Bioactive Self-Assembly Hydrogel for the Treatment of Intervertebral Disc Degeneration

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Abstract

Severe back pain caused by intervertebral disc degeneration (IVDD) and disc herniation effects up to 35% of people within their lifetime, of which 10% of cases lead to sufferers being chronically disabled. The impact of these conditions has been estimated in terms of social-economic cost, to be in excess of €12 billion per year in the UK alone. While, the biological processes that cause IVDD are not currently fully understood, IVDD can be characterised as progressive degeneration of the nucleus pulposus tissue, driven by a combination of inflammatory immune processes, enzymatic degradation of extracellular matrix components and changes in cellular phenotype and expression. While there is currently no suitable long-term treatment for IVDD, there has been a new drive of research in the field of tissue engineering and regenerative medicine in an attempt to find a potential treatment for this condition. The aim of this study is to develop a novel bio-active self-assembly hydrogel capable of \textit{in situ} formation as a potentially injectable treatment for IVDD. The design and intended formulation of this hydrogel was based on both the previous work of (Reches and Gazit, 2003) on the self-assembly peptide, Fmoc-F$_2$, and the design of a novel peptide from the active site of human insulin like growth factor -1.

The Fmoc-F$_2$ and sIGF-1 peptide were synthesised using conventional liquid and solid phase peptide synthesis methods, respectively. The produced peptides were analysed for their content, yield and purity by HPLC and mass spectrometry. Subsequently, the Fmoc-F$_2$ peptide hydrogels were formulated and studied to explore the mechanisms of self-assembly, material properties and nanostructure. Furthermore, the peptide hydrogel was tested for its ability to support the growth of primary bovine chondrocytes and for its biocompatibility and potential toxicity of its degradation products. The produced sIGF-1 peptide was also investigated to assess its potential dose curve effect on the growth and expression of both primary bovine chondrocytes and human mesenchymal stem cells. Together, the sIGF-1 peptide and Fmoc-F$_2$ hydrogels were integrated to create a bio-active hydrogel capable of supporting primary bovine chondrocyte migration and extracellular matrix deposition \textit{in vitro}.

Results confirmed the successful synthesis of both peptides obtaining high purities suitable for biological investigation. Study of the material properties of Fmoc-F$_2$ hydrogel found the material to be viscoelastic in nature, while possessing a complex nanostructure based upon the assembly of beta-sheets formed of a helical or twisted type stacking of peptide monomers, consistent with current literature. Biological investigation found no significant material toxicity to primary bovine chondrocytes, the cells adopting a spread morphology on the surface of 2D hydrogels. The \textit{in vitro} testing of the novel sIGF-1 peptide showed the ability of the peptide to elicit a significant dose sensitive increase cellular growth in both primary bovine chondrocytes and human mesenchymal stem cells. 3D cellular culture of formulated bioactive hydrogel found primary bovine chondrocytes were capable of migration and proliferation through the hydrogel matrix. Cell encapsulation in 3D hydrogels showed the significant deposition of extracellular matrix components.

In conclusion, the formulated sIGF-1 peptide loaded Fmoc-F$_2$ hydrogel has been shown to have suitable rheological and biological properties for application in the minimally-invasive treatment of IVDD.
Publications & Dissemination


- In vitro bioactivity of IGF-1 mimetic peptide on human mesenchymal stem cells: a therapeutic agent for avascular tissue regeneration, J. Lacey, S. Meikle, A. Guildford, G. Philips and M. Santin - 9th World Biomaterials Congress (WBC), Chengdu, China, 1-5 June (2012)


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Declaration

I declare that the research contained within this thesis, unless otherwise stated within text, is the original work of the author. The thesis has not been previously submitted in any form to any other university in fulfilment of any degree or qualification, nor has any of its material contained within.

Joseph Lacey  
date
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AF</td>
<td>Annulus fibrous</td>
</tr>
<tr>
<td>b-FGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CEP</td>
<td>Calcified end plate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CRD</td>
<td>Cell receptor domain</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial cell growth factor</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMB</td>
<td>Diethyl methylene blue</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Minimal Essential Medium</td>
</tr>
<tr>
<td>Fmoc-</td>
<td>Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>Fmoc-F&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Fmoc-diphenylalanine</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>HA</td>
<td>Hyuronic acid</td>
</tr>
<tr>
<td>HBTU</td>
<td>N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGF-BP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor – beta</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>il-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IVD</td>
<td>Intervertebral disc</td>
</tr>
<tr>
<td>IVDD</td>
<td>Intervertebral disc degeneration</td>
</tr>
<tr>
<td>KS</td>
<td>Keratin sulphate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MD</td>
<td>Mesodifferentiated</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
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<td>------------------------------------------------</td>
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<tr>
<td>MTS</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
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<tr>
<td>NP</td>
<td>Nucleus pulposus</td>
</tr>
<tr>
<td>NR</td>
<td>Nerve root</td>
</tr>
<tr>
<td>PAA</td>
<td>poly acrylic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBC</td>
<td>Primary Bovine Chondrocytes</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly ethylene glycol</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time – polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Spinal cord</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TWIST 1</td>
<td>class A basic helix-loop-helix protein 38</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
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Chapter 1: Introduction
1.1 Background & overview

Severe back pain caused by intervertebral disc degeneration (IVDD) and disc herniation effects up to 35% of people within their lifetime, of which 10% of cases lead to sufferers being chronically disabled (Maniadakis, 2000). The impact of these conditions has been estimated in terms of social-economic cost, to be in excess of €12 billion per year in the UK alone (Maniadakis, 2000). While there is no clear definition, IVDD is often described as an accelerated aging process causing a reduction or change in the size and morphology of the nucleus pulposus (NP) (Urban and Roberts, 1995). This change leads to a loss in the mechanical properties of the disc, starting a process of degeneration driven by multi-factorial changes in cellular growth, expression, inflammation and vascular invasion (Urban and Roberts, 2003, Zhao, 2007). The progression of this condition significantly impacts on the quality of life of hundreds of thousands of people every year and is therefore an essential area of current research. In an attempt to find an alternative to prosthetic discs researchers have focused on the development of biomaterial hydrogels for the delivery of chondrocytes or stem cells in order to regenerate the damaged tissue (Collin et al., 2011, Halloran, 2008). While there has been much improvement in this area of research, there are currently no acceptable long term treatments for IVDD. However, recent developments have greatly contributed to both the understanding of the biological processes of degeneration and the biomaterial properties required in order to achieve regeneration (Zhao, 2007).

Recently, there have been a number of new approaches to the treatment of IVDD, of which the most promising is the research into injectable hydrogels for the delivery of chondrocyte-based cell therapies (Collin et al., 2011, Crevensten et al., 2004, Halloran, 2008). Although, presenting a significant step forward, the production of
the biomaterial used relies on naturally derived extracellular matrix (ECM) components from animal sources which carry the risk of transmittable disease and batch-to-batch variation (Collin et al., 2011).

To address the problems of the current material approaches there has been a surge in research focusing on the use of peptides which mimic components of the ECM (Yan, 2010). This important area of research, branched from the classical study of proteins and peptides, aims to isolate and utilise short amino acid sequences responsible for the successful control of cellular processes within the body. Specific sequences such as RGD and YISGR have been used to mimic cellular binding domains on specific ECM proteins, fibronectin and laminin respectively (Yan, 2010). These modifications have been made in an attempt to control cellular phenotype and expression, a major limitation in the use of chondrocytes and stem cells for the treatment of a range of conditions (Dawson et al., 2008). In addition to the modification of conventional biomaterials with peptide bio-ligands, peptides have recently been used to create self-assembling nanostructure materials in the form of hydrogels, of which the most simple is comprised of a sequence just two amino acids long (Yan, 2010). This new class of materials have a number of advantages over naturally derived biomaterials such as collagen, as they can be produced in a controllable and cost-effective manner and do not carry the risks of transmittable diseases, poor batch to batch reproducibility and high production costs commonly associated with animal derived products. This class of materials are most commonly referred to as self-assembly peptide hydrogels, due to their ability to rapidly self-assemble through π-π interactions (Jayawarna et al., 2009). Specifically, one form of self-assembly biomaterial which can be considered relatively new to the field, is Fluorenylmethyloxycarbonyl (Fmoc)-diphenylalanine (Fmoc-F₂) hydrogels. First
reported by Jayawarna et al., 2006, Fmoc-F₂ self-assembly gels have since been studied in terms of their chemical and material properties by a small number of research teams (Yan, 2010). However, in a more recent publication the biocompatibility of Fmoc-F₂ based materials was investigated for its properties as a substrate for 3D cell culture (Jayawarna et al., 2009). This novel research demonstrated the ability of this class of material to support the growth of chondrocytes while inhibiting the growth of fibroblasts (Jayawarna et al., 2009). This research, while being only a preliminary investigation, demonstrates the potential for the use of Fmoc-F₂ hydrogels in cartilaginous tissue engineering and the treatment of IVDD.
1.2 The intervertebral disc & degeneration

1.2.1 Anatomy & function

The IVDs are cartilage-like tissues that are located between the vertebrae of the spine, playing an essential role in the mobility of all vertebrates. They serve the function of shock absorbers, capable of complying with the forces that are applied to the spine. (Shankar, 2009, Zhao, 2007). The structure of the intervertebral disc can be divided primarily into two clearly defined sections, (i) the annulus fibrosus (AF); comprised of vascularised fibrotic tissue and (ii) the nucleus pulposus (NP); formed of a dense cartilaginous matrix maintained and regulated by chondrocyte-like cells (Shankar, 2009) (Figure 1.1).

![Figure 1.1 A schematic view of a spinal segment and the intervertebral disc. The figure shows the organisation of the disc with the nucleus pulposus (NP) surrounded by the lamellae of the annulus fibrosus (AF) and separated from the vertebral bodies (VB) by the cartilaginous end-plate (CEP). The figure also shows the relationship between the intervertebral disc and the spinal cord (SC), the nerve root (NR), and the apophyseal joints (AJ). (Adapted from Urban and Roberts, 2003, Arthritis Research & Therapy Vol. 5 No. 3)
The AF encircles the NP serving as a protective layer of vascularised fibrotic tissue that holds the NP in place while facilitating movement and compression of the IVD (Shankar, 2009). The NP however, is formed of a dense matrix comprised of proteoglycans and collagens that are produced and maintained by a sparse population of chondrocyte-like cells (Shankar, 2009). The ECM of the NP is comprised predominantly of collagen type II forming the basic scaffold of the gel-like matrix (Zhao, 2007). Collagen II is a helical protein that is cross-linked by collagen IX, creating a tightly bound mesh that entraps the chondrocytes while restricting the swell limit of the tissue. In addition, there is an extensive range of glycosaminoglycans (GAGs) and proteoglycans that play essential roles in the function and activity of the ECM of the NP (Zhao, 2007). The main non-collagenous component of the NP is aggrecan, a proteoglycan which is highly cross-linked by GAGs such as chondroitin sulphate (CS) and keratin sulphate (KS) (Zhao, 2007). The negatively charged GAG chains create a high osmotic potential in the NP by binding cations, creating an tissue osmotic pressure of between 0.1 and 0.3 mPa, slightly higher than that of cartilage (0.02-0.2 mPa) (Maroudas and Bannon, 1981), (Basser et al., 1998), (Setton et al., 1995). This High pressure osmotic pressure drives re-hydration of the tissue after loading allowing for restoration of disc height. The ECM of NP differs significantly form that of other cartilages processing a much greater proteoglycan to collagen ratio of 27:1, While hyaline cartilage typically has a ratio of 2:1 (Mwale et al., 2004) In addition to cartilage matrix proteins, there are a wide range of biochemical messengers; such as insulin-like growth factor 1 (IGF-1) and transforming growth factor-beta (TGF-β) which help to regulate cell growth, volume and protein expression (Veilleux and Spector, 2005). The maintenance of this matrix is essential, as changes in the specific types of collagens or the ratio of
proteoglycans can significantly diminish the mechanical strength of the disc (Videman et al., 2007). These will be discussed in greater depth in the following sections. In addition, the third and also very important area of the intervertebral disc is the cartilaginous end-plate (CEP). The CEP is a thin layer tissue that serves as an interface between the vertebrae and the nucleus pulposus (Urban and Roberts, 2003). The CEP facilitates the transfer of nutrients from the vascularised bone to the avascular NP (Shankar, 2009). As the disc ages the CEP becomes progressively more calcified and the supply of nutrients to the intervertebral disc are reduced (Kealey et al., 2005, Modic and Ross, 2007).

