2B27 on standard gelatinised TCP, with and without 1mg/ml HS failed. This is thought to be due to cell density issues and the inherent variability of the differentiation protocol used.
4.4.2.3 WT1 Neural Differentiation

To determine if ppAm microfibre scaffolds could be used to enhance neural differentiation of wildtype mouse ES cells, WT1 ES cells, were cultured in N2B27 on pre-soaked ppAm microfibre scaffolds in 4 conditions: +HS+FGF4, +HS-FGF4, -HS+FGF4 and -HS-FGF4. Scaffolds were prepared as described briefly in Figure 106 and culture conditions were the same as EXT1^-/- ES cell neural differentiation.

As with EXT1^-/- cells, WT1 cells grew in large cell aggregates on the scaffold surface. A limited amount of βIII tubulin was present in all culture conditions at day 6, with more extensive networks present in +HS+FGF4 and +HS-FGF4 conditions (Figure 109).
After 8 days, βIII tubulin demonstrated heterogeneous staining, with differences between conditions not as easily discerned as in EXT1−/− cultures. However, cell aggregates in +HS+FGF4 and +HS-FGF4 conditions had grown and joined together creating areas covered with cell masses (Figure 110). On scaffolds without immobilised HS, aggregates were present but had not grown to the same extent as scaffolds with HS. In conditions +HS+FGF, +HS-FGF and –HS+FGF, βIII tubulin was present within the cell aggregates and in neural processes which sprouted out of the aggregates to form networks across the scaffold surface. These processes were also present in –HS-FGF, but to a lesser extent with some aggregates negative for βIII tubulin expression.

At day 8, Map2 expression in all conditions was limited to the cell aggregates with little staining observed in neural extensions (Figure 111). Expression within the aggregates appeared to be disorganised with aggregates negative for Map 2 present in +HS+FGF and –HS-FGF culture conditions.

Attempts to differentiate WT1 ES cells in N2B27 on standard gelatinised TCP, with and without 1mg/ml HS failed. Once again, this is thought to be due to cell density issues and the inherent variability of the differentiation protocol.
Figure 110. Day 8 neural differentiation of WT1 cells on pre soaked microfibre scaffolds +/- HS +/- FGF4 – βIII tubulin staining. ppAm microfibre scaffolds were soaked and prepared as described in Figure 106. WT1 cells were seeded and cultured as described for EXT1^- cells. 2ng/ml FGF4 was added to N2B27 media from day 0 of neural differentiation and maintained at the same concentration throughout the differentiation period. Samples were fixed at day 8 of culture in N2B27 and stained for βIII tubulin.
Figure 111. Day 8 neural differentiation of WT1 cells on pre-soaked microfibre scaffolds +/- HS +/- FGF4 –Map2 staining. ppAm microfibre scaffolds were soaked and prepared as described in Figure 106. WT1 cells were seeded and cultured as described for EXT1^-/- cells. 2ng/ml FGF4 was added to N2B27 media from day 0 of neural differentiation and maintained at the same concentration throughout the differentiation period. Samples were fixed at day 8 of culture in N2B27 and stained for Map2 (green).
4.4.3 Human ES Cell Culture

In a preliminary assessment of the ability of ppAm microfibre meshes to support human ES cell culture, HUES7 human ES cells were seeded onto ppAm microfibre scaffolds +/-HS in MEF conditioned hES media +10ng/ml FGF2. Cells were maintained on immortalised MEFs in human ES media +10ng/ml FGF2 prior to seeding. When HUES7’s were ready to be split, two wells of a 6 well plate were trypsinised for approximately one minute and detached from the well surface. Cells were then spun down and resuspended in fresh MEF-conditioned media +10ng/ml FGF2. As HUES7 ES cells do not grow well when disassociated into single cells, the cell suspension was comprised of loose aggregates. One quarter of the cell suspension was seeded onto each ppAm microfibre scaffold.

1.5cm by 1.5cm ppAm microfibre scaffolds were immobilised in 24 well plates using Scaffdex CellCrowns and incubated overnight in either PBS or HS (1µg/cm²). Before cell seeding, scaffolds were incubated overnight in unconditioned hES media. Media was changed every day except for the first day after seeding to allow the cells to settle and attach to the surface. Samples were sacrificed at day 3 and day 5 of culture and stained for Oct4 and actin.

ppAm microfibre scaffolds with and without immobilised HS supported the attachment of HUES7 ES cells. After three days, cells were growing in Oct4 positive colonies on both surfaces. However, although Oct4 expression was detected on both meshes it appeared to be greater in colonies growing on ppAm microfibre scaffolds without immobilised HS (Figure 112).
Figure 112. Day 3 HUES7 cell culture on ppAm microfibre meshes +/- HS in conditioned hES media + 10ng/ml FGF. Samples were fixed in 4% paraformaldehyde at day 3 and stained for Oct4 (red) and Actin (green).

By day 5, colonies had grown in size and Oct4 expression was present on ppAm microfibre meshes with and without HS (Figure 113). Colonies growing on ppAm microfibre meshes with immobilised HS had areas where Oct4 was not present. This is in comparison to ppAm microfibre meshes without HS, where Oct4 was present throughout the cell population.

Very little cell spreading was observed on the scaffolds at both days, with actin mainly restricted to a cortical arrangement within the cell colonies.
Human ES cells were seeded onto ppAm microfibre scaffolds +/- HS in unconditioned media +100ng/ml FGF2 to test the ability of scaffolds to support human ES cells in defined culture media. Cells and scaffolds were prepared as described above, except the cells were resuspended in unconditioned media +100ng/ml FGF2. Cells were fixed and stained for Oct4 expression at day 3 and day 5.

At day 3, very few ES cells were present on ppAm microfibre scaffolds +/- HS. The majority of the cells on the ppAm microfibre scaffolds +/-HS were Oct4 positive and were rounded with little cell spreading (Figure 114).
<table>
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<tr>
<th>Day 3 – Unconditioned Media + 100ng/ml FGF</th>
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**Figure 114.** Day 3 HUES7 cell culture on ppAm microfibre meshes +/-HS in unconditioned hES media + 100ng/ml FGF. Samples were fixed in 4% paraformaldehyde at day 3 and stained for Oct4 (red) and Actin (green).

Larger colonies were present on ppAm microfibre scaffolds +HS at day 5 of culture (Figure 115). Cells growing in colonies were positive for Oct4 with limited cell spreading across the scaffold surface. ppAm microfibre scaffolds –HS had few substantial colonies present on the surface after 5 days. Despite cells remaining Oct4 positive with limited cell spreading, little cell growth had occurred on the scaffold surface.
Figure 115. Day 5 hES cell culture on ppAm microfibre meshes +/-HS in unconditioned hES media + 100ng/ml FGF. Samples were fixed in 4% paraformaldehyde at day 5 and stained for Oct4 (red) and Actin (green).
4.5 Discussion

Plasma polymerisation of allyl amine onto electrospun PLGA meshes dramatically altered the surface chemistry of the microfibre scaffolds without altering the fibrous architecture of the mesh. This is in agreement with previous research in which electrospun PLGA scaffolds were coated with plasma polymerised acrylic acid. Despite a dramatic increase in hydrophilicity, there was no change in pore size, fibre diameter, porosity or mechanical properties (Park et al., 2007).