1.2.2 Chondrocyte phenotypes and signalling

There are a number of different chondrocyte sub-phenotypes, the main type found in healthy adult cartilage is most commonly referred to as the differentiated phenotype. The differentiated phenotype is typically characterised by cellular expression of high levels of collagen II and IX and aggrecan while presenting with a spherical or ellipsoid-like morphology with a diameter between 2-3 µm (Benya and Shaffer, 1982) (Stockwell, 1971). The differentiated phenotype is ultimately controlled by sox-9 transcription factor which inhibits the β-catenin and wnt pathways (Akiyama et al., 2004). Changes in this regulation have been shown to result in a change in cell phenotype to a fibroblast-like expression and morphology referred to as the dedifferentiated phenotype (Brodkin et al., 2004). This subtype exhibits rapid proliferation rates unlike differentiated chondrocytes which are not proliferative cells. Furthermore, dedifferentiated cells have a marked decrease in the expression of cartilage specific ECM molecules with an increase in collagen I ratio over collagen II (Brodkin et al., 2004). This process is well documented with a number of papers
suggesting possible signalling pathways through the up-regulation of Twist1, leading to the inhibition of sox-9 regulation of the differentiated phenotype through the β-catenin pathway (Gu et al., 2013) (Stokes et al., 2002). The process of de-differentiation occurs during in vitro 2D culture of chondrocytes and has been well documented. The findings of Brodkin et al., 2004 demonstrated this process, finding that after 1 week of 2D monolayer culture a mixture of differentiated and dedifferentiated phenotypes were obtained. While at passages later than 2 weeks all cells were found to be dedifferentiated (Brodkin et al., 2004). This important findings highlights the importance of cell passage number when studying chondrocytes in vitro. If the intention of the study in to investigate the effect of a treatment on differentiated cells then the use of earlier passages is important; whereas if the study purpose is to achieve re-differentiation of de-differentiated chondrocytes, then it is important that later passages are used. While dedifferentiated chondrocytes have a reduced level of ECM molecule expression they also up-regulate the expression of MMP’s such as MMP 3 and MMP 13 (Goessler et el., 2005) that degrade collagen II of the ECM, essential to any cartilaginous tissue formation.

In addition to the de-differentiated phenotype there is a third recently discovered non-embryonic chondrocyte phenotype referred to as an intermediate, mesodifferentiated (MD) subtype (Qusous & Kerrigan, 2012). This phenotype is seen between the phenotype shift of differentiated to dedifferentiated during the first 9 days of in vitro monolayer culture. This MD phenotype is similar to dedifferentiated chondrocytes in that they proliferate and also present with a fibroblastic morphology (Qusous & Kerrigan, 2012). However, their expression of
ECM molecules differs significantly from dedifferentiated, expressing higher levels amount of collagen II and sox-9 (Qusous & Kerrigan, 2012).

1.2.3 Biochemical process of intervertebral disc degeneration

While IVDD is often described as an accelerated ageing process, the molecular process behind this condition is somewhat more complex, with a multitude of cytokines and growth factors playing a significant role in affecting the cellular phenotype and production of matrix components (Zhao, 2007). While a fully coherent biochemical process for IVDD has yet to be elucidated, a number of publications have shown a variety of factors that may play a synergistic role in development of IVDD. Although there seems to be no single specific cause of IVDD, the initiation of the disease is strongly linked with excessive or abnormal mechanical loading of the intervertebral disc, that can be a cause of poor work related practices or recreational sports (Latham, 1994, Videman T, 2007, Videman et al., 2007, Zhao, 2007). This excessive or abnormal loading is thought to cause damage of the nucleus pulposus leading to a process of inflammation and tissue remodelling (Zhao, 2007). The release of inflammatory cytokines interleukin-1 and -6 (II-1 and II-6) and tumour necrosis factor - alpha (TNF-α) induce an inflammatory process (Zhao, 2007). In addition to this process, an increased production of growth factors including basic-fibroblast growth factors (b-FGF) and platelet derived growth factor (PDGF) is seen (Tolonen J et al., 2006, Tolonen J. et al., 1995). From this phase, the increased levels of growth factors have been shown to cause a significant change in cellular expression and phenotype, leading to a more fibroblastic or de-differentiated phenotype from the cellular population (Tolonen J et al., 2006). This has been characterised primarily as a change in the composition of the NP ECM, with increased expression of collagen type I and a down-regulation of collagen type
II, proteoglycans and GAGs (Beard et al., 1981, Cs-Szabo et al., 2002, Shankar, 2009). This process of biochemical and morphological change is very similar to those seen in osteoarthritis, where collagen type I expression increases while collagen type II decreases with significant changes in cell morphology (Quinn and Hunziker, 2005).

In addition to the down-regulation of key matrix components, there is a significant increase in matrix metalloproteases (MMP), such as MMP 13 which breaks down collagen type II and the proteoglycans of the ECM (Crean et al., 1997, Zhao, 2007). The changes in the ECM composition significantly reduce the mechanical strength and water content of the disc contributing to greater damage (Cs-Szabo et al., 2002). Furthermore, morphological changes in the chondrocyte-like cells are observed. The spherical cells observed in healthy discs change to elongated cells with a spindle-like morphology similar to those of fibrocartilage (Quinn and Hunziker, 2005). This series of events in turn leads to a tissue remodelling process, the outcome of which is the invasion of vascularised scar tissue (Modic and Ross, 2007, Zhao, 2007). As the mechanical stability of cartilaginous tissues far exceeds that of fibrotic tissues, this invasion of vascularised fibrotic tissue drives further inflammation through increased cellular damage, the result of which is extreme pain through inflammation of the spinal cord (Zhao, 2007). The process as a whole leads to the clinical observation by MRI which is referred to as “black disc” an example of which can be seen in Figure 1.2. The appearance of a black disc shown in Figure 1.2, is due primarily to the significant reduction in water content of the nucleus pulposus (Morgan, 1999). This is due largely to the decrease in the levels of proteoglycans and GAGs within the ECM of the nucleus pulposus (Hardingham, 1995).
1.2.4 Proteoglycans, Hyaluronic acid and Glycosaminoglycans

Proteoglycans and GAGs play a combined role in healthy discs where they bind cations such as Na\(^+\) and K\(^+\) through interaction with the sulphated functional groups of the GAG chains (Kiani, 2002). The interaction of GAGs with the related cations creates an osmotic potential which drives the hydration of the tissue (Kiani, 2002). This ability to rehydrate and swell the tissue following loading is one of the key properties in the mechanical strength and flexibility of the IVD (Kiani, 2002). Similar to the behaviour of a water bed, the IVD is capable of redistributing load from different angles of compression. In considering this factor, it is a key...
consideration that any potential future treatment will require a strategy to increase the level of GAGs that significantly diminish with degeneration.

As previously mentioned there are a number of proteoglycans and GAGs that are required to maintain the normal functioning of the IVD. Among the proteoglycans, the most abundant in cartilage and the IVD is aggrecan, making up approximately 50% of the NP by dry mass (Buckwalter, 1982). Aggrecan is a large proteoglycan formed of 2132 amino acids divided into 5 distinct domains, G1, G2, KS,CS and G3 each with very different functions (Kiani, 2002). The N-terminal G1 domain serves as an immunoglobulin functioning region, acting as a site for cell recognition, cell adhesion and hyaluronic acid binding (Grover, 1994). While the function of G2 domain is less well understood it is believed to play a significant role in the binding of the CS and KS before modification of the G3 domain (Kiani, 2002). In addition to this function the G2 domain, also referred to as the inter-globulin domain (IGD), contains a number of proteolytic cleavage sites liable to specific MMPs (Hardingham, 1995, Mort, 1998). Proteolytic cleavage at this site results in the loss of the G1 region responsible for cell anchorage to the protein and the cross-linking of the proteoglycan to the HA chains of the ECM (Hardingham, 1995). The third domain is the KS region which as the name suggests is the main region of aggrecan responsible for the binding of KS, with up to 30 chains of 10 kDa KS able to bind for each protein (Doege, 1991). The fourth domain known as the CS region, similar to the KS domain it also binds GAGs but specifically CS and in much greater numbers with approximately 100 CS chains bound through post-translational modification for each protein (Krueger, 1990). The binding of CS chains have been found to be a result of repeating sequences of serine-glycine with occasional spacing of acidic or hydrophobic amino acids (Krueger, 1990). Due to the highly reactive OH group of
serine amino acid, it is likely that the GAGs have a strong interaction with this functionality which could serve as a possible strategy for the binding of GAGs and cations in potential biomaterial treatment approaches. The final G3 domain is composed of two separate protein structures, an endothelial growth factor (EGF)-like region and a cell receptor domain (CRD) region. The EGF region of the protein contains the structures of both EGF-1 and EGF-2 proteins that have been found to elicit a growth promoting bioactive effect (Fulop, 1993). This finding highlights the important biochemical activity of aggrecan in the control of chondrocyte-like cells of the NP and other cartilaginous tissues. In addition to this functionality, the CRD region of the G3 domain has been found to play an essential function in the binding of CS to the proteoglycans during post-translational modification (Li H, 1993). The removal of this section of the domain through the introduction of a stop codon to the responsible gene has been found to block the binding of CS chains to aggrecan (Li H, 1993). The loss of this section of the proteoglycan has also been found to cause the condition known as chondrodystrophy, which causes sufferers to have poor development of bone and cartilage leading to reduced length of limbs. (Li H, 1993). The role of aggrecan in cartilaginous tissue is extremely important, not just for the binding of GAGs, but also in the control of cell biochemical behaviour and the mechanical properties of the tissue. Moreover, the regulation of aggrecan synthesis and production is greatly affected by the mechanical forces experienced by the cell (Wright et al., 1997). Referred to as mechanotransduction, chondrocytes detect the mechanical force in the form of extracellular pressure through surface intergrins, specifically the α5β1subunit (Wright et al., 1997). With increasing pressure an increase in aggrecan expression occurs in order to maintain the ECM integrity. Aggrecan also effects the mechanical properties of cartilage through its ability to
cross-link the matrix component HA and provide cell binding and anchorage domains, significantly increasing the mechanical strength of the ECM (Kiani, 2002). The long HA chains of the cartilaginous ECM also play an important role in the flexibility of this tissue by serving as a viscoelastic polymer with chain-chain binding through electrostatic interactions.

During degeneration there is a significant reduction in aggrecan which, as previously discussed, has further implications for the fate of the tissue (Cs-Szabo et al., 2002). Interestingly as aggrecan levels reduce, other proteoglycans, such as versican and lumican increase (Zhao, 2007). As these proteins are also affected by MMPs, the reduction of aggrecan may be a combined effect of both enzymatic degradation and down-regulation. Versican is most commonly found in newly forming blood vessels, and is produced primarily by fibroblasts (Zheng et al., 2004). The significant increase in these proteoglycans in IVDD indicates a change in the cell behaviour but is also indicative of the development and formation of new blood vessels and fibrotic tissue (Zheng et al., 2004). The formation and ingrowth of vascularized and fibrotic tissue can result in significant inflammation at later stages as the newly formed fibrotic tissue does not have the mechanical properties to withstand the forces applied to the IVD, resulting in cell damage.