The hydrophobic electrospun mesh was transformed into a more hydrophilic structure, with initial WCA reduced and the water droplets spreading over and into the mesh. Increased hydrophilicity is in accordance with previous research modifying surfaces by plasma polymerisation (Alexander and Duc, 1999; Park et al., 2007; Prabhakaran et al., 2008) and coating with amine functional groups (Harsch et al., 2000; Keselowsky et al., 2003). XPS analysis confirmed the altered surface chemistry, with ppAm causing a marked decrease in oxygen content (from 34.2 at.% to 5.28 at.%) and introducing nitrogen (5.28 at. %). High-resolution analysis of the core C 1s signal of uncoated scaffolds revealed three peaks at intervals of 2 eV, corresponding to C-H, C-O and C(=O)-O groups within the polymer chain (Barry et al., 2005; Ogaki et al., 2008). High-resolution analysis of core O 1s level revealed two oxygen environments that corresponded with the oxygen containing environments within the high-resolution C 1s spectrum. As the C-C/C-H bond is only present within PLA it was possible to calculate the molar fraction of PLA within the XPS sampling depth (Ogaki et al., 2008). The molar fraction was 89%, slightly greater than the 85:15 PLA: PGA ratio of the PLGA used. This has previously been observed and has been attributed to the adsorption of adventitious carbon from the atmosphere or the orientation of the polymer chains (Ogaki et al., 2008). Recent work by Cui et al. (2008) observed an increase in the C-H/C-C environment in electrospun meshes compared to solvent cast films suggesting the electrospinning process orientated the methyl groups to the surface of the fibres.

Fitting the high-resolution C 1s peak of ppAm microfibre meshes was more complex. Plasma polymerisation is a highly variable process, affected by parameters such as substrate surface chemistry, reactor geometry and plasma power (Alexander and Duc, 1999; Morosoff, 1990; O'Toole et al., 1996; O'Toole and Short, 1997). Therefore, it is difficult to predict which chemical environments will be present and in what proportions. To the best of our knowledge, no other research group has plasma polymerised allyl amine onto electrospun PLGA scaffolds. Therefore, the work by Barry et al. (2005) was used as a template for peak fitting high-resolution spectra as the study coated ppAm onto 3D PDLA sponges.

In agreement with Barry et al., plasma polymerisation of allyl amine dramatically reduced the intensity of the C-O and C(=O)O environments detected in PLGA. The presence of these oxygen environments could be due to the X-ray beam penetrating through the ppAm layer and detecting the underlying PLGA. The N/C ratio of the ppAm layer is used to predict the thickness of the ppAm coating on flat surfaces with a value of 0.20 – 0.24 indicative of a full thickness coating greater than the ~10nm XPS sampling depth (Mahoney et al., 2004; Robinson et al., 2008; Whittle et al., 2000). The N/C ratio of coated samples ranged between 0.12-0.21 with an average of 0.15 (SD: 0.032). However, the N/C ratio will not give an accurate estimation of coating thickness due to the 3D nature of the fibre mesh. Oxygen could also have been incorporated into the coating during plasma
polymerisation as the reactive species combined with residual oxygen or H\textsubscript{2}O within the reactor (Barry et al., 2005; Shard et al., 2004; Whittle et al., 2000). Persistent reactive species residing within the coating could have also incorporated oxygen when the treated samples were exposed to the atmosphere (Whittle et al., 2000).

As with Barry et al., the high-resolution carbon spectrum could be well fitted with 5 peaks, including the two oxygen environments discussed above. Plasma polymerisation of allyl amine introduced a peak at +1.0 eV relative to the C-H environment, corresponding to C-N bonds. Unfortunately it is not possible to distinguish between primary, secondary and tertiary amines (Barry et al., 2005).

A peak at +3.0 eV was also required to adequately fit the C 1s spectrum indicating amides were present within the coating (Barry et al., 2005; Dehili et al., 2006; Mahoney et al., 2004). These could have formed due to oxygen in the reaction chamber and in the atmosphere as described above. They may have also occurred due to reaction of radical species with the surface groups present on underlying PLGA (Barry et al., 2005). The amide peak was proportional in intensity to a third peak within the high-resolution oxygen environment present at a lower binding energy (531.2 eV). This peak is tentatively allocated to this chemical environment as it has not been previously reported in the literature.

The high-resolution oxygen peaks of ppAm microfibre meshes were particularly difficult to fit. Although the C(=O)-O environments were proportional in both C 1s and O 1s, the C-O environment was disproportionally large in the C 1s compared to the O 1s indicating further de-convolution of the C 1s peak was necessary. A sixth peak at +1.5eV was therefore added to the fit proposed by Barry et al. and was attributed to the presence of imines previously reported in ppAm deposited films (Dehili et al., 2006; Harsch et al., 2000; Mahoney et al., 2004; Shard et al., 2004).

The high-resolution spectrum of nitrogen was fitted with a single symmetrical peak representative of amine, imine and amide groups detected in the ppAm layer. The peak was at a lower energy level of 399.4 eV, which is indicative of a higher proportion of amine and imine functionalities in comparison to amides (Dehili et al., 2006; Whittle et al., 2000). This supports the peak fitting of the core C 1s signal. Mahoney et al. plasma polymerised allyl amine onto 96 well microtitre plates and successfully immobilised heparin in a form accessible to protein binding (Mahoney et al., 2004). The presence of amine functionalities with lower concentrations of imines and amides is in agreement with this research.

XPS analysis was limited to the top surface of the microfibre scaffolds and did not give an indication of the extent of ppAm deposition within the mesh. Barry et al. revealed a lowering of nitrogen content within the centre of porous PDLLA scaffolds, indicating plasma polymerisation was limited by the diffusion of reactive species (Barry et al., 2005). However, the scaffolds were porous cylinders approximately 10mm in diameter and 3mm thick. Considering the microfibre scaffolds used in this study have a maximum recorded diameter of 7.99 µm, and that both sides were treated with ppAm, it is highly possible that a ppAm was uniform throughout the scaffold. However, further analysis will be necessary to confirm this assumption.

RP-HPLC analysis revealed HS bound to the surface of ppAm coated microfibre scaffolds via a non-covalent ionic interaction. Previous research on microtitre Epranex plates revealed the interaction between the negatively charged oligosaccharides and the positively charged allyl amine
surface can be disrupted by 1M NaCl (Mahoney et al., 2004). In this study, 4M NaCl was used successfully to remove HS from the scaffold for analysis. Initial analysis revealed a 1cm² section of ppAm microfibre scaffold bound a maximum of 1-2µg HS. This value varied during subsequent experiments, reaching a maximum of 2.52µg in unsoaked scaffolds. This could be attributed to variance in scaffold structure and ppAm coating. Each step involved in preparing samples for RP-HPLC, such as desalting, heparinase digestion, and AMAC labelling, could also have introduced variability into the RP-HPLC data.

Despite this variability, selective binding of 6-0-sulphated species at concentrations above the scaffold saturation level (>5µg) was consistently observed. This was most evident in an increased binding of HS containing a higher proportion of the tri-sulphated species UA2S-GlcNS6S and to a lesser extent the di-sulphated UA-GlcNS6S. As the interaction with the allyl amine surface is ionic in nature, the increased negative charge present on HS chains containing tri-sulphated species could have provided a selective advantage when binding to the positively charged surface. The increased binding of HS containing UA-GlcNS6S compared to other di-sulphated disaccharides suggests that the 6-0-sulphate plays a fundamental role in mediating the selective binding.

However, binding to ppAm surfaces is influenced by but not dependent on the sulphation motifs present on the heparin/HS chain. Completely unsulphated HA and heparin stripped of 2-O, 6-O and N-sulphation can all be immobilised in a functional form on ppAm (Clark et al., 2006). At concentrations below the saturation level, it appears that the ppAm microfibre scaffolds equally bound all species within the heterogeneous HS preparation. This is in agreement with recent research revealing the capacity of 100% allyl amine coatings to bind an array of different GAGs, including HA, DS and C-6-S (Marson et al., 2009).

The Link module of TSG-6 has been repeatedly used to assess the capacity of immobilised GAGs to engage in protein interactions (Clark et al., 2006; Mahoney et al., 2005; Mahoney et al., 2004; Marson et al., 2009; Robinson et al., 2008) and has been successfully used to resolve the heparin binding residues within the Link module (Mahoney et al., 2005). Link_TSG-6 binds both heparin and hyaluronan via distinct regions, with heparin binding mediated via a series of positively charged residues distinct from the HA binding site. The proposed method of interaction involves dimerisation of the Link module upon heparin binding (Mahoney et al., 2004). The ability of bA-Link_TSG-6 to bind heparin immobilised on the surface of ppAm plates was confirmed by a dose dependent increase in absorbance with increasing levels of applied heparin (Mahoney et al., 2004). Absorbance values of unsoaked ppAm microfibre scaffolds were equivalent to Epranex plates and began to plateau at 1000ng applied heparin. This indicates that the heparin was bound in a conformation that was flexible and free from the scaffold surface enabling engagement of protein interactions. Pre-soaking ppAm microfibre scaffolds in PBS approximately halved TSG-6 binding. However, RP-HPLC analysis did not indicate reduced binding of HS, in fact both RP-HPLC and radiolabelling data suggested soaked scaffolds bound slightly more HS and exaggerated the selective binding of HS chains with high 6-O-sulphation. These combined results indicate soaked scaffolds bind the equivalent amount of HS/heparin but in a conformation that is not as free to interact with ligands such as bA-Link_TSG-6. A similar inconsistency between the amount of immobilised heparin and binding ability has been previously reported by Robinson et al. (2008). Using plasma polymerised chemical gradients of octadiene and allyl amine, XPS analysis revealed
increased binding of heparin with increasing concentrations of allyl amine. However, a significant drop in bA-Link_TSG-6 binding was observed at the maximum heparin binding point of 100% allyl amine. It would appear that although heparin can bind to positively charged ppAm surfaces, it is important to ascertain whether it is bound in a form available for optimal protein interaction.

It is clear from the altered binding capacity of bA-Link_TSG-6 that the surface chemistry of ppAm microfibre meshes and subsequent immobilisation of HS is dramatically altered by exposure to aqueous solution. Previous research has suggested similar questions over the stability of plasma polymerised surfaces. Barry et al. reported thinning of ppAm coatings after washing revealing the underlying PLA substrate, which was exaggerated by lower plasma power (Barry et al., 2005). Chu et al. (2006) used ethanol to extract soluble, non-crosslinked components from deposited ppAm and concluded that a longer deposition time and increased plasma power enhanced crosslinking creating a more stable layer. However, the alteration of plasma parameters should be done with care as increasing plasma power induces more monomer fragmentation compromising the retention of monomer functional groups (Alexander and Duc, 1999; O'Toole et al., 1996; O'Toole and Short, 1997). Another possibility for enhancing stability is the incorporation of octadiene, a hydrocarbon monomer that has enhanced the stability of acrylic acid plasma deposits in aqueous solution (Alexander and Duc, 1999). It is clear that further modification of plasma polymerisation parameters and analysis of pre-soaked surfaces will be necessary to optimise ppAm deposition on PLGA microfibres.

Phage display derived ScFv antibodies specific for particular sulphation motifs within the HS chain were investigated for their ability to bind HS/heparin immobilised on the scaffold surface. The differential binding of specific ScFv antibodies towards specific sulphation motifs made them an excellent tool in assessing whether the HS/heparin was bound in a conformation that exposed these epitopes to ligand interaction. Importantly, they also enabled the comparison of GAG chains which were known to have distinct patterns of ScFv binding in solution to detect if these cell-specific differences were maintained once the GAGs were immobilised. In the present study heparin binding ScFv antibodies HS4C3 and NS4F5 were successfully applied to heparin immobilised on Epranex plates, revealing a dose dependent increase in absorbance with applied heparin and very little non-specific binding. The development time was reduced to 10 minutes compared to TSG-6 binding, which had a 20 minute development window. This could be due to binding of multiple ScFv antibodies to a single heparin chain (Ten Dam et al., 2006) combined with binding of multiple secondary antibodies to the VSV-G tag. As with TSG-6 binding, this indicated that the heparin was bound with sulphation motifs retained and in a conformation accessible for ligand interaction. A dose dependent increase in absorbance was also observed when both antibodies were applied to heparin-coated ppAm microfibre scaffolds. However, the assay was complicated by non-specific binding of both antibodies and the instability of the ppAm layer. Moving the scaffold before addition of development reagent revealed that the scaffolds themselves were able to bind NS4F5, with binding increasing as more heparin was applied. There was also drastically reduced absorbance values for scaffolds incubated with just PBS, indicating the non-specific binding observed was due to the unstable ppAm layers that had detached from the scaffold and adhered to the inside of the wells.
To prove the differential binding of ScFv antibodies to specific sulphation motifs within the bound HS, RB4EA12 and EV3C3 were applied to immobilised HS isolated from either Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) or E14 (wild type) ES cells. Cell lines lacking both Sulf1 and Sulf2 enzymes have previously shown a significant increase in 6-O-sulphation, with higher levels of UA2S-GlcNS6S, UA-GlcNS6S and UA-GlcNAc6S within the HS chain (Lamanna et al., 2006; Lamanna et al., 2008). RP-HPLC analysis revealed that the Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) ES cell HS contained a higher percentage of tri-sulphated disaccharides compared to E14 HS. Both RB4EA12 and EV3C3 have previously displayed differential binding to Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS compared to wildtype controls (Lamanna et al., 2006). RB4EA12 requires 6-O-sulphation for high affinity binding therefore an increase in binding was observed on the surface of double knock out MEFs. In comparison, EV3C3 binding (which is inhibited by 6-O-sulphation) decreased (Lamanna et al., 2006). Considering the differences in HS composition between Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) ES cell HS and E14 HS confirmed by RP-HPLC analysis it was predicted that a similar differential binding activity would be observed with HS immobilised on ppAm treated surfaces. This was confirmed by greater binding of RB4EA12 to Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS immobilised on Epranex plates and ppAm microfibre scaffolds compared to E14 HS. This suggested differential binding of ScFv antibodies to specific sulphation motifs that were presented in an accessible form by the immobilised HS. This was further supported by EV3C3 binding to HS preparations immobilised on Epranex plates. EV3C3 binding to Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS was almost 50% of that recorded for RB4EA12 binding after 20 minutes development. However, drastically reduced binding of EV3C3 to E14 HS was observed with absorbance readings at almost negligible levels. This contrast in binding suggests differential binding of ScFv antibodies is occurring, but not in a manner entirely predicted by their recognition epitopes. It could be hypothesised that although E14 HS has lower 6-O-sulphation, it lacks other elements necessary for successful EV3C3 binding. However, flow cytometry data comparing E14 and Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) mouse ES cells has revealed little/no difference in binding of EV3C3 (Dr. Rebecca Holley, personal communication).

The differences between the E14 and Sulf1\(^{-/-}\)/Sulf2\(^{-/-}\) HS preparations may alter their immobilisation on the ppAm which in turn would affect their ability to react with the ScFv antibodies. However, this is unlikely considering the variety of GAG preparations that have been successfully immobilised in an accessible form on ppAm microtitre plates (Clark et al., 2006; Marson et al., 2009). The differences in ScFv binding observed may be due to the contrasting environments of the HS. Flow cytometry analyses HS on the surface of the cell, in dramatic contrast to extracted HS immobilised on ppAm microfibre meshes and plates. Initial experiments therefore suggest that whilst some of the cell-type specific differences in HS patterning observed using the ScFv antibodies for flow cytometry are maintained when the HS is immobilised there may also be some loss or change in the epitopes displayed.

ScFv binding to heparin immobilised on ppAm microfibre scaffolds indicates that specific sulphation motifs are largely unaltered and presented in an accessible form reactive with ScFv antibodies. This has important implications as specific sulphation patterning within HS chains regulate the affinity of binding to growth factors such as FGF1, FGF2 and FGF4 (Ashikari-Hada et al., 2004; Guimond et al., 1993; Maccarana et al., 1993; Pye et al., 2000; Sugaya et al., 2008; Turnbull et al., 1992). The availability of these motifs and the retention of their differential binding capacity is further supported by the contrasting binding of RB4E12 and EV3C3. However, more detailed
analysis utilising defined HS oligomers of defined sequence will be necessary to further investigate this conclusion and the dramatically reduced binding of EV3C3 to immobilised E14 HS.