1.2.5 Collagens

There are a number of collagens present in the IVD, but there is a clear distinction of collagen types in the different regions of the disc. In healthy discs, collagen type I is found most prominently in the annulus, while collagen type II is the main collagen found in the NP, increasing in concentration closer to the centre of the NP (Nerlich et al., 1998, Zhao, 2007). However, the NP also contains collagen type IX, which
plays an important role in stabilising the ECM through cross-linking of matrix components (Müller-Glauser et al., 1986). The structure of collagen type II is homotrimeric in nature giving rise to helical fibres that can be cross-linked by collagen IX creating a dense mesh ECM capable of withstanding the high compressive forces applied to the IVD (Stockwell et al., 1991). During degeneration, the collagens of the NP change dramatically, with a decrease in collagen type II and IX, and an increase in collagens type I, V, VI and X (Boos et al., 1997, Zhao, 2007). These changes have a significant effect on the mechanical integrity of the IVD due to the reduction in the cross-linked collagen fibres and a shift to collagen I (Müller-Glauser et al., 1986). The reason for the effect of collagen type I on chondrocyte differentiation is not yet fully understood, but it is believed that a change in cell-matrix interaction induces dedifferentiation (Zhao, 2007). Whilst some biomaterial treatment strategies seek to replace the collagen matrix of the IVD following degeneration (Collin et al., 2011, Halloran, 2008), the biological processes that have given rise to the degeneration of the ECM must also be considered in order to achieve regeneration. As in the case of the natural ECM of the NP, despite its highly specialised and organised structure, degeneration still occurs. Therefore, any collagen-based biomaterial is likely to suffer the same fate unless the biological determinants of IVDD are controlled. In consideration of these findings, the role of MMPs and growth factors must be considered to develop future treatments for the regeneration of the collagen matrix.
1.2.6 *Growth factors*

The effects of changes in growth factor expression seen in IVDD are significant, with a number of growth factors playing important roles. Arguably the growth factor most responsible for the progression of IVDD is basic-fibroblast growth factor (b-FGF) (Ellman et al., 2008). In healthy discs b-FGF is for the most part undetectable or at very low levels within the NP (Zhao, 2007). However, during degeneration the levels of b-FGF have been shown to dramatically increase (Tolonen J et al., 2006, Vincent et al., 2002). While b-FGF has been found to have a potent mitogenic effect on chondrocytes (Rosselot et al., 1994), a number of other studies have shown this to have a negative effect on cellular phenotype and expression of this cell type (Ellman et al., 2008). The increased levels of b-FGF in NP have been shown to stimulate the up-regulation of MMP-13 (Peng et al., 2006), down regulation of proteoglycans and inhibition of bone morphogenetic protein (BMP-7) (Li x et al., 2008). In addition to these findings, b-FGF has also been shown to increase the production of type I collagen in bovine NP cells (Tsai Tsung-Ting et al., 2007). These multiple effects of b-FGF could explain how IVDD can rapidly progress with significant remodelling of the NP ECM. From the work of Vincent et al. (2002), b-FGF is rapidly released following damage to cartilaginous tissues. This release if sufficient, could potentially initiate changes in chondrocyte expression to induce matrix remodelling which may lead to IVDD. While there are mixed reports surrounding its use as a therapeutic agent to tissue regeneration, b-FGF has been shown to be effective *in vivo* at increasing disc height. However, these findings alone show no clear indication as to the matrix content or as to whether this increased tissue volume is of fibroblastic origin. Similar in its action, platelet derived growth factor - BB (PDGF-BB) has also been found to have similar mitogenic and phenotypic influence on chondrocytes.
(Weiser et al., 1999). These two growth factors seem to play a pivotal role in degeneration of the intervertebral disc by stimulating the change in cellular expression and phenotype. In consideration of these findings, it could be argued that research into antagonists or inhibitors of these growth factors may provide a potential treatment for IVDD, as well as a number of other conditions affected by these factors. Indeed, there have been a number of publications reporting an effective PDGF-BB antagonist peptide (Brenn et al., 1997a, Brenn et al., 1997b, Patel et al., 1999). However, no studies have yet been conducted to assess the ability of this antagonist to inhibit the differentiation effect of PDGF-BB on chondrocyte cell types.

1.2.6.1 Insulin-like growth factor 1 (IGF-1) as a therapeutic agent

IGF-1 is a 7.5 kDa protein that is capable of eliciting a range of cellular expression changes in a wide variety of tissue and cells types (Humbel, 1990). IGF-1 production and regulation in cartilage is control through autocrine/paracrine signalling (Schlechter et al., 1986) (Tsuruzoe et al., 2001). The endogenous production of IGF-1 by chondrocytes is regulated through negative feedback via IGF-1 receptor activation. IGF receptor activation triggers phosphorylation of tyrosine kinase, in turn activating insulin receptor substrate protein, a docking protein which acts on a range of signalling pathways (Tsuruzoe et al., 2001). This pathway is extremely important to regulation of a range of cell survival factors, key in regulating ECM changes in response mechanical loading of cartilaginous tissues. During the development of IVDD a reparative process initiated with the increased production of IGF-1. This increase is believed to be in response to the decrease in levels of proteoglycans that occurs during degeneration and has been shown to up-regulate collagen type II and aggrecan, while down-regulating the production of MMP 13 and
inhibiting the signalling pathways for the cytokine IL-1 (Mengshol et al., 2000, Wang et al., 2003, Zhang M et al., 2009). Despite this increased production of IGF-1, the ECM remodelling process still occurs. However, as a result of the importance of IGF-1, it has become a therapeutic molecule for the development of new treatments for cartilaginous tissue regeneration (Jaklenec et al., 2008). One specific example of this is the work of (Jaklenec et al., 2008) in which IGF-1 has been incorporated into PLGA microspheres as an injectable drug delivery system to stimulate cartilage tissue regeneration. While this treatment approach represents a novel step forward in cartilaginous tissue regeneration, it may not be successful in inducing repair or inhibiting degeneration due to the up-regulation of IGF-1 regulatory binding proteins (IGF-BP). During degeneration IGF-BP’s, specifically IGF-BP3 are up-regulated nullifying the biological activity of IGF-1 (Olney et al., 1996). The effect and cause for this up-regulation of IGF-BP3 has been the subject of a large amount of research to understand whether this up-regulation is a primary cause or a contributing factor to degeneration. However, currently little is known about its full role in cartilaginous degeneration (Olney et al., 1996). IGF-BP3 in normal cartilage accounts for the binding of more than 80% of the available IGF-1 rendering it inactive while bound. The specific structural mechanism by which IGF-BP3 binds to IGF-1 is not yet fully understood. However, from a number of protease fragment interaction studies of IGF-BP3 with IGF-1 found that both and N and C terminal regions of IGF-BP3 are required for high affinity binding (Hwa et al., 1999) but currently the IGF-1 domain or peptide sequence that facilitates the binding with IGF-BP3 is not known (Hwa et al., 1999) therefore although IGF-1 growth factor represents a potential therapeutic mechanism for repair it’s inhibition by IGF-BP’s may mean the full protein is unlikely to be an effective treatment alone.
While, the full IGF-1 protein may not be a viable treatment for IVDD, its sub-protein structures and regions provide insight for the design of potential new drugs. The structure of IGF-1 (see Figure 1.3) can be divided into 5 sub regions, each with specific roles. From the research by (Rinderknecht and Humbel, 1978) the active site responsible for specific activation of IGF-1 receptors on the cell membrane has been identified.

Figure 1.3 Human IGF-1 molecular structure diagram. Arrow indicates active site (C region), pink lines represent disulfide bonding, N and C terminal region (required for IGF-BP3 binding highlighted in pink). Adapted from Vajdos FF, Ultsch M, Schaffer ML, Deshayes KD, Liu J, Skelton NJ, de Vos AM. Crystal Structure of Human Insulin-like Growth Factor-1: Detergent Binding Inhibits Binding Protein Interactions. Biochemistry. 2001 Sep 18;40(37):11022-9
The active site of IGF-1 is a 12 amino acid sequence from amino acids 30-41 of the full protein (Rinderknecht and Humbel, 1978). The peptide sequence was identified from the full 70 amino acid sequence of IGF-1, but has currently not been investigated for its biological activity. This peptide sequence may have potential as a possible bioactive agent as it may be able to bind to the IGF-1 receptor while bypassing the regulation of IGF-BP’s. This is hypothesised due to the sequences lack of the original IGF-1 N and C terminal domains required of IGF-BP binding, while possessing the required sequence for activation of the IGF-1 receptor (Hwa et al., 1999) (Rinderknecht and Humbel, 1978). If the active site sequence of IGF-1 is capable of eliciting the same biological response as the full protein then this short peptide sequence could have great potential as a therapeutic agent for the treatment of IVDD. Due to its small size, the active site peptide could potentially be able to diffuse more freely through the dense matrix of cartilaginous tissues and may also bypass the regulatory IGF-BP’s that inhibit IGF-1 induced repair.

1.2.7 Matrix metalloproteases & cytokines

As previously discussed MMP’s play an important role in the regulation of the ECM in healthy discs. However, the up-regulation of these enzymes in IVDD significantly contributes to the progression of the condition (Zhao, 2007). While there are a great number of MMP’s produced in IVDD, arguably the most destructive is MMP-13 which breaks down collagen type II, resulting in the loss of mechanical strength of the cartilaginous tissues (Crean et al., 1997). The up-regulation of MMP-13, among many others, is seen in IVDD and osteoarthritis in response to cellular damage (Crean et al., 1997). Although, there has been no clearly defined pathway for MMP up-regulation (Zhao, 2007), the publication by (Mengshol et al., 2000) found the MMP-13 production to be mediated by the inflammatory cytokine IL-1, with
increased levels of IL-1 stimulating up-regulation of MMP-13. The release of cytokines such as IL-1 and TNF-α in response to cellular damage within the cartilaginous tissue is an important stage in the reparative process. However, the excessive release of these cytokines in chronic conditions such as IVDD significantly contributes to the progression of the disease. The cyclic effect of the tissue remodelling is stimulated by the release of cytokines which, in turn, up-regulate MMP production (Mengshol et al., 2000), resulting in ECM degradation, thus increasing the direct mechanical stress on the chondrocyte-like cells contributing to cellular damage (Lee et al., 2002). In addition to this effect, the presence of IL-1 has also been shown to significantly down-regulate the cellular production of aggrecan (Radons et al., 2006). The loss of this production combined with the increased rate of matrix degeneration demonstrates the central role of IL-1 in IVDD and its importance as a potential target for the development of future treatments. The search for therapeutic inhibitors of IL-1 is a current area of research which to date has only produced one successful inhibitor, diaecerin, that is currently available for use within the US (Fidelix et al., 2009). While there are mixed reports of its efficacy in clinical trials as oral tablet form delivery (Fidelix T et al., 2009), diacerein has been shown to be highly effective in in vitro studies where the bioavailability is significantly higher (Fidelix et al., 2009). However, while there are a limited number of direct inhibitors of IL-1, IGF-1 has been shown to have a significant inhibitory effect on IL-1 activity (Wang et al., 2003). This effect is achieved through the indirect inhibition of the IL-1 cytokine through the up-regulation of IL-1 receptor II (Wang et al., 2003). While the up-regulation of a receptor for a given cytokine may intuitively be associated to an increased response to the given cytokine, in the case of IL-1 receptor II, its function is as a “decoy
receptor”. This term is used to describe a receptor that reduces the effect of its binding factor by binding the molecule, but not activating the down-stream process that leads to its effect (Wang et al., 2003). This effect of IGF-1 demonstrates its role in controlling inflammation within cartilaginous tissues. However, its levels are not significant enough in IVDD to stem the progression of the condition (Zhao, 2007). Considering this, IGF-1 may be a potential therapeutic agent in the treatment of IVDD and osteoarthritis.
As previously discussed in Section 1.2.2, there are a wide range of biochemical changes that occur in IVDD. From the review of currently available literature, a summary of these changes are shown in Table 1.1 and Figure 1.4.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Changes occurring in IVDD</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Cytokines</strong></td>
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<tr>
<td>IL-1a</td>
<td>Increase</td>
<td>(Le Maitre et al., 2005a)</td>
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<tr>
<td>IL-1b</td>
<td>Increase</td>
<td></td>
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<tr>
<td>IL-6</td>
<td>Increase</td>
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<td>TNF-α</td>
<td>Increase</td>
<td>(Zhao, 2007)</td>
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<tr>
<td>IL-1-R1</td>
<td>Increase</td>
<td>(Wang et al., 2003)</td>
</tr>
<tr>
<td>MMP - 1,2,3,7,8,9 &amp; 13</td>
<td>Increase</td>
<td>(Crean et al., 1997)</td>
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<td>MMP’s</td>
<td></td>
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<tr>
<td>MMP - 19</td>
<td>Decrease</td>
<td>(Gruber et al., 2005)</td>
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<tr>
<td>ADAMTS-4</td>
<td>Increase</td>
<td>(Hatano et al., 2006)</td>
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<tr>
<td><strong>Growth Factors</strong></td>
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<td>B-FGF</td>
<td>Increase</td>
<td>(Le Maitre et al., 2005b)</td>
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<td>PDGF</td>
<td>Increase</td>
<td>(Weiser et al., 1999)</td>
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<td>IGF-1</td>
<td>Increase</td>
<td>(Le Maitre et al., 2005b)</td>
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<td>TGF-β</td>
<td>Contradictory findings</td>
<td>(Konttinen et al., 1999)</td>
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<td>(Cs-Szabo et al., 2002)</td>
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<td>Lumican</td>
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<td>(Boos et al., 1997)</td>
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<tr>
<td>Collagen IX</td>
<td>Decrease</td>
<td>(Boos et al., 1997)</td>
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Table 1.1 Summary of biochemical changes occurring in IVD
Figure 1.4 Summary of biochemical changes in healthy and degenerated disc – positively associated factors in blue, negatively associated factors in red.
1.3 Current and future approaches to the treatment of IVDD

The current treatment options available to patients suffering with IVDD are limited to two stages of treatment. Firstly, a pharmacological approach to treatment is employed which focuses on the use of common non-steroidal anti-inflammatory drugs (NSAID’s) such as ibuprofen and diclofenac combined with physiotherapy (Group, 2009, Kraemer, 2008). While this treatment stage is effective in treating very mild degeneration, it often provides insufficient alleviation from pain and also fails to address the underlying processes of the condition (Kraemer, 2008). The second phase of treatment is surgical intervention, this stage is initiated when sufferers experience exceptional pain and are essentially unable to manage basic day to day tasks (Kraemer, 2008). The surgical intervention stage currently consists of two options (Kraemer, 2008):

(i) Disc arthroplasty: where the disc is removed and replaced with a prosthetic disc an example of which can be seen in Figure 1.5.