Radiolabelling data revealed $^{3}$H HS was retained by the ppAm microfibre scaffolds when incubated at 37°C in PBS and N2B27 over 10 days. The presence of HS on the scaffolds after 10 days incubation has important implications for cell culture, indicating that any biological activity provided by the immobilised HS will be maintained for at least 10 days. Counts detected during media changes and in PBS washes revealed $^{3}$H HS was continually being lost from the samples over the incubation period. The fact that this slow erosion of $^{3}$H HS was not translated into loss of counts from the scaffolds themselves suggested $^{3}$H HS was bound to the eppendorfs via detached ppAm coating. Subsequent analysis confirmed the coating did in fact detach from the scaffold and adhere to the tubing. However, once this unstable layer was removed, no further detachment occurred with very little binding of $^{3}$H HS to tubing. Further analysis will be needed to ascertain how much $^{3}$H HS is lost to the surrounding media during long term incubation after scaffolds are pre-soaked in PBS to remove the unstable ppAm.

Cells remained viable on the ppAm microfibre meshes +/- HS attaching after 24 hours and growing over 7 days culture in standard ES media. There appeared to be a greater degree of cell spreading on ppAm coated meshes compared with uncoated microfibre scaffolds. Enhanced attachment and spreading on ppAm coated surfaces has been recorded by a number of groups (Hamerli et al., 2003; Harsch et al., 2000) including Barry et al. (2005). Modification of PLA sponges with ppAm encouraged fibroblast adhesion, proliferation and migration into the porous scaffold. This could be attributed to the altered surface chemistry and increased hydrophilicity of the scaffold surface (Webb et al., 1998) and also the possible adsorption of a different array of serum proteins (Keselowsky et al., 2003; Whittle et al., 2002; Zhang and Feng, 2007). However, cells still primarily grew in aggregates indicating that the heaped morphology of the cells on the scaffold surface is largely driven by the micro-fibrous architecture of the mesh.

LIF withdrawal revealed marked differences in gene expression of cells cultured on PLGA scaffolds compared to gelatinised TCP and ppAm TCP +/- HS. The most unusual disregulation occurred in FGF5 expression, which is present in the primitive ectoderm (Rathjen et al., 1999). On ppAm TCP surfaces, expression was detected at the earlier time point of D2. However, expression levels were comparable to gelatin controls and were maintained until day 6. On scaffolds constructed using PLGA, including ppAm coated meshes, both the timing and strength of expression was altered with very little expression detected on uncoated microfibres and spin coated films. The disregulation of ectoderm lineages was confirmed by the altered expression profile of neuroectoderm marker NF68kD. The disregulation was more prominent on the fibrous meshes, with lower expression levels on ppAm coated surfaces and an early peak in expression at day 2 on uncoated microfibre scaffolds. Disregulation in AFP and TTR expression on all scaffolds also indicated impaired/ altered endoderm formation. However, mesoderm formation remained similar between surfaces as confirmed by relatively uniform T-Bra expression. This suggests specific alteration of ectoderm and endoderm differentiation on the PLGA scaffolds. As differentiation on ppAm TCP remained largely similar to gelatin controls it could be concluded the architecture and the surface chemistry of the PLGA scaffolds motivated the observed disregulation. However, it should be noted that cells were
trypsinised off the scaffolds before RNA extraction. Therefore, it cannot be ruled out that this selectively detached certain cell phenotypes from the scaffold surface.

No obvious difference in expression profiles was observed between cells cultured on ppAm scaffolds with and without immobilised HS. However, cells cultured on ppAm surfaces +HS demonstrated enhanced proliferation compared to the equivalent surface without immobilised HS. As the cultures were comprised of mixed cell phenotypes, growth factors such as FGF4, FGF5 and VEGF would have been secreted by the differentiating/differentiated cells. The presence of HS may therefore have facilitated growth factor binding promoting increased survival and proliferation.

Initial analysis of neural differentiation revealed unsoaked ppAm microfibre scaffolds functionalised with immobilised HS rescued the neural differentiation capacity of EXT1<sup>-/-</sup> ES cells, as revealed by positive βIII tubulin and Map 2 staining. This indicated the HS immobilised on the scaffold surface was biologically active and in a form competent to influence cell behaviour. In contrast, cells seeded on uncoated microfibre scaffolds did not survive the differentiation process despite preconditioning in standard ES media. The switch to N2B27 media is a cytotoxic event that is largely dependent on cell density (Ying et al., 2003b). If the uncoated microfibre scaffolds did not promote efficient attachment of ES cells and effectively promote their proliferation (as suggested by data in section 3.2.4) then the ability of ES cells to withstand the harsh N2B27 environment would be significantly reduced. It would appear that the ppAm coating itself was able to sustain the attachment and growth of EXT1<sup>+</sup> ES cells as aggregates were present on the surface of ppAm microfibre meshes without HS after 8 days culture in N2B27. As initial analysis was conducted before the stability of the coating came under scrutiny, it cannot be ruled out that HS associated with detached ppAm contributed to the rescue of neural differentiation. However, pre-soaked scaffolds were still able to promote neural differentiation suggesting HS immobilised on the scaffold surface was biologically active and largely responsible for the neural differentiation that took place. However, further analysis will be necessary to determine whether HS bound to soaked scaffolds is retained in a similar manner as unsoaked scaffolds.

Addition of FGF4 to the surrounding media gave varying results between soaked and unsoaked ppAm microfibre scaffolds. On the unsoaked scaffolds, FGF appeared to have a synergistic effect with HS promoting the formation of developed neural networks that were more independent of the cell aggregates from which they originated. On pre-soaked scaffolds there was little difference between +HS+FGF4 and +HS-FGF4 conditions, with βIII tubulin staining highlighting equally extensive neural processes. This could be attributed to the differences in surface chemistry and HS immobilisation between soaked and pre-soaked scaffolds or could be representative of the inherent variability of the neural differentiation process. EXT1<sup>+</sup> cells cultured in –HS+FGF4 developed irregular structures with limited neural process development across the scaffold surface. This could be due to the increased concentration of FGF4 compensating for the lack of HS, as FGFs still have low affinity for FGFRs in the absence of heparin/HS (Harmer, 2006). However, -HS-FGF4 conditions were positive for βIII tubulin staining on both soaked and unsoaked scaffolds. This limited neural differentiation, which was restricted to a few aggregates with irregular, branching structures, could be due to contamination by GAGs present within the standard ES media in which the scaffolds were pre-conditioned and the cells were seeded. Attempts were made to precondition...
and seed the cells in GAG-free N2B27 media. However, under these conditions the cells did not survive the differentiation procedure.

βIII tubulin staining enabled the extensive neural processes on the scaffolds to be clearly visualised and differences between the conditions easily discerned. However, Map2 staining was not as clear cut. The localisation of Map2 staining to the cell aggregates and origins of neural processes is in agreement with its localisation to the cell body and dendrites (Dehmelt and Halpain, 2005). This staining profile reinforces the observation that neural differentiation occurs within the aggregates, with βIII tubulin positive neural processes extending over the scaffold as differentiation progresses. Whilst positive staining was observed in all conditions on unsoaked scaffolds, a sharp divide between ppAm microfibre scaffolds with and without HS was observed on pre-soaked scaffolds. The difference between the two staining profiles could be attributed to the variability of the neural differentiation process. However, the positive staining present on soaked ppAm microfibre scaffolds +HS highlighted organised, well developed structures and re-emphasised the ability of the HS to promote neural differentiation.

Neural differentiation of mouse ES cells in N2B27 medium is promoted by autocrine and/or paracrine signalling by FGF4 (Stavridis et al., 2007; Ying et al., 2003b). FGF4 signalling via FGFRs is facilitated by HS (Aviezer et al., 1999; Guimond et al., 1993; Sugaya et al., 2008) with an apparent specific requirement for N-, 6-O and 2-O sulphation within the HS chain (Ashikari-Hada et al., 2004; Sugaya et al., 2008). EXT1−/− cells and ES cells deficient in N-sulphation (NDST1/2−/− ES cells) have impaired differentiation and cannot form EBs with organised germ layers (Lanner et al., 2009; Lin et al., 2000). However, both cell phenotypes can be rescued by the addition of exogenous heparin to the cell culture medium (Johnson et al., 2007; Lanner et al., 2009). The extensive neural differentiation of EXT1−/− ES cells on ppAm microfibres with immobilised HS confirms the biological activity of the HS on the scaffold surface. The enhanced neural differentiation present at day 6 in +HS+FGF conditions, together with the more extensive neural processes present at day 8 on unsoaked scaffolds, suggests that the immobilised HS worked in concert with FGF4; facilitating its binding to the FGFRs on the cell surface and enhancing differentiation towards a neural lineage. However, further analysis of downstream signalling products such as phosphorylated Erk1/2 will be necessary to confirm this (Stavridis et al., 2007; Sugaya et al., 2008).

At present it is unclear whether the signalling complexes of FGF4, HS and FGFR are endocytosed or if the signalling complex is held in a fixed at the plasma membrane due to the immobilisation of the HS on the scaffold surface. If the latter is the case, the complex would likely have a greater half life and an enhanced signalling capacity. Activated FGFR signalling complexes are removed from the plasma membrane by clathrin mediated endocytosis. Covalent immobilisation of growth factors has previously led to enhanced and sustained signalling compared to soluble forms, hypothetically due to inhibited internalisation and growth factor localisation at the cell surface (Fan et al., 2007). However, internalised FGF (notably FGF2) are translocated to the nucleus where they interact with a number of proteins to regulate ribosomal gene transcription and biogenesis (Sorensen et al., 2006). Not only does this contribute to the cell behaviour, but in some cases is essential for the mitogenic response (Sorensen et al., 2006). However, covalently immobilised FGF2 has previously enhanced cell proliferation and attachment to polyamide meshes compared to soluble FGF2 (Nur
et al., 2008). As the assessment of HS retention using radiolabelled HS was conducted without any cells, it is unclear what signalling dynamics are occurring. It therefore remains to be determined whether the HS immobilised on the surface remains attached to the fibres or is detached as the cells dynamically alter their microenvironment.

Pre-soaked ppAm microfibre scaffolds were also able to support the differentiation of wildtype ES cells. As with EXT1<sup>−/−</sup> ES cells, WT1 cells formed large spherical aggregates on the scaffold surface indicating that such behaviour during neural differentiation was not cell type specific but due to the morphology and surface chemistry of the ppAm microfibre mesh. However, WT1 cells formed more clustered aggregates on the scaffold surface which appeared to hinder the formation of independent complexes of neural extensions previously observed with EXT1<sup>−/−</sup> cells. This could be attributed to improved attachment, survival and growth of the WT1 cells compared to HS deficient EXT1<sup>−/−</sup> cells. If this is the case then optimisation of cell seeding density may improve the extension of neural processes over and into the scaffold.

The aggregation of mouse ES cells on electrospun scaffolds during neural differentiation was also observed by Xie et al. (2009) on both randomly and aligned fibre meshes. In concurrence with WT1 and EXT1<sup>−/−</sup> differentiation, a network of neural processes extended out from the cell masses and over the scaffold surface. The study also seeded the scaffolds with EBs formed with RA and cultured the constructs in neural basal media containing B27 (1:50 dilution). Neural differentiation led to Tuj1/βIII tubulin positive processes extending from the EB and forming networks very similar to those presented here. When cultured on the aligned scaffolds, the processes followed the alignment of the fibres and demonstrated enhanced neurite extension (Xie et al., 2009). In comparison, neural processes on random fibres appeared not to follow individual fibres and were apparently dominated by chemokine signalling. This appeared to be the case with cells cultured on ppAm microfibre meshes. The neural processes extended from aggregates forming a close knit network. Particularly with EXT1<sup>−/−</sup> ES cells, extensions were observed originating from one cell mass and then weaving across the scaffold to wrap around a neighbouring aggregate. SEM analysis also revealed extensions crossing over multiple fibres rather than relying on a particular path of fibres for guidance.

In the study by Xie et al., three populations of cells were observed migrating from the central EB mass at the end of the culture period; oligodendrocytes (O4<sup>+</sup>ve), astrocytes (GFAP<sup>+</sup>ve) and neurons (Tuj1/βIII tubulin<sup>+</sup>ve). It is entirely possible that such subpopulations were formed in the current study. As the monolayer differentiation protocol first introduced by Ying et al. (2003b) does not select against the formation of other cell phenotypes, it is also possible that mesodermal and endodermal cells were present. Further analysis for specific markers will be necessary to determine the populations present on ppAm microfibre scaffolds with and without immobilised HS.

βIII tubulin staining of WT1 cells at day 6 of neural differentiation was largely the same between conditions, with only a limited number of aggregates extending neural processes in +HS+FGF4 conditions compared to EXT1<sup>−/−</sup> cells. WT1 ES cells also formed neural processes that extended out of the aggregates and over the scaffold surface, with more extensive βIII tubulin staining present on scaffolds functionalised with HS and on scaffolds -HS but supplemented with exogenous FGF4. The localisation of Map2 to aggregates and origins of neural processes is in agreement with EXT1<sup>−/−</sup>
differentiation and the distribution of Map2 within neurons (Dehmelt and Halpain, 2005). Map2 staining of cells cultured in +HS+FGF4 conditions revealed weaker expression compared to +HS-FGF4 and -HS+FGF4. Considering βIII tubulin staining remained high, the weaker Map2 expression could be due to the tight clustering of aggregates described above obscuring the detail of the more refined Map2 positive cell constructs. Although no obvious difference in βIII tubulin staining was observed between +HS-FGF4 +HS+FGF4 conditions, the limited neural differentiation of cells cultured in -HS-FGF indicates that immobilisation of HS on the scaffold surface does enhance neural differentiation of wildtype ES cells on ppAm microfibre meshes.

The final aim of the study was to conduct a preliminary investigation of whether immobilised HS enhanced the attachment, proliferation and self-renewal of human ES cells on ppAm microfibre scaffolds. Unsoaked ppAm microfibre scaffolds were cytocompatible supporting the attachment and growth of the human ES cell line, HUES7. The greatest level of attachment and growth was observed when cells were seeded and cultured in MEF conditioned media +10ng/ml FGF2. Cells formed large compact Oct4 positive ES cell colonies after 5 days of culture, with only a limited number of Oct4-ve cells present on ppAm microfibre scaffolds +HS. In comparison, very little attachment was observed when cells were seeded in unconditioned media +100ng/ml FGF2. At the end of the 5 day culture period, only small loose colonies were present on the surface with areas deficient in Oct4 expression. The increased binding in MEF conditioned media may be due to the difference in composition compared to unconditioned media. MEFs secrete proteins such as albumin and an array of HSPGs including perlecan and syndecan-4 (Levenstein et al., 2008). The adsorption of these secreted products to the scaffold surface may have enhanced human ES cell attachment and proliferation. This is supported by the traditional requirement of ECM components for hES cell attachment. Previous substrates have included matrigel (Xu et al., 2001), laminin (Li et al., 2005b) and fibronectin (Amit et al., 2004; Lu et al., 2006). Human ES cells can be supported in unconditioned media supplemented with 100ng/ml FGF2 when cultured on matrigel (Levenstein et al., 2008; Xu et al., 2005). However, the absence of MEF secreted products and the lack of FCS within the media would have greatly restricted the formation of an adsorbed protein layer for hES cell attachment to ppAm microfibre scaffolds.