(ii) Spinal fusion: were the vertebrae are fixed in place using a metal fixator which is screwed directly into the vertebrae.

1.3.1 Pharmacological intervention

The pharmacological treatment of IVDD is an important stage and whilst often not successful in the treatment of more severe degeneration, this form of treatment can provide sufficient alleviation of the symptoms of IVDD for many patients (Kraemer, 2008). As previously discussed, there are a number of different medicinal approaches to the treatment of IVDD. Firstly, the use of basic NSAIDs such as ibuprofen and diclofenac to reduce inflammation and pain (Group, 2009, Kraemer,
These compounds work as selective cyclooxygenase (COX) inhibitors, specifically COX-1 and 2 enzymes responsible for the production of prostaglandins which induce inflammation (Kraemer, 2008, Rao and Knaus, 2008). If this treatment is found to be ineffective, the second stage of pharmacological intervention is the administration of corticosteroids such as prednisolone (Group, 2009, Kraemer, 2008). However, long-term treatment with corticosteroids is limited due to the risk of induction of osteoporosis, a common side effect associated with prolonged treatment with these drugs (Group, 2009, Kraemer, 2008). While suitable prophylaxis with calcium and vitamin D supplementation can be used to reduce the risks, this treatment option frequently fails to provide adequate alleviation of pain from inflammation (Kraemer, 2008). The next line of treatment is the use of opioid drugs such as Tramadol to reduce pain (Group, 2009). Opioid drugs work as extremely potent analgesics by having combined action on both neurotransmitter receptors and the release and uptake of relevant neurotransmitters (McDonald and Lambert, 2008). In the case of Tramadol, commonly used in the treatment of IVDD, increased serotonin release is stimulated while the re-uptake of norepinephrine is inhibited (Frink et al., 1996). While the use of pharmacological intervention may provide relief for many sufferers of IVDD, the long-term side effects and inability to address the underlying mechanism of IVDD, means that this stage of treatment often fails (Group, 2009, Kraemer, 2008).
1.3.2 Surgical intervention, surgical devices and spinal fusion

Whilst prosthetic disc devices have improved significantly since the development of “ball bearing” style devices, long-term clinical results are poor with prosthetic disc failure being common (Freeman and Davenport, 2006, LeMaire et al., 2005). Currently, there are only two prosthetic disc devices approved for use by the Food and Drug Administration (FDA) (CareAllies, 2007) – CHARITÉ artificial disc (DePuy Spine, Inc.) and PRO-DISC-L total disc replacement (Synthes Spine, Inc.). However, there are a number of other prosthetic disc devices in late stage clinical trials, such as the Maverick disc™ (CareAllies, 2007). Although these devices have been reported to provide significant improvements in the quality of life for sufferers of IVDD (Freeman and Davenport, 2006), there are a wide range of side effects and morbidities associated with their implantation (LeMaire et al., 2005). The failure rate of the most common disc Charité design is estimated to be approximately 10% (LeMaire et al., 2005, Shim et al., 2007). While the reasons for the failure of these devices may vary, commonly reported causes are poor osteo-integration leading to implant migration (LeMaire et al., 2005) and accelerated degeneration in adjacent discs (Shim et al., 2007). In addition to this problem the presence of a large foreign object in such an anatomically sensitive site leads to immune activation causing inflammation of the local tissue. This in-turn causes further pain and discomfort due to the stimulation of the nearby nerve terminals and spinal cord (LeMaire et al., 2005). Further to these issues, prosthetic discs are still unsuccessful in equally distributing mechanical loading through the spine (Murtagh et al., 2009). Numerous publications have shown that the poor distribution of mechanical force through these devices causes accelerated degeneration in other discs further up the spine (Freeman and Davenport, 2006, LeMaire et al., 2005).
1.3.3 Pre-clinical treatment strategies and research

Due to the limitations and issues with current treatments for IVDD, a new field of research has emerged focusing on the development of regenerative strategies for the treatment of IVDD. The main stream of which has concentrated on the development of injectable cellular constructs with the intention of regenerating the NP of the IVD. Current strategies have used a range of bio-mimetic and naturally derived biomaterials in order to encapsulate stem cells to provide them with a 3D microenvironment for the regeneration of the tissues. One such example is the work by (Collin et al., 2011) who utilised an injectable collagen type II gel matrix to support the growth of rat NP cells. While the application of this form of approach represents a step forward, it still fails to address the biological factors that have driven the process of degeneration. Furthermore, the limitations of this treatment arise in part from the requirement of implantation. In order to inject a cell loaded
biomaterial into the NP of a degenerated disc, a procedure called discectomy is first required in order to remove all or a large proportion of the NP of a degenerative disc. This is achieved by making a small access bore hole within the annulus and cutting out the NP tissue (Kraemer, 2008). The injection of a cell loaded scaffold into the newly created cavity is considered the optimal approach for currently developing treatments. However, this procedure in itself creates a number of complications that could inevitably lead to the failure of the injected or implanted cellular construct. Firstly, the procedure results in a fissure within the annulus, through which the injected or implanted construct, is likely be extruded when pressure is returned to the disc (Kraemer, 2008). While there are a number of strategies currently being developed to seal the annulus, this problem is still the main limitation associated with this approach. Furthermore, any remaining degenerative tissue not removed during discectomy could potentially influence the cellular phenotype of any implanted cells. This clinical situation is likely to have a significant impact on the outcome of any treatment when considering the increased expression of fibroblast growth factors and inflammatory cytokines that occur in degenerative NP tissue (Kraemer, 2008). In addition, the further trauma associated with surgical discectomy is likely to increase the expression of cytokines such as IL-1, shown to down regulate proteoglycan synthesis and increase MMPs production (Zhao, 2007), that results in the breakdown of components essential to the regeneration of cartilaginous tissues (Arner and Pratta, 1989, Le Maitre et al., 2005a, Mengshol et al., 2000). Although some promising results have been found with the use of cell loaded material constructs, adequate regeneration is yet to be achieved (Whatley and Wen, 2012). In consideration of these factors, the use of cell based therapy treatments should be used in conjunction with bioactive treatment, where growth factors and
anti-inflammatory agents control both cell phenotype and the biological mechanisms that drive degeneration.

1.4 Chondrocyte culture and 3D tissue engineering

The *in vitro* culture of primary chondrocytes and NP chondrocytes is considered a major limitation for regenerative medicine strategies for the treatment of IVDD. The limitation lies in the loss of phenotypic control of chondrocytes once isolated from the body. Once isolated, chondrocytes dedifferentiate rapidly, losing their native spherical morphology and down-regulating the expression of essential ECM components, collagen II and aggrecan progressively over a 14 day period (Brodkin et al., 2004). From current literature, the restoration of this expression has not yet been achieved, therefore the question, how can cartilaginous tissues be grown or regenerated if cell phenotype cannot be retained outside of the body would seem to be an essential one. However, the re-differentiation of chondrocytes has been demonstrated over short time intervals when cells are cultured in 3D biomaterial constructs (Benya and Shaffer, 1982). Although not all expression is restored, the 1982 publication by Benya *et al*., demonstrated that 3D culture in agarose could effectively increase collagen type II expression. The reason for agaroses ability to conserve the differentiated phenotype is currently not well understood. However, the work by Yoon *et al*, 2002, found differentiation to be controlled by Protein kinase C α and ERK signalling through regulation of cell adhesion molecules, in response to the substrate environment (Yoon *et al*, 2002). Other biomaterials such as silk fibroin scaffolds have been shown to retain GAG expression in 3D culture up to 14 days (Talukdar et al., 2011). This work also illustrates the importance of cell seeding density to the formation of a self-supporting tissue. A range of different cell densities were investigated in the study, revealing a significant difference in cellular
behaviour and cell survival with changes in cell density (Talukdar et al., 2011). However, the work utilised cellular seeding densities of up to 100 million cells/ml in order to achieve good cell viability. While beneficial to cell survival, the phenotypic influence of an abnormally high seeding density, more than 6 times that of native human cartilage and more than 10 times that of the NP (Maroudas et al., 1975, Stockwell, 1971), may be adverse to the formation of ECM. In consideration of this finding it would seem important that future studies aim to populate 3D scaffolds with the native cell density of cartilaginous tissue or with only a slightly greater number, so to allow for more rapid tissue formation. Another widely used material for the 3D culture of chondrocytes is alginate, like agarose, alginate is a polysaccharide biopolymer, reported for its ability to preserve chondrocyte phenotype ex-vivo (Häuselmann et al., 1994). However, due to the nature of alginate and agarose chondrocytes embedded in these gels have not be found to produce self-supporting tissues (Benya and Shaffer, 1982), (Häuselmann et al., 1994). This may be an effect on the density of the matrix that limits that ability of the cell to lay down a complex ECM structure. Furthermore chondrocytes are not capable of producing agarase enzymes in order to digest and remodel the 3D construct in order to form cartilaginous tissue.

In an attempt to regenerate the NP specifically, a number of different natural and synthetically derived materials have been employed in injectable cell scaffold treatment concepts. One example of this is the recent work of Brona et al. (2011) which investigates the influence of long-term culture of NP chondrocytes in alginate hydrogels and the mechanical properties of the cell construct in comparison to the native NP. While the results showed promising result of expression of proteoglycans and collagen, the long-term stability of the constructs was not sufficient for the
formation of a self-supporting tissue (Brona et al., 2011). These findings and the comments of the authors raises the important limitation for consideration. In order to regenerate a tissue a construct must be sufficiently stable in order to facilitate the formation of new tissue while degrading at a rate that allows the growing tissue to replace the biomaterial. This issue has also been highlighted in the recent publication by (Collin et al., 2011) in which polyethylene glycol (PEG) polymers were used in combination with type II collagen to create stronger hydrogels capable of longer incubation times. However, hydrogels formed with PEG were found to elicited a fibroblastic differentiation and expression within the cell population (Collin et al., 2011). The reasons behind the phenotypic influence of many biomaterials is still not fully understood, however, in an attempt to develop new biomaterials to overcome the limitations of conventional polymers, researchers have developed and modified new materials with proteins and peptides in order to achieve a greater phenotypic control and material properties.