It has previously been reported that MEF secreted HSPGs stabilise endogenous and exogenous FGF2, protecting it from degradation and enhancing signalling (Levenstein et al., 2008). It was hoped that immobilised HS on the surface of the ppAm microfibre scaffolds would stabilise FGF2 and form signalling complexes with FGFR2 on the cell surface (Eiselleova et al., 2009; Pye and Gallagher, 1999). This enhancement of signalling and the stabilisation of the FGF2 molecule could potentially reduce or eliminate the need for FGF2 supplementation, as supported by previous studies (Furue et al., 2008; Levenstein et al., 2008). The most marked difference between scaffolds with and without immobilised HS was observed when cells were cultured in unconditioned media +100ng/ml FGF2. Cells on ppAm scaffolds +HS formed larger Oct4+ve colonies compared to cells cultured without immobilised HS. This supports the hypothesis that immobilised HS is bioactive, able to facilitate growth factor signalling and to influence ES cell behaviour. Colonies cultured in MEF-CM were largely similar between scaffolds with and without immobilised HS, with a slight reduction in Oct4 expression at day 5 on ppAm microfibre scaffolds +HS. This could suggest a competitive relationship between the immobilised HS and the HSPGs secreted by MEFs. The
binding of FGF2 by immobilised HS is sufficient to promote hES cell proliferation in unconditioned media. However, in MEF conditioned media the immobilised HS may reduce the amount of FGF2 available for higher efficiency signalling via MEF secreted HSPGs. However, the preliminary nature of the study, restricted by time and reagents, necessitated that only one replicate per culture condition was analysed. Therefore, further analysis will be necessary to determine the optimal conditions for human ES cell propagation on ppAm microfibre scaffolds with and without immobilised HS.

The results described above confirm that plasma polymerisation of allyl amine onto PLGA microfibre scaffolds resulted in a more hydrophilic mesh that was capable of binding heparin and HS in a manner that was accessible for ligand binding. The GAGs appeared to retain characteristic sulphation epitopes and were biologically functional and competent to support ES cell differentiation. HS/heparin was stably bound by non covalent ionic interactions and was retained by the scaffolds for at least 10 days when incubated in N2B27 and PBS. Except for a minor degree of selective binding of HS chains enriched in 6-O-sulphation at concentrations above the 1-2µg/cm² saturation level, the scaffolds were capable of binding all forms of HS within a heterogeneous HS preparation. Immobilised HS/heparin was bound in a form accessible to the link module of TSG-6 and sulphation epitope specific ScFv antibodies. Despite bA-Link_TSG6 binding raising questions over the accessibility of HS/heparin immobilised on soaked scaffolds, both soaked and unsoaked ppAm microfibre scaffolds were capable of rescuing the neural differentiation capacity of EXT1−/− ES cells. Neural differentiation was extensive, with neural processes extending across the scaffold surface and into the mesh itself creating a 3D culture environment. The addition of FGF4 accentuated neural differentiation on ppAm microfibre scaffolds +HS, suggesting that the immobilised HS may act synergistically with FGF4 and participate in FGF: HS: FGFR complexes. In addition, the ppAm microfibre scaffolds were cytocompatible with human ES cells, supporting the attachment and growth of large Oct4+ve colonies in MEF conditioned media. The full implications of this research and the potential for further development and application will be discussed in the next section.
5 Discussion and Future Directions

Both human and mouse ES cells are currently cultured almost exclusively in flat 2D conditions, with no flexibility in terms of surface structure or 3D architecture. The culture of human ES cells remains largely undefined, with a reliance on high levels of FGF2 and ECM coated TCP, conditions under which the ability to support human ES cells demonstrates a high degree of variability. Biomaterial scaffolds have shown promise in enhancing ES cell pluripotency, proliferation and differentiation by providing an environment more reminiscent of that experienced in vivo. If ES cells are to be propagated on a large scale or differentiated towards mature adult cell phenotypes and highly organised tissues, novel scaffolds with controllable, reproducible architecture are necessary.

Within the ICM and during development, cells are governed by growth factors and cell: cell, cell: ECM interactions. The fundamental importance of ECM cues is demonstrated during EB development where the deposited basement membrane provides an instructive, organised fibrous substratum for ES cell differentiation. A diverse array of fabrication techniques has developed a range of scaffolds with distinct architecture, each aiming to replicate the in vivo environment from which the cultured cells are derived. Electrospinning is a versatile and, in the case of ES cells, underexplored technique that produces non woven meshes comprised of nm scale fibres with a high surface area: volume ratio and high porosity (Huang et al., 2003; Li et al., 2002b). The versatility of electrospinning lies in the ability to spin a wide array of synthetic and natural polymers (either together or separately) and produce meshes with different dimensions and fibre organisation, simply by varying spinning parameters or altering fibre collection methods. Synthetic polyesters are currently at the forefront of electrospinning as they provide mechanical stability, reproducibility, tailored degradation rates and flexibility in terms of co-spinning with natural polymers and post synthetic modification. The initial aim of the study was to assess the capacity of electrospun PLGA fibres to support either ES cell propagation or differentiation. Not only would the electrospun meshes provide topography more reminiscent of the ECM experienced in vivo, but the scaffolds would also provide flexibility in terms of surface structure and scaffold architecture, with the potential for 3D culture.

The second aim was to incorporate heparin/HS in a biologically functional form that was free to participate in protein interactions and which presented the sulphation motifs necessary to facilitate growth factor-receptor binding and therefore mediate cell fate. The importance of HS in ES cell development has been underscored by a number of studies. In culture, ES cells appear to manipulate their immediate microenvironment by expressing unusually low sulphated HS, which becomes decorated with specific sulphation motifs upon differentiation (Baldwin et al., 2008; Johnson et al., 2007). These modifications potentially function to regulate differential binding to growth factors such as FGF2 and VEGF. By anchoring functional HS to electrospun scaffolds, it may be possible to replicate and accentuate the natural modifications ES cells make to native HS. The incorporation of HS/heparin with electrospun scaffolds has been explored with limited success. To date, HS/heparin has not been successfully immobilised on electrospun scaffolds in a fully functional form. The combination of the two has the potential to provide a highly flexible system for cell culture. By binding specific HS species and altering electrospinning parameters to produce
scaffolds with particular dimensions, a scaffold tailored for stem cell culture and differentiation into specific cell types can be created.

Electrospun PLGA scaffolds and spin coated films were able to sustain the attachment and growth of mouse ES cells. Although attachment appeared to be compromised compared to gelatinised TCP, which may have contributed to the observed reduced proliferation, the scaffolds sustained mouse ES cells in a pluripotent state for up to 17 days. Distinct differences in morphology were observed between the two surfaces, with ES cells on microfibre meshes adopting a more rounded and heaped appearance compared to the increased spreading present on spin coated films. In disagreement with previous research (Nur et al., 2006), the observed differences in morphology did not translate into differences in proliferation or pluripotency with both scaffolds sustaining ES cells to the same degree. However, electrospun scaffolds represent a more versatile system for cell culture. As demonstrated in this study, by altering parameters such as flow rate, polymer concentration and applied voltage it is possible to increase fibre diameter creating a thick mesh with the potential to create a 3D culture environment. E-cadherin\textsuperscript{\textsuperscript{-/-}} ES cells proved a useful tool in assessing the ability of electrospun meshes to support cell infiltration. Cell infiltration below the first layer of fibres was observed in meshes with an average fibre diameter of 2.43\,\mu m and 3.27\,\mu m. Although pore size was not addressed in this study, electrospun meshes with an approximate average fibre diameter of 2\,\mu m and 3\,\mu m have an estimated pore size of 10.5\,\mu m and 14.9\,\mu m respectively (Pham et al., 2006). The minimum pore size requirements for ES cells has not yet been determined, however meshes with an average fibre diameter of approximately 5\,\mu m and average pore size of >20\,\mu m facilitated full infiltration of MSCs (Pham et al., 2006). Therefore, although cell infiltration was observed, it is likely that the critical fibre diameter and subsequent pore size for full penetration into the scaffold was not met. However, infiltration into the scaffolds may be further enhanced by culturing in a flow perfusion bioreactor, which has previously increased cellular infiltration 5 fold compared to static culture conditions (Pham et al., 2006).

It could be argued that increasing fibre diameter compromises the ECM mimicking architecture of electrospun meshes. However, the cross-section views of both microfibre and macrofibre scaffolds demonstrated the stark difference in scaffold dimensions. Whilst the macrofibre fibres deposited to form a mesh 106.25\,\mu m in thickness, the microfibre meshes formed a sheet thinner than the width of a cell. The differences between the two constructs highlights the problems electrospun meshes face relating to their potential as 3D scaffolds. One of which is a lack of cell migration into the scaffold centre and another is the difficulty in producing a mesh with enough volume to support multiple layers of cells. The thickness of the macrofibre scaffolds presented here combined with the observed initial infiltration supports the ability of macrofibre scaffolds to realise the 3D potential of electrospun meshes. One of the many advantages of plasma polymerisation is that 3D scaffolds, such as porous sponges, can be effectively coated due to diffusion of reactive species into the scaffold interior, facilitating cell infiltration (Barry et al., 2005). ppAm coating of macrofibre scaffolds may not only facilitate cell infiltration, but could also create a heparin/HS functionalised, degradable 3D scaffold with the potential to support the differentiation of stem cells into mature cell types and organised, multilayered tissue (Park et al., 2009).

Heparin/HS was successfully non-covalently immobilised in a form accessible to ligand binding and presented the essential sulphation epitopes necessary for their biological function. Bound HS
rescued the neural differentiation capacity of \( \text{EXT1}^{-/-} \) ES cells and worked in synchrony with FGF4 to enhance neural differentiation on the scaffold surface. The immobilisation of heparin/HS in a fully accessible form competent to direct cell fate is in contrast to studies where covalent immobilisation has rendered the GAGs unable to facilitate growth factor binding (Park et al., 2009). By using ionic immobilisation, stable at physiological salt concentrations, the heparin/HS molecules remain unaltered and retain their biological activity. The immobilisation of HS may indeed enhance the signalling capacity of growth factors as FGF2 covalently anchored to electrospun polyamide meshes enhanced fibroblast proliferation, neurite outgrowth and human ES cell attachment (Nur et al., 2008). Covalent immobilisation of EGF also provided greater and more sustained ERK phosphorylation in MSCs compared to soluble forms, possibly due to inhibition of ligand-receptor internalisation and increased local concentration of EGF at the cell surface (Fan et al., 2007). The immobilisation of heparin/HS on the scaffold surface could have similar effects, enhancing binding of growth factors such as FGF2 and FGF4 by localising them at the cell surface and preventing internalisation, as well as increasing their stability in culture. Assessments of phosphorylation levels of ERK and FGFRs, will help to unravel the signalling events which occur during neural differentiation on ppAm microfibre scaffolds +/- HS and to determine if signalling via FGF4 is enhanced and sustained when HS/heparin is in an immobilised form.

An assessment of the signalling events occurring during human ES cell culture on ppAm microfibre scaffolds would also determine whether immobilised HS facilitates FGF2 signalling. Considering the stabilising role of MEF secreted HSPGs in supporting FGF2 signalling during human ES cell propagation (Levenstein et al., 2008) and the enhanced FGF2 signalling facilitated by exogenous heparin in certain media compositions (Furue et al., 2008), electrospun meshes functionalised with immobilised HS/heparin hold great potential in human ES cell culture. The immobilised HS has the potential to stabilise, enhance and sustain FGF2 signalling, therefore reducing the amount of FGF2 necessary to maintain human ES cells. ppAm microfibre meshes with and without immobilised HS supported the attachment and growth of human ES cells in MEF conditioned media, with Oct4 positive aggregates observed after 5 days. Although the ppAm scaffolds demonstrated reduced human ES cell attachment and growth in unconditioned media, an improvement was observed when HS was immobilised on the surface. The reduced attachment and proliferation in unconditioned media could be attributed to the absence of MEF secreted ECM proteins such as laminin and fibronectin (Braam et al., 2008). The adsorption of such elements to the ppAm microfibre scaffolds may have promoted the improved cell attachment/growth of human ES cells observed in MEF conditioned media. This could be overcome by adsorption of fibronectin, as adsorbed fibronectin has previously sustained human ES cells (Amit et al., 2004; Baxter et al., 2009) and facilitated attachment of neural stem cells to ppAm coated PLGA microspheres (Bible et al., 2009). Adsorbed fibronectin combined with assisted and enhanced signalling of FGF2 by immobilised HS has the potential to create a more defined environment for human ES cell culture.

Covalent immobilisation of FGF2 on the surface of polyamide meshes enhanced the attachment of human ES cells and largely retained its biological activity after storage at 4°C over 5 months, demonstrating its potential as a commercial product (Nur et al., 2008). However, immobilisation of heparin/HS on ppAm scaffolds has a number of advantages over tethering of specific growth factors. One of which is the comparatively inexpensive nature of HS/heparin compared to FGF2.
and its higher stability in storage. Also, although at present HS and heparin are largely isolated from tissue such as porcine mucosa, recent advances in creating synthetic, defined heparinoids means that heparin/HS immobilised ppAm meshes will meet the requirements for an animal-free human ES cell culture environment. Advances in the creation of synthetic GAGs include the use of bacterial enzymes for the synthesis of unmodified GAG chains (Sismey-Ragatz et al., 2007) and the creation of an artificial Golgi using microfluidics and functionally active enzymes such as 3-OST (Martin et al., 2009). In addition, once polymerisation parameters are optimised, scaffolds can be coated with ppAm with relative ease. The only subsequent stage is soaking with HS/heparin in PBS, an elegantly simple method compared to the multi-step process necessary for covalent immobilisation.

As HS/heparin assists the binding of an array of growth factors the scaffolds are applicable to a variety of culture systems. Covalently immobilised heparin has previously been used in an attempt to enhance chondrogenic differentiation of MSCs by facilitating TGFβ1 signalling (Park et al., 2009). Considering electrospun PLGA scaffolds have been successfully applied to the chondrogenic differentiation of MSCs (Xin et al., 2007), the ppAm electrospun scaffolds functionalised with HS/heparin may serve to further enhance chondrogenic differentiation. MSCs are often maintained in media supplemented with FGF2 and have previously been sustained in a multipotent state by electrospun PLGA scaffolds (Xin et al., 2007). This combined with the fact MSCs are derived from the bone marrow, an environment rich in PGs, suggests ppAm microfibre scaffolds functionalised with HS may have promise in sustaining MSCs. In addition, VEGF has been previously incorporated into biomaterial scaffolds in an effort to increase vascularisation (Kanczler et al., 2010). Immobilised HS may enhance angiogenesis, creating vascularised tissue from cells seeded within the scaffold and also assisting vascular integration with host tissue.