1.5 Peptide Hydrogels

The term hydrogel is used to describe a three dimensional network of natural or synthetic cross-linked polymer chains capable of binding large amounts of water. Similar to the ECM in physical properties, hydrogels have high water content and can in some cases be modified to interact with specific peptides and proteins essential to cellular adhesion and growth. There is an extensive range of hydrogels most of which are formed from polymers such as poly lactic acid (PLA), poly acrylic acid (PAA), poly ethylene glycol (PEG) and poly hydroxyl ethyl methyl acrylamide (pHEMA). In addition to conventional polymer hydrogels, there has in recent years been a new development in peptide and protein-based hydrogels (Matsona and
Stupp, 2012). While possessing the obvious benefits of non-toxic and non-immunogenic degradation products i.e. amino acids, peptide based hydrogels also allow for specific tailoring of the material to evoke a desired material and biological effect (Jayawarna et al., 2009, Matsona and Stupp, 2012). The research area of self-assembly peptides has stemmed from the origins of β-amyloid protein research. By understanding the interactions of specific self-assembly amino acid sequences, researchers have designed short peptides capable of self-assembling to form nanostructure materials capable of supporting cellular growth (Matsona and Stupp, 2012). While this area of research is still relatively new, there are a great number of different self-assembly peptide materials that have been discovered. The shortest sequence of which is made of just two amino acids (diphenylalanine) (Yan, 2010). The self-assembly of this peptide has been studied by a number of groups with the findings showing the molecular organisation to be in the form of the stacking of β-sheets through π-π interactions (Yan, 2010). The use of this class of hydrogel for biomedical applications was first proposed by (Aggeli et al., 1997) in which the study of Lysβ-21 peptide sequence, found in the egg white protein lysozyme, was reported. This peptide sequence was one of the first found to form hydrogels in aqueous formulations and is considered to be the driving force behind the new generation of self-assembly peptide hydrogels (Semino, 2008). Furthermore, the discovery of the RADA sequence by (Zhang S et al., 1994) revolutionised the approach to tissue engineering by developing novel peptide hydrogels capable of supporting the growth of mammalian cells (Zhang S et al., 1994). From the elucidation of the assembly mechanism of alternate hydrophilic-hydrophobic amino acid chains, a large range of new self-assembly sequences have been discovered and utilised in the tissue engineering field (Matsona and Stupp, 2012).
1.6 Fmoc-F<sub>2</sub> Hydrogels

Recently a new class of hydrogel has emerged based on the self-assembly of the Fmoc-F<sub>2</sub> peptides. From the previous research of (Reches and Gazit, 2003) the development of Fmoc-F<sub>2</sub> hydrogels has been studied for a range of material and biological properties. The structure of Fmoc-F<sub>2</sub> consists of the smallest self-assembly peptide unit, F<sub>2</sub>, first discovered in 2003 as the core structural sequence in b-amyloid protein aggregation. While this sequence alone can self-assemble to form nanotube structure at acidic pH. The introduction of a Fmoc- functionality first reported by (Mahler et al., 2006) significantly altered the self-assembly properties of this peptide. These modifications lead to the pH sensitive self-assembly of peptide monomers to form nanofibres through π-π interactions (Mahler et al., 2006, Smith et al., 2007). While the self-assembly of these nanofibres at concentrations of 1-3 mg/ml result in the formation of viscoelastic hydrogels that are stable under physiological conditions (Jayawarna et al., 2009, Mahler et al., 2006) (Smith et al., 2007). The formation nanofibres has been discovered to be achieved through the assembly of interlocking twisted β-sheets (Smith et al., 2007).

These peptide hydrogels have been shown to be capable of supporting the growth of a number of different cell types (Jayawarna et al., 2009, Mahler et al., 2006), while having the potential for bio-functionalization with specific peptide structures (Jayawarna et al., 2009, Matsona and Stupp, 2012). This research achieved specific modification of the nanofibre structure through the introduction of Fmoc-RGD and Fmoc-RGE into the molecular self-assembly (Zhou et al., 2009). While this method...
of structural modification was found to disrupt self-assembly at concentrations greater than 30% M/M, the paper found significant improvement in both cell adhesion and proliferation with the addition of these sequences in the culture of anchorage dependant human dermal fibroblasts (Zhou et al., 2009). However, the modification of Fmoc-F₂ hydrogels is not required for the growth of all cell types, from the work of (Jayawarna et al., 2009), Fmoc-F₂ hydrogels have been shown to possess unique properties, being able to support the growth of chondrocytes while inhibiting the growth of fibroblastic cell types (Jayawarna et al., 2009). This cell selective property may provide the potential to create self-assembling scaffolds capable of supporting the growth of cartilage tissue formation while inhibiting the growth of fibroblastic scar tissue. While the current research has demonstrated this cell selective behaviour in 2D in vitro study, this behaviour may not be exhibited in 3D studies or in the in vivo environment. However, from the publication by Mahler et al., 2006 the 3D culture of bovine chondrocytes, has shown that chondrocytes cultured in 3D present with a spherical type morphology, similar to that of cells in their native cartilage. Whether this morphological change is a result of phenotypic changes, perhaps in the form of chondrocyte de-differentiation to a chondritic type expression is currently unclear. As the 2006 paper (Mahler et al., 2006) does not give results of gene or protein expression, cell viability or cell proliferation. Therefore, further investigation is required into these properties in order to achieve a greater understanding of the longer term effects on cell behaviour and to explore the potential benefits of this material in the treatment of IVDD. In addition, this class of biomaterial has yet to be investigated for its immunogenic potential, and important issue for consideration. The original discovery of the F₂ core self-assembly sequence in β-amyloid protein aggregation and assembly shares similarity in both a structure.
and assembly mechanism with that of Fmoc-F$_2$. As a result it could be argued that while these subunits of the β-amyloid proteins are significantly smaller and simpler, they may have the potential to elicit an immune response similar to that seen in plaque formation seen in Alzheimer's patients. However, while β-amyloid plaques are insoluble and precipitate, the self-assembly hydrogels are stable under physiological conditions. Although immunological-material response studies have not been performed on this class of materials, Fmoc-F peptide monomer has been studied for its anti-inflammatory properties (Yen et al., 2009). The result of this research revealed Fmoc-F to inhibit human neutrophil production of super anions and esterase secretion released during inflammation response (Yen et al., 2009). In consideration of this finding it may be postulated that Fmoc-F$_2$ hydrogels may not elicit an inflammatory response but may in fact have anti-inflammatory response as the material is degraded in vivo.
1.7 Project Aims and Objectives

As discussed previously, IVDD is a multi-factorial progressive condition driven by a combination of inflammation, mechanical stress and cellular differentiation resulting in changes in ECM composition. In order to address these issues, any potential treatment would require a multi-faceted approach to control the individual factors that influence the progression of IVDD. This project aims to develop a bioactive hydrogel for the treatment of IVDD. This is proposed to be achieved through the development of a hydrogel capable of repeatable self-assembly to be used in the form of either an injectable hydrogel or as a tissue engineering construct for the ex-vivo growth of a replacement IVD. This is proposed to be achieved through the development and application of Fmoc-F$_2$ based bioactive self-assembly peptide hydrogel.

**Hypothesis:** *Fmoc-F$_2$ Self-assembly hydrogels loaded with IGF-1 C peptide sequence can serve as an injectable 3D cellular scaffold for cartilaginous tissue regeneration.*

1.7.1 Aims

1. To produce a self-assembly peptide hydrogel capable of setting at physiological conditions
2. To test the biocompatibility of hydrogel
3. To synthesise bioactive peptide to control biochemical processes of degeneration
4. To investigate bioactivity of the synthesised peptide
5. To investigate the potential of the combined hydrogel/bioactive peptide formulation to control chondrocyte phenotype *in vitro*
1.7.2 Development of an Fmoc-F$_2$ hydrogel scaffold for IVD tissue regeneration  

(Aims 1 and 2)

The development of an injectable hydrogel scaffold capable of retaining chondrocyte phenotype is considered the main objective within this treatment strategy (Aim 1). This is envisaged to be achieved through the development of a self-assembly peptide nanostructured hydrogel based on the self-assembly mechanism of Fmoc-F$_2$. While this material does not possess the mechanical strength of other materials, it has been shown to retain the morphology of chondrocytes in both 2D and 3D culture (Jayawarna et al., 2009). Furthermore, Fmoc-F$_2$ hydrogel has also been demonstrated to inhibit fibroblast cell growth (Jayawarna et al., 2009), a beneficial property in blocking the invasion of scar tissue which occurs in IVDD. This approach is envisaged to be utilised either as a scaffold for the ex vivo growth of cartilaginous tissue, or as an injectable gel capable of in situ setting. The latter approach would serve to mimic the behaviour of the IVD with its pressurised “water bed-like” behaviour, capable of sustaining equal distribution of mechanical load. However, the development of an annulus sealing device required for this approach is considered to be outside of the scope of this research project. Due to the self-assembling properties of the hydrogel and its liquid nature when non-assembled, this type of hydrogel may have the added benefit of being injected with minimal removal of existing or degenerated NP. Therefore, in this work the produced hydrogel was tested with chondrocytes and relevant cell types in order to investigate their potential to be used in the treatment of IVD (Aim 3).
1.7.3 Strategies for the regulation of biological factors (Aims 3 and 4)

While the biomaterial approaches to IVD regeneration can demonstrate an \textit{in vitro} competency, supporting the culture of relevant cell types, \textit{in vivo} studies have yet to yield conclusive evidence of regeneration. Due to this concern, many research groups have focused on the delivery of growth factors and bioactive molecules in order to regenerate the IVD (Ellman et al., 2008). However, there has been limited focus on the use of IGF-1 despite its potential to inhibit the effect of a number of factors implicated in the progression of IVDD. The synthesis and testing of the peptide active region C of IGF-1 was here performed in an attempt to provide a synthetic novel bioactive molecule with the potential to up-regulate the expression of proteoglycans and collagen type II production (Aim 3). It is envisaged that this peptide can be used either within a hydrogel construct or as a separate treatment to slow or reverse the progression of IVDD. Moreover, the peptide, while increasing matrix production, may also inhibit the effects of pro-inflammatory factors that jeopardise to phenotypic status of implanted cells. However, currently there is no literature on the use or the activity of the specific active region of the IGF-1.
Chapter 2: Peptide Synthesis and Characterisation
2.1 Introduction

The chemical synthesis of peptides was first discovered and performed in 1901 by Emil Fischer who first synthesised the di-peptide glycine-glycine (Kunz, 2002, Orster, 1920). While the methods for the synthesis of peptides have changed and improved since Emil Fischer’s discovery, the basic amino acid coupling reactions remain the same. Amino acids are coupled together through a condensation reaction between the amine and carboxylic acid groups to form a peptide bond (Nilsson et al., 2005). The reaction of amino acids together in a specific sequence allows for the formation of chains of amino acids called peptides. A great number of peptides have specific bio-activity such as the RGD peptide, a well-known integrin-binding sequence and one of the most common sequences found in mammalian cells, has specific activity as a binding agent for cell anchorage proteins allowing for cell adhesion (Ruoslahti, 1996).

In order to create synthetic peptides for biological applications, careful control of the chemical reactions involved is required in order to produce complex peptide structures. The development and improvements in peptide chemistry in recent years are largely due to the historic discovery of the complex peptide drug Enfuvirtide used in the treatment of HIV (Albericio, 2000). This discovery led to the improvement in methods of synthesis and the considerable reduction in cost of peptide synthesis reagents. Peptide synthesis can be achieved through a variety of different methods however; the two core methods are liquid phase and solid phase synthesis. Liquid phase synthesis is performed in liquid, commonly organic solvents such as DMF, while solid phase synthesis utilises a solid resin with which each
amino acid in sequence is reacted. Each method has its own significant advantages over the other. In the case of liquid phase synthesis, peptides can be synthesised reproducibly and on large scale. However, the synthesis of long peptide sequences would be extremely time consuming (Albericio, 2000). Conversely, solid phase synthesis methods can produce long peptide sequences quickly and at high purities. However, solid phase synthesis is significantly more expensive and performed at a lesser scale (Albericio, 2000).

2.1.1 *Amino acid coupling reaction*

Amino acids can be reacted to form a peptide bond with the use of many different forms of chemical catalysts to promote the condensation reaction between carboxylic acid and amine groups (Albericio, 2000). However, reagents $N,N$-diisopropylethylamine (DIPEA) and O-benzotriazole-$N,N,N'$-$N'$-tetramethyl-uronium-hexafluoro-phosphate (HBTU) are most commonly used for the synthesis of biological peptides due to the absence of toxic by-products (Albericio, 2000). DIPEA is a basic compound which acts as a hydrogen acceptor, the presence of DIPEA in equal molarity as an amino acid leads to the removal of hydrogen from the carboxylic acid group creating a reactive COO$^-$ functionality. The now negatively charged amino acid can react with the HBTU reagent through the reaction mechanism shown in Figure 2.1. The reaction with HBTU leads to the formation of an unstable ester bond which is in-turn hydrolysed by the donation of hydrogen to from the NH$_2$ group of the alternative amino acid forming a peptide bond with water as a by-product (Albericio, 2000).
Figure 2.1 HBTU reaction mechanisms: The chemical diagram shows the process of HBTU binding to the carboxylic acid side group of an amino acid. Once bound the HBTU forms a ester linkage with the amino acid which can later be substituted for an amine side group of another amino acid.

2.1.2 Solid phase Fmoc-peptide synthesis

Solid phase Fmoc-peptide chemistry works specifically through the use of amino group modified resin beads (Albericio, 2000). Amino acids are reacted sequentially to the surface of the resin beads with controllable chain growth using amino group protection with fluorenylmethyleneoxycarbonyl (Fmoc-) protected amino acids (Albericio, 2000) (Figure 2.2). This protecting group prevents uncontrollable chain reactions of amino acids during the coupling (Albericio, 2000). Following the amino acid coupling reaction, the Fmoc- protecting group can be removed using treatment with a basic solution such as piperidine, which then allows for the next required amino acid to be added to the peptide chain (Nilsson et al., 2005). While peptide synthesis is often referred to as “click” or “Lego” chemistry due to its ease and quick reaction times, peptide chemistry can often be extremely difficult as some specific
sequences, those containing repeating alanine, proline or cysteine can lead to incomplete peptide fragments. This is due in part to poor reaction efficiency and folding of sequences caused by these amino acids. Therefore many peptide sequences require optimisation of standard methods in order to achieve successful synthesis (Nilsson et al., 2005). However, the cost effective synthesis of short peptide sequences of 2 or 3 amino acids can be achieved more efficiently using liquid phase synthesis methods.

![Diagram of solid phase chemical synthesis](image)

**Figure 2.2 Solid phase chemical synthesis diagram**, adapted from (Hayes, 2002) the diagram demonstrates the process of controllable peptide formation by the reaction of one amino acid at a time with a resin bead. The amino acid side groups are protected using a base liable chemical group (X).
2.1.3 *Liquid phase peptide synthesis*

Liquid phase peptide synthesis is performed using two amino acids that have either the amine or carboxylic acid group protected by a chemical modification such as, Fmoc- or methyl functionality. The two amino acids are reacted together through a condensation reaction using coupling reagents such as the described HBTU and DIPEA which activate the carboxylic acid group of one amino acid allowing it to react with the unprotected amine group of the other (Albericio, 2000). The produced peptides can then be collected from the reaction solution by precipitation or rotary evaporation. This method of synthesis would therefore be more appropriate for the production of Fmoc-F$_2$ based self-assembly peptides as it does not require a strong acid treatment to collect the peptide as require by solid phase methods (Albericio, 2000). Strong acid treatments used for the cleavage of peptides in solid phase synthesis can hydrolyse weaker bonds such as ester bonds of Fmoc-protected amino acids (Atherton and Sheppard, 1989).

2.1.4 *IGF-1 C region active site peptide design*

The role of growth factors in the regulation and repair of cartilaginous tissue is arguably very important to controlling ECM production, cellular growth and phenotype. In consideration of the previously discussed biochemical processes of IVDD (Section 1.2.2), the design and synthesis of the IGF-1 C region active site peptide for incorporation into this treatment is one of the primary aims of this chapter. The 2D chemical structure of this peptide sequence can be seen in Table 2.1 below has a number of chemical properties which allows it to take on a coiled shape in the native full protein. However, when isolated from the full protein whether this
peptide still possesses the ability to coil is unclear and the effect this will have on the activity of this peptide is also unknown.

2.1.5 Chapter aims

This chapter aims to investigate the synthesis, purification and analysis of the specific peptides for application in the combined treatment approach as outlined in Chapter 1. The peptides will be divided into two categories, those for self-assembly hydrogel formation and bioactive peptides. A list of all the peptides for synthesis and their relative chemical structures and molecular weights are detailed in Table 2.1 (Self-assembly peptides) and Table 2.2 (Bioactive peptides)
<table>
<thead>
<tr>
<th>Peptide Structure</th>
<th>Intended Purpose</th>
<th>Molar Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-F&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Core self-assembly monomer for the formation of hydrogels</td>
<td>534.61</td>
</tr>
<tr>
<td>IGF-1 (C-region sequence)</td>
<td>Stimulation of ECM production and cell proliferation</td>
<td>1265.6</td>
</tr>
</tbody>
</table>

**Table 2.1 Summary of peptide structures**: the peptide chemical structures to be synthesised, Fmoc-F<sub>2</sub> and sIGF-1 peptide with their corresponding molecular weight and intended purpose for study. Chemical structures were produced using Chemdraw 2011.
2.2 Materials and methods

2.2.1 Computational modelling of synthesised peptides

2.2.1.1 Computational modelling of Fmoc-F$_2$ self-assembly

Computational modelling of the structure of the Fmoc-F$_2$ peptide was performed in order to greater understand the molecular interactions between monomer units involved in the self-assembly process of Fmoc-F$_2$ hydrogels. The Fmoc-F$_2$ structure was constructed using Spartan ’10 software and discrete monomers were modelled for their lowest stable energy state in the presence of H$_2$O in order to find stable conformational assembly. Computational methods followed literature precedent (Manandhar et al., 2011). Equilibrium geometries for the monomer and dimer were determined by full conformational searches of 1000 possible structures followed by molecular mechanics energy minimization methods (using the Merck Molecular Force Field). Calculations were made using Spartan ’10 installed on a desktop computer equipped with Intel Xenon Dual Quad Core CPUs running at 2.33 GHz.

2.2.1.2 Computational modelling of IGF-1 active region 3D structure

Computational modelling of IGF-1 active site peptide was performed in order to greater understand the structure of the IGF-1 active site peptide when isolated from the full IGF-1 protein. IGF-1 active region peptide (GYGSSSRRAPQT) was constructed using ChemDraw 2D 2010 (Cambridgesoft, UK) and modelled in chemDraw 3D 2010 (Cambridgesoft, UK) in order to find the lowest energy state structural conformation.
2.2.2 *Fmoc-F₂ peptide synthesis reaction*

The synthesis of Fmoc-F₂ was performed using equimolar concentrations of Fmoc-Phe-OH (Merck, UK) and NH₂-Phe-OH (Merck, UK) with the addition of 7 ml of 0.45M 2 - (1H - benzotriazole - 1 - yl) - 1,1,3,3 - tetramethyluronium hexafluorophosphate (HBTU) in dimethylformaldehyde (DMF) and 3.5 ml of 33 % N,N-diisopropylethylamine (DIPEA) in DMF. The reaction was performed using 100 ml excess DMF and was incubated at 25 °C under magnetic stirring for 2 hours. Following the coupling reaction, the produced peptide was precipitated on excess de-ionised H₂O and centrifuged in 25 ml aliquots at 3500 rpm for 25 minutes. The excess supernatant was aspirated and the collected peptide was washed using 4 ml ethanol and vacuum dried over night at -70 °C. The produced peptide was then analysed using electro spray mass spectrometry (BRUKER), high performance liquid chromatography–ultra violet detector (HPLC-UV) and ion trap mass spectrometry detectors (LC-MS) (BRUKER) (method described in Section 2.2.4).

2.2.3 *Solid phase synthesis of bioactive peptides*

Peptides were synthesised following the manual solid phase synthesis method detailed below.

0.5 mg of Tenta gel–NH₂ resin beads were added to a 10 ml fritted polypropylene syringe and swollen in DMF for 15 minutes. 0.4 mM of acid liable Fmoc protected amide linker was reacted with the resin for 45 minutes at room temperature using a solution of 0.4 mM HBTU and 0.8 mM DIPEA in 3 ml of DMF. The rink amid linker was then de-protected using 3 x 2 minute washes with 20% piperidine in DMF. The resin was then washed using 4 x 5 ml of DMF; all Fmoc-protected amino acids were reacted with resin individually following the previous steps described.
Following the reactions for the specific peptides sequence listed in Table 2.1, peptides were washed with 3 x 5 ml DMF, dichloromethane and methanol and cleaved using a cleavage mixture of 88% trifluoroacetic acid (TFA), 5% H$_2$O, 5% phenol and 2 % triisopropylsilane (TIPS) for 3 hours at room temperature. The cleavage mixture was then passed through a glass wool packed pasture pipette and precipitated in 10 ml of ice cold diethyl ether. The precipitate was then collected by centrifuge at 3500 rpm for 20 minutes the pellet was then washed with 3 x 3 ml of diethyl ether collecting by centrifugation. The collected peptide was then dried using nitrogen flow until a constant mass. The produced peptides were analysed using electro spray time of flight mass spectrometry (MS) (BRUKER) and HPLC-UV (method described in Section 2.2.4) or when available Liquid chromatography-mass spectrometry (LC-MS).

2.2.4 Peptide analysis

Synthesised peptides were analysed using gravimetric readings, mass spectrometry (MS) and high performance liquid chromatography analysis (HPLC) using the following methodologies. Gravimetric sample readings (SM) of collected peptides were recorded and calculated from maximum theoretical reaction yield (TM) in order to obtain % reaction yield by mass using the equation below in Equation 2.1.

\[
\text{Equation 2.1}
\]

\[
\text{(SM ÷ TM)} \times 100 = \% \text{ Yield}
\]

MS analysis was performed by diluting the produced raw peptides in MS-grade methanol (Fisher, UK) to obtain a working concentration of 1 µg/ml. The working solution was then injected at a rate of 180 µl/hour into a Bruker micro time of flight
mass spectrometer (BRUKER). Readings were taken over 2 minutes of injection between 150-3000 m/z. The readings were recorded and expressed as relative intensity against samples mass divided by charge (m/z). HPLC analysis was performed using a semi-automated waters HPLC-UV system (Waters, UK) using an analytical grade HPLC column (Phenomenex, UK). All readings were recorded at 223 nm wavelength for excitation of peptide bonds with 10 µl injections of 1 mg/ml of raw peptide samples in di-H2O. A moving solvent gradient was used from 100 % H2O to 100 % acetonitrile over 20 minutes, with a flow rate of 1 ml/minute. The purity of peptide samples was calculated by peak area. Peptides requiring purification were purified by repeat collections of peak elution using 100µl injections of peptide samples using a semi-preparative HPLC column on a semi-automated waters HPLC-UV system (Waters, UK). LC-MS analysis was used when available to provide HPLC peak mass identification. Samples were injected at a volume of 10µl at a concentration of 1 mg/ml. Samples were eluted using a 50:50 methanol to water solvent ratio.
2.3 Results

2.3.1 3D computational modelling of peptide structure

2.3.1.1 Computational modelling of Fmoc-F₂ monomer assembly

The results of 3D computational modelling found the steric energy for Fmoc-F₂ monomer to be 249.47 kJ mol⁻¹ while in the lowest energy conformation dimers had a steric energy of 425.53 kJ mol⁻¹. The energy of stabilisation due to dimer formation is therefore:

\[ 425.53 \text{ kJ mol}^{-1} - (2 \times 249.47 \text{ kJ mol}^{-1}) \text{ kJ mol}^{-1} = -73.41 \text{ kJ mol}^{-1} \]

Consequently, Fmoc-F₂ is more stable when assembled as a dimer than two discreet monomers by -73.41 kJ mol⁻¹. From this finding, Fmoc-F₂ peptide monomers were modelled at lowest energy state conformations forming a vertically-stacked alignment of the Fmoc- functional groups forming an axial point. The aggregated molecules were found to orientate, fitting tightly together in a helical confirmation with an approximate helical angle of 50-60 ° between molecules (Figure 2.3).
Figure 2.3 Computational model of Fmoc-F₂ monomer interactions – Monomers were modelled using Spartan '10 software. Fmoc-F₂ monomers were arranged in a vertical stack and modelled to achieve the lowest available energy state. At the lowest energy state the Fmoc-F₂ molecules were found to align in a helical structure.
2.3.1.2 Computational structural modelling of sIGF-1 peptide

3D computational modelling of the IGF-1 active site peptide found the peptide to have a “coil like” structure at the lowest energy state under vacuum conditions. No additional folding or overlapping of sub molecular structure was observed. The Lowest molecular energy state was found to be -75.32 Kcal/M (Figure 2.4).