The system is also very versatile in that a wide array of GAGs can be immobilised (Marson et al., 2009). The scaffold could be tailored with specific HS sequences, which promote certain growth factor: receptor interactions; essentially replicating and enhancing the HS alterations that occur during ES cell differentiation. For instance, immobilisation of HS rich in the HS4C3 high binding epitope IdoA(2S)-GlcNS(6S,3S) may facilitate differentiation of mouse ES cells towards haemangioblast progenitors and endothelial cells ((Baldwin et al., 2008), see section 1.2.3). In mouse ES cell culture, BMP signalling assists in maintaining pluripotency by inhibiting neural differentiation. The scaffold could be decorated with HS specifies which promote BMP signalling (Takada et al., 2003) over FGF, differentially regulating growth factor interactions and serving to maintain mouse ES cell pluripotency. For human ES cell culture, FGF2 signalling could be enhanced by the immobilisation of HS rich in N- and 2-O-sulphation (particularly UA-GlcNS-UA-GlcNS-IdoA2S) for FGF2 interaction and in 6-O-sulphation (particularly the trisulphated IdoA(2S)-GlcNS(6S)) for FGFR binding. Immobilised HS could also be tailored to simultaneously facilitate noggin signalling (Viviano et al., 2004), repressing BMP4 signalling and further enhancing human ES cell pluripotency (Xu et al., 2005).

The successful rescue of EXT1−/− ES cells and the development of extensive neural processes highlights the potential of the scaffold in neural tissue engineering. The high porosity and the ability to manipulate fibre architecture are just two reasons why electrospun meshes hold great promise as tubular conduits for nerve regeneration. The inherent permeability of electrospun P3LGA
enabled diffusion of nutrients whilst simultaneously creating a barrier to unwanted tissues (Bini et al., 2004). In addition, culture on electrospun PLA scaffolds formed from aligned microfibres enhanced the neurite extension of neural stem cells, the growth of which aligned with the fibres of the scaffold (Yang et al., 2005). The optimum parameters for enhanced neural differentiation of ES cells on ppAm microfibre scaffolds remains to be determined. Wild type ES cells did not form as extensive neural processes compared to EXT1\textsuperscript{-/-} and instead appeared to form clusters of cell aggregates. However, the aggregated morphology of ES cells on the scaffolds appears to be an inherent characteristic of ES cells culture on microfibre meshes and has been observed by previous researchers (Xie et al., 2009). Adjusting cell density may address this issue and enable greater extension of neural processes in wildtype ES cells. Previous studies have reported aligned neural processes when mouse ES cells differentiated on aligned meshes. Optimisation of ppAm scaffolds for neural differentiation should explore whether the combination of aligned ppAm microfibre scaffolds together with immobilised HS function to further enhance neural differentiation of mouse ES cells. The ppAm microfibre scaffolds could also be applied to the neural differentiation of human ES cells. Differentiation of human ES cells into dopaminergic and motoneurons requires treatment with sonic hedgehog (SHH) (Suter and Krause, 2008), with differentiation towards a dopaminergic cell fate also requiring FGF8 (Li et al., 2005a; Suter and Krause, 2008). As the activity of both factors are modulated by HS interactions, neural induction of human ES cells on random or aligned ppAm scaffolds functionalised with HS may enhance differentiation towards a more mature cell phenotype.

Plasma polymerisation proved an effective method of dramatically altering the surface chemistry without affecting scaffold dimensions. This is in agreement with previous studies that report little/no impact on scaffold architecture or mechanical properties of PLGA electrospun meshes after plasma treatment with acrylic acid (Park et al., 2007). Although the mechanical properties of the mesh were not assessed in this particular study, it may be prudent to address this characteristic considering its impact on stem cell behaviour. Engler et al. (2006) reported that the matrix stiffness of polyacrylamide gels induced the differentiation of human MSCs, in the absence of supplementary growth factors, towards cell phenotypes which reside in tissue with similar mechanical properties. In addition, Evans et al. (2009) reported altered mouse ES cell differentiation with varying substrate stiffness, with increasing stiffness promoting mesodermal and osteogenic lineages.

The use of \textsuperscript{3}H HS confirmed the retention of HS by ppAm microfibre scaffolds over a 10 day incubation period in N2B27 and PBS, a result not previously reported for electrospun meshes. This is in agreement with previous studies that report little/no impact on scaffold architecture or mechanical properties of PLGA electrospun meshes after plasma treatment with acrylic acid (Park et al., 2007). Although the mechanical properties of the mesh were not assessed in this particular study, it may be prudent to address this characteristic considering its impact on stem cell behaviour. Engler et al. (2006) reported that the matrix stiffness of polyacrylamide gels induced the differentiation of human MSCs, in the absence of supplementary growth factors, towards cell phenotypes which reside in tissue with similar mechanical properties. In addition, Evans et al. (2009) reported altered mouse ES cell differentiation with varying substrate stiffness, with increasing stiffness promoting mesodermal and osteogenic lineages.

The application of \textsuperscript{3}H HS also highlighted the instability of the ppAm coating. The progressive loss of \textsuperscript{3}H HS into the surrounding media during the 10 day incubation period originated from detached ppAm coating adhered to the inside of the sample tube rather than from the scaffold itself. This was
concluded as no loss in $^3$H HS was observed from the isolated scaffolds, despite the cumulative loss of counts into the surrounding media. Soaking scaffolds overnight removed the unstable elements within the ppAm layer, as confirmed by $^3$H HS analysis. Removal of the unstable layers should therefore have eliminated the HS leaching from ppAm fragments attached to the inside of the well. When repeating the differentiation of EXT1$^{+/−}$ ES cells, numerous efforts were made to ensure that the culture conditions were not contaminated with detached ppAm. Scaffolds were soaked overnight in PBS, transferred to fresh PBS solution for mounting in CellCrown holders and transferred to fresh wells after HS immobilisation. Therefore, the ability of soaked ppAm scaffolds to rescue the neural differentiation capacity of EXT1$^{+/−}$ ES cells suggests cells were responding to HS bound to the scaffold surface. Further research including an assessment of HS retention of soaked scaffolds during incubation in media, will serve to confirm this conclusion.

The effect of soaking in PBS/aqueous solution on the surface chemistry and the immobilisation of HS should also be investigated further. Although soaked scaffolds bound the equivalent amount of HS and rescued the neural differentiation of EXT1$^{+/−}$ ES cells, bALink_TSG-6 binding data suggested that, compared to unsoaked scaffolds, HS was not bound in an optimal form for ligand binding. This can potentially be verified using XPS analysis to quantify the amount of bound HS paired with repeated bA-Link_TSG-6 binding assays, as conducted by Marson et al. (2009). XPS analysis of soaked scaffolds will also determine what alterations are occurring at the scaffold surface and assist in optimising coating parameters to create a more stable coating. Alteration of plasma deposition parameters should be done with care to minimise loss of functional amine groups. However, the incorporation of the hydrocarbon monomer octadiene has previously stabilised plasma polymerised acrylic acid in aqueous solution (Alexander and Duc, 1999). It has also been used to create ppAm gradients, with optimal heparin binding observed at 60-90% ppAm (Marson et al., 2009). Therefore, incorporating octadiene may enhance ppAm coating stability without adversely affecting GAG immobilisation.

Microfibre meshes represent highly flexible scaffolds for ES cell culture, supporting ES cell proliferation and pluripotency at levels comparable to gelatin. Fibre dimensions were manipulated with ease by altering spinning parameters, creating meshes with increased fibre diameter and thickness, encouraging cell infiltration and potentially providing a 3D environment for ES cell propagation/differentiation. The retention of $^3$H HS by unsoaked scaffolds and immobilisation of heparin/HS in a form accessible to ligand binding is a breakthrough in the incorporation of HS/heparin into biomaterial scaffolds with previous attempts resulting in loss of heparin from electrospun fibres (Casper et al., 2005; Luong-Van et al., 2006) and an inability to facilitate growth factor receptor binding after covalent immobilisation (Park et al., 2009). In the current study immobilised HS and heparin were free to participate in protein interactions and presented the sulphation epitopes implicated in mediating biological activity. The ability of immobilised HS to direct cell fate and work in synchrony with growth factor binding has extensive implications in both mouse and human ES cell culture. The success and ease of HS immobilisation on electrospun ppAm microfibre scaffolds in a biologically active and accessible form creates a highly versatile scaffold, where both fibre architecture and GAG species can be tailored to govern ES cell behaviour.
References