![Computational model of IGF-1 active site peptide 3D structure](image)

*Figure 2.4 Computational model of IGF-1 active site peptide 3D structure – Peptide was modelled using ChemDraw 3D software in a vacuum environment to achieve the lowest available energy state for all bonds, peptide presented with a “coil-like” structure similar to that of the active site in the native IGF-1 protein.*
2.3.2 *Mass spectrometry and HPLC-UV analysis of Fmoc-F_2 peptide*

The MS results presented in Figure 2.5 A on the following page, show the peptide products produced for the raw product of the liquid phase synthesis reaction to produce Fmoc-F_2. The main peak corresponds to Fmoc-F_2+H_2O (553 m/z) with no reaction precursors detected Figure 2.5 A. However, additional peaks corresponding the Fmoc-F_3 (700 m/z) and Fmoc-F_4 (944 m/z) were also detected, as well Fmoc-F_2 dimer and trimer. The HPLC purified Fmoc-F_2 peptide MS results presented in Figure 2.1 B shows a single large peak corresponding to the MW of Fmoc-F_2 + Na showing successful purification of Fmoc-F_2 and a much greater purity from the raw product presented in Figure 2.5 B. However, the by-products F_2 (313 m/z) and F + sodium adduct (179 m/z) were detected, although in trace amounts.
Figure 2.5 Mass spectrometry analysis of Fmoc-F$_2$ raw synthesis product: HPLC purified. Mass spectroscopy analysis was performed at a peptide concentration of 10 µg/ml using methanol injection solvent at a rate of 180 µl/minute. Readings were recorded over 1 minute and readings were taken for suitable mass ranges to include all products detected. Peaks were recorded and expressed as mass/charge.
Figure 2.6 Mass spectrometry analysis of Fmoc-F₂ purified synthesis product: HPLC purified. Mass spectroscopy analysis was performed at a peptide concentration of 10 μg/ml using methanol injection solvent at a rate of 180 μl/minute. Readings were recorded over 1 minute and readings were taken for suitable mass ranges to include all products detected. Peaks were recorded and expressed as mass/charge. Plot shows purified Fmoc-F₂ synthesis product with a corresponding mass of 557 with a 1+ charge.
HPLC-UV analysis of the purified Fmoc-F$_2$ peptide showed the elution of a single peak at 80% acetonitrile (Figure 2.7). The obtained peptide was found to have a purity exceeding 98% by peak area, supporting the finding of high sample purity from mass spectrometry analysis.

**Figure 2.7 HPLC-UV analysis of Purified Fmoc-F$_2$.** HPLC analysis was performed using 10 µl injections of 1mg/ml peptide sample. The HPLC mobile phase solvents consisted of a gradient of acetonitrile/water at a rate of 1ml/minute. Solvent gradient was run from 50:50 to 100:0 acetonitrile/water over 20 minutes with a retention time of approximately 5 minutes.
2.3.3 Mass spectroscopy and HPLC-UV analysis of IGF-1 peptide

The results from MS analysis of the IGF-1 C region peptide synthesis product (MW: 1264) revealed successful synthesis of the full peptide sequence with significant peaks for the full mass corresponding to $1^+$ and $2^+$ charge, 1265 m/z and 633 m/z (Figure 2.8). Also a peak corresponding to the full sequence minus the last two amino acids was identified with $1^+$ charge of 1109 m/z.

Figure 2.8 Mass spectrometry analysis of IGF-1 mimetic peptide - Mass spectrometry analysis was performed at a peptide concentration of 10 µg/ml using methanol injection solvent at a rate of 180 µl/minute. Readings were recorded over 1 minute and readings were taken for suitable mass ranges to include all products detected. Peaks were recorded and expressed as mass/charge.
The HPLC-UV analysis of the produced IGF-1 peptide revealed good separation of the two products with the full molecule found to elute at 9.9 minutes, approximately 5-10 % acetonitrile (Figure 2.9). The peptide was found to have a raw purity of 86.8 % by peak area. The second peak elution was at 14.6 minutes is most likely to be to full sequence minus the last two amino acids as supported by the MS analysis (Figure 2.8). Primary peptide peak was isolated by the purification method (Section 2.2.4).

Figure 2.9 HPLC-UV analysis of produced IGF-1 peptide: HPLC analysis of purified IGF-1 peptide: HPLC analysis was performed using 10 µl injections of 1mg/ml peptide sample in H2O. HPLC mobile phase solvents consisted of a gradient of water/acetonitrile at a rate of 1 ml/minute. Solvent gradient was run from 100:0 to 0:100 water/acetonitrile over 20 minutes. Elution of products was detected at 9.9 and 14.6 minutes using UV detection at 220 nm wave length.
The purified peptide was analysed using the semi-preparative HPLC column and found to have a purity of 94.6% by peak area (Figure 2.10). Furthermore, the purified peak was found to have a different elution time from the non-purified sample, eluting at 3 minutes. However, this reduction retention time is likely due to the use of a semi-preparative separation column of different dimensions and with a different mobile phase for the final analysis.

**Figure 2.10 HPLC analysis of purified IGF-1 peptide:** HPLC analysis was performed using 10 μl injections of 1mg/ml peptide sample in H2O. The HPLC mobile phase solvents consisted of a gradient of water/acetonitrile at a rate of 1 ml/minute. Solvent gradient was run from 80:20 to 50:50 water/acetonitrile over 12 minutes. Purified peptide was detected at 2.5 minutes.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Raw product yield</th>
<th>% yield</th>
<th>% Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-F₂</td>
<td>2.2g</td>
<td>67.2</td>
<td>98.7%</td>
</tr>
<tr>
<td>IGF-1 peptide</td>
<td>116.2 mg</td>
<td>91.8</td>
<td>94.6%</td>
</tr>
</tbody>
</table>

*Table 2.2 Summary of peptide synthesis product yield and purity*
2.4 Discussion

The 3D molecular structures of the produced peptides were modelled in order to greater understand the molecular organisation of these structures. In the case of the Fmoc-F\(_2\) peptide, the computational modelling aimed to assess the way in which peptide monomer units interact and assemble to form nanofibres. From the results of the 3D computational modelling of Fmoc-F\(_2\), the monomers were found to occupy the lowest energy level when assembled in a helical-like confirmation with the Fmoc-group serving as the axial point of the helical assembly (Figure 2.3). Additional computational modelling of both discreet and paired monomers in an environment of water molecules found the monomeric units to acquire lower bond entropy when paired than as discreet units, with a reduction in bond energy of -73.41 kJ mol\(^{-1}\), equating to a 14.7 % reduction in total bond energy. This finding demonstrates that it is more energetically favourable for Fmoc-F\(_2\) peptides to assemble than remain as discreet peptides in and aqueous solution. This finding further supports the theory outline in the previous publications (Smith et al., 2007, Yan, 2010) in which the mechanism of assembly is proposed to occur in stages of peptide aggregation and then organised assembly.

The computation modelling of the sIGF-1 peptide was performed in order to assess its 3D conformational structure for comparison with the native protein. As previously discussed, the active site sequence of IGF-1 possesses a coiled-like conformation in the IGF-1 full protein. However, it is not known whether this conformation is retained if the overall secondary structure of the protein is removed from the peptide sequence thus affecting its binding affinity to the cell receptor. From the results of the computational modelling the IGF-1 peptide was found to assume a coiled conformation at the lowest available energy state (Figure 2.4). While
this result is promising as it suggests that the native structure is conserved, it could be argued that the structure is modelled in a vacuum environment and that its conformation may change in the aqueous environment. While this is a realistic criticism, the computational model used assumes a neutral charge of the peptide, thus modelling its structure as it would be at neutral pH. As the peptide is intended to be used in a pH neutral buffered solution, modelling the peptide in an aqueous model would utilise the addition of water molecules around the structure which would facilitate hydrogen bonding but would no longer in effect give a neutral pH simulation. Only the inclusion of parameters taking into account ions would give a more comprehensive prediction of peptide folding.

The analysis of synthesised peptides using MS, HPLC and gravimetric analysis was performed in order to confirm the successful production of the target molecules and to assess the purity and yield at which they were obtained. The results of synthesis of the Fmoc-F$_2$ peptide obtained by liquid phase method, showed the successful synthesis of Fmoc-F$_2$ at the $^{1+}$ charge of the full mass + H$_2$O (553 m/z). While the raw synthesis product contained significant contamination of the by-product Fmoc-F$_3$, at the mass of 700 m/z, a number of other unknown by-products were detected at lower levels. These by-products are likely to be un-reacted conjugates of longer amino acid chains of Fmoc-Fn as well as of the coupling reagents in there activated states. However, following HPLC purification the peptide sample for Fmoc-F$_2$ was found to have a purity exceeding $>94\%$ by peak area. The mass of the peptide was confirmed by the detection of the $^{1+}$ charge of the full mass of Fmoc-F$_2$ + sodium (557). The presence of the sodium ion adduct is likely to be a result of the isolation method, which relies on the precipitation of the peptide on water. In addition of
Fmoc-F$_2$ a number of trace contaminants were also detected at the mass of 179 and 313 were detected at very low intensities. The mass of these impurities correspond to mass of phenylalanine + sodium and di-phenylalanine respectively. The detection of phenylalanine in the end product is likely due to incomplete reaction of the precursors. However, the intensity of phenylalanine in the Fmoc-F$_2$ product is extremely low and is therefore unlikely to affect the behaviour of Fmoc-F$_2$. The results of this synthesis, demonstrate this method to be both cost effective and relatively rapid. While the product required purification, future methods could potentially be optimised to introduce an additional protecting group to the NH$_2$ group of the un-protected phenylalanine. This optimised protocol, while adding an additional step in to the synthesis by removing the protective group, would allow the elimination of possible chain reactions of phenylalanine generating undesired, longer Fmoc-F$_n$ chains.

The manual solid phase synthesis of the IGF-1 active site peptide was successful with the desired end product being the main product of the reaction (Figure 2.7). The synthesis of the human mimetic IGF-1 C region peptide results of MS analysis detected two main significant peaks corresponding to the $^{1+}$ and $^{2+}$ charges of the full 12 amino acid sequences (Figure 2.7). However, there was a significant peak also detected that corresponded to the full sequence minus the last two amino acids. While the full molecule was found to be the main product of the synthesis, with MS and HPLC-UV analysis revealing a raw purity of 86.7 %, further purification of this peptide achieved 94.6 % purity. From these results it can be concluded that the C region IGF-1 peptide can be synthesised using conventional solid phase synthesis methods, a finding that has yet to be reported in literature. Furthermore the purity of this peptide and that of Fmoc-F$_2$ are sufficient for further biological study.
2.6 Conclusions

In summary, the findings of this chapter show the successful synthesis of Fmoc-F$_2$ and sIGF-1 peptides required for the combined treatment approach, completing thesis aim two outlined in Section 1.5. Furthermore, these findings demonstrate that the previously unreported novel peptide, sIGF-1peptide can be successfully synthesised using conventional peptide synthesis methods and retains a coiled like 3D structure of the native sequence. While the findings of computational analysis of Fmoc-F$_2$ further supports those of current literature. From these findings the produced peptides have been investigated further to assess their material and biological properties in the following chapters.
Chapter 3: Self-assembly Hydrogel

Formulation and Characterisation
3.1 Introduction

The production of self-assembly nanostructured materials from aromatic peptides through π-π interactions requires the tuning of the solvent/gelling systems in which the peptide is initially dissolved (Smith et al., 2007). In fact it is common for highly aromatic peptides to be highly insoluble in water, making the formation of hydrogels from these peptides extremely difficult (Da Violante et al., 2002). In order to overcome these issues researchers in this field have relied on the use of strong bases such as sodium hydroxide to dissolve the peptide through de-protonation of functional groups (Smith et al., 2007). This method works by removing the hydrogen from the COOH functional group of the peptide leaving a negatively-charged oxygen molecule. This can then interact freely with water molecules through charge related interactions, in turn holding the peptide in solution (Figure 3.1). The hydrogels are then formed by decreasing pH with the addition of strong acid into the range of pH 7.5-8.0, within which the hydrogel self-assembles (Yan, 2010). Whilst this strategy can be successful in the formation of hydrogels, the control of pH can prove difficult as slight changes in pH can cause the disassembly of the hydrogel structure and precipitation of the peptide. Alternatively, if the alkaline solution is too strong then the ester bonding of the Fmoc protecting group can be broken by hydrolysis (Albericio, 2000). In order to tackle these issues recent publications have reported the use of overnight incubations in growth medium in an attempt to buffer the hydrogel prior to the culture of cells (Jayawarna et al., 2009, Raeburn et al., 2012). However, this method does not provide reproducible control of gel formation and can still lead to the formation of micro-domains of acid or base solutions (Raeburn et al., 2012). Other strategies for achieving peptide solubility and hydrogel formation include the use of organic solvents at low percentage (Da Violante et al., 2002,
Raeburn et al., 2012) or heating treatment at 70 °C for a number of hours. However, these methods have clear limitations, as with the use of organic solvents such as DMSO or methanol, cytotoxicity is a significant risk even at low concentrations (Da Violante et al., 2002). While heat treatment does not intrinsically carry any risk of toxicity, there is a significant risk of peptide hydrolysis which could potentially result in a range of peptide fragments which may disrupt the gel formation or uncontrollably change the material properties of the hydrogel. In addition peptide fragments may have a number of different biological activities that would be unknown and potentially toxic.

Figure 3.1 Theoretical interaction of Fmoc-F$_2$ with water.

The formation of micro domains with the use of acid-base pH controls can be potentially harmful to cells as isolated regions of high or low pH can have a significant impact on the metabolism of the cell and function of membrane proteins (Yoon et al., 2008). In order to address these issues it is important that this research focuses on the development of controllable buffering systems to reliably form hydrogels and nanomaterials without the risk of pH shock to cells or biological systems. In order to address this issue the development of a working buffer system
is an important requirement for the future development of peptide hydrogel treatments.

3.1.1 Self-assembly mechanisms

The Fmoc-F₂ based hydrogels are formed through the self-assembly of Fmoc-F₂ monomers in aqueous solution at pH 7.5 to form nanofibres through π-π interactions (Smith et al., 2007). The structure and self-assembly mechanisms are believed to be based on the hydrophobic interactions of phenol and Fmoc- domains of the phenylalanine interacting with corresponding domains on opposing monomers (Yan, 2010). This process results in the formation of monomer chains assembling to form beta-sheets (Smith et al., 2007). Following this, further assembly through π-π interactions between β-sheets results in the formation of nanofibres (Smith et al., 2007, Yan, 2010) (Figure 3.2). The monomer structure is formed of an Fmoc-protecting group coupled to a chain of at least two phenylalanine amino acids.
The formation of β-sheets can be detected through the appearance of a negative “Cotton effect” in CD spectra analysis. The “Cotton effect” is defined as a shift in the polarisation of light through the molecular alignment of the nano structure, shifting light from negative to positive in a wave-like pattern; this behaviour has been shown to be indicative of β-sheet assembly (Elie and Wilen, 1994). However, current literature focusing on the self-assembly mechanisms of Fmoc-F$_2$ have postulated β-sheet assembly, but are yet to achieve successful detection of a negative Cotton effect (Smith et al., 2007, Yan, 2010).
3.1.2 Chapter aims

This chapter aims to investigate the self-assembly properties of synthesised peptides and the nanostructured hydrogels that can be formed from them. To achieve this, the chapter will focus on the development of buffering systems and preparation methods to form hydrogels that overcome the issues of acid-base micro domains. From this work the materials formed will be analysed and characterised using physical and chemical characterisation methods such as scanning electron microscopy (SEM), rheometry and circular dichroism (CD), with the intention of further understanding how modifications of the peptide structure have in turn affected the core material in terms of structure, size and behaviour.
3.2 Materials and Methods

3.2.1 Production of Fmoc-F$_2$ hydrogel through drop wise addition of acid and base

Fmoc-F$_2$ hydrogels were formed by using the method developed by (Jayawarna et al., 2009). The produced Fmoc-F$_2$ was dissolved in sodium hydroxide adjusted H$_2$O (pH 12) to obtain a 3 mg/ml solution. The solution is then neutralised with the drop-wise addition of 0.5 M HCl and incubated at 25 °C for 1 hour to allow gel formation. The hydrogels produced using this method were imaged using laser confocal microscopy at 5 and 20 minute assembly times and scanning electron microscopy (SEM) (see Section 3.2.2 for experimental details). Sample preparation was repeated in triplicate. Inversion test was performed on hydrogel by transferring 1 ml of the produced hydrogel into a 5 ml glass vial. The hydrogel aliquots were left to stand for 10, 20, 30, 45 and 60 minute intervals. After setting time points the vial was turned upside down, hydrogels that remain in contact with the setting surface and retain set shape are classed as passing inversion test for the given time point.

3.2.2 SEM and confocal analysis of Fmoc-F$_2$ nanostructured hydrogels

SEM imaging analysis was performed on Fmoc-F$_2$ prepared hydrogels using the method detailed in Section 3.2.1 and using buffer system 6 outlined in Section 3.2.3. Following hydrogel setting after 20 minutes 25 µl of each hydrogel type was transferred by pipette to the surface of a carbon-coated metallic stub. The samples were left under sterile air flow overnight to dry. The dried hydrogel was then coated with a 2 nm platinum coating using a Quantum orbital sputter coater (Quorum Technologies Ltd.). The samples were then imaged using a sigma field emission gun scanning electron microscope (Carl Zeiss Ltd. UK). A series of images of the nanostructured materials were taken at equal magnifications and available
resolutions dependent on samples. Using the SEM systems image analysis software provided (Carl Zeiss Ltd. UK) measurements of nanofibre diameters were recorded. A total of 9 fibres per randomly selected areas of 3 different samples were measured and reported as mean value ±SD. Tukey’s t test analysis was performed to compare samples.

3.2.3  Laser confocal imaging of hydrogel fibres

Confocal laser scanning microscopy is a form of microscopy that obtains high resolution optical images by using focused laser excitation of samples (Pawley, 2006). This process allows for a higher resolution of florescence samples as it only excites light within the given samples area and plane that is being images. Confocal imaging was performed in order to resolve the structure of the hydrogel in the hydrated state as conventional florescence microscopy was not capable of resolving the hydrogel structure. Confocal imaging was performed on 50 µl gel samples assembled on glass slides at 5 and 20 minute self-assembly time points. Sample areas were chosen at random. Excitation was achieved a 405 nm wavelength with a laser power of 80% and a scan rate of 400 Hz with a Z-stack depth of 90 µm. Images were captured using a 62 x power optic using a Leica Confocal microscope (Leica Microsystems, UK). Sample fields were imaged over short durations and no bleaching of the samples was observed. Poor reproducibility of imaging was experienced due to difficulty in focusing on samples believed to be a result of the dynamic nature of the material. For this reason measurement analysis was not performed as is was often not possible to image samples within study sets.
3.2.4 Development of buffer systems for Fmoc-F₂ based hydrogels

Due to the limitations in pH control with the use of drop-wise addition of acid to neutralise the hydrogel solution, a series of buffer preparations were devised using the previously discussed publications (Robinson, 1968) as a guide for the ratio of different buffering solutions. In combination with the dissolution method detailed in Section 3.2.1 [sodium hydroxide adjusted H₂O (pH 12)] the buffer solutions detailed in the Table 3.1 were examined for their suitability in the preparation of Fmoc-F₂ hydrogels by observation of hydrogel clarity, recorded hydrogel pH, where or not the peptide was soluble or precipitation occurred, and whether the hydrogel was capable of passing inversion test in a 1 ml volume in a 5 ml glass vial following 20 minutes incubation at 20 °C (see details of inversion test in section 3.2.1).

<table>
<thead>
<tr>
<th>Number</th>
<th>Buffer system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3.3mM NaOH: 10 x PBS (10:1)</td>
</tr>
<tr>
<td>2.</td>
<td>3.3mM NaOH: 6x PBS (10:1)</td>
</tr>
<tr>
<td>3.</td>
<td>3.3mM NaOH: 4x PBS (10:1)</td>
</tr>
<tr>
<td>4.</td>
<td>3.3mM NaOH: 4x PBS (10:2)</td>
</tr>
<tr>
<td>5.</td>
<td>3.3mM NaOH: 10 x PBS: 20% acetic acid (10:1:0.02)</td>
</tr>
<tr>
<td>6.</td>
<td>3.3mM NaOH: 6x PBS: 20% acetic acid (10:1:0.02)</td>
</tr>
</tbody>
</table>

Table 3.1 Table of experimental buffer systems
3.2.5 *Circular Dichroism spectroscopy*

Circular dichroism spectroscopy (CD) is a photometric technique used to assess the molecular organisation of nano materials and proteins. CD experiments were performed using 3 mg/ml pre-prepared Fmoc-F$_2$ hydrogel. Hydrogel was analysed using a 15 $\mu$l quartz glass-well slide with readings taken from 180-320 nm wavelength. The polarisation of light was recorded and was plotted against wavelength (n=3). CD experiments were conducted at the University of Sussex under the supervision of Louise Serpell and Kyle Morris.

3.2.6 *Rheological analysis of hydrogels*

Rheological analysis was performed to achieve a deeper understanding of the physical properties of the Fmoc-F$_2$ hydrogel. The samples were prepared using the optimised buffering system; the peptide was first dissolved in solution A and buffered using solution B buffer system 6 as outlined in Table 3.3. The hydrogel were allowed to set at room temperature and disrupted by vortex mixing prior to analysis. A volume of 100 $\mu$l of the disrupted hydrogel was then transferred by pipette to the plate of the rheometer and incubated for 10 minutes to allow for re-assembly. Oscillating shear stress sweep analysis was performed on the hydrogel samples at 37 °C with an increasing shear stress from 0.05-20 Pa with a frequency of 1 Hz for 60 seconds (n=3), following shear sweep analysis new samples were analysed using an Oscillating frequency sweep from ranging from 0.1 to 10 Hz with a static stress of 0.2 Pa.
4.2.2 Statistical analysis

All data sets that were found to be normally distributed were analysed using two-tailed Tukey’s t-test analysis unless otherwise stated in text. Significance was determined using a significance level of 0.05 ie. Values greater 0.05 were not considered to be statistically significant. All statistical analysis was performed using PRISM. Where multiple comparisons were required two-way ANOVA was used with post-hoc Turkey’s t-test analysis.
3.3 Results

3.3.1 Production of Fmoc-F$_2$ hydrogel through drop wise addition of acid

The Fmoc-F$_2$ hydrogels, produced using a previously published method (Jayawarna et al., 2009), were transparent with a large number of bubbles becoming entrapped by the high viscosity of the hydrogel (Figure 3.3). While able to pass the inversion test after 45 minutes, the hydrogel had a low viscosity when disrupted by pipetting or agitation still maintaining the ability to rapidly reassemble when static conditions were reintroduced. These properties make the gel highly suitable for injectable applications.

![Figure 3.3 Photograph of Fmoc-F$_2$ hydrogels during inversion test. Image taken after 45 minute setting at room temperature following inversion test procedure using a cannon 5D digital camera.](image-url)
The self-assembly process in the acid-base drop-wise addition preparation method was observed by confocal laser microscopy. Although difficult to capture, as the process is active, the findings demonstrate the effect that remnant acid domains can generate when using a strong acid in the preparation of the hydrogel (Figure 3.4). It is proposed that rapid assembly of large aggregates of peptides localised around areas of low pH, thus creating acid-entrapped domains. As the self-assembly process progressed with time, fewer peptide spherical aggregates were observed while a greater number of assembled fibres became visible (Figure 3.4B). A short time lapse video was capture of this process over 6 seconds, the video showed fibres to move and grow rapidly outward from the spherical clusters. However, reproducible images of this process were difficult to acquire.

![Figure 3.4 Auto-fluorescence confocal imaging of Fmoc-F$_2$ hydrogel](image)

**Figure 3.4 Auto-fluorescence confocal imaging of Fmoc-F$_2$ hydrogel**

A- after 5 minutes self-assembly, B- after 20 minutes self-assembly. Hydrogels prepared were images at x 62 magnification optic, samples were excited at 405 nm and imaged using a Leica confocal microscope system. Image A shows a large number of spherical formation particulates of peptide after 5 minutes of assembly, Image B shown a greater amount of nanofibres present after 20 minutes of assembly.
3.3.2  *Hydrogel buffer system optimisation*

The hydrogel buffer optimisation studies (Table 3.2) revealed that buffer system 6 composed of 3.3 mM NaOH: 6x PBS: 20% acetic acid (10: 1: 0.02) was found to be the most suitable by allowing dissolution of the peptide in its primary solution, self-assembly of hydrogel, pass of inversion test and adequately buffering within a neutral pH range. Similar results were achieved with buffer systems 4 and 5. However, in these buffers sufficient self-assembly was not achieved as the hydrogel failed inversion test after 1 hour (Table 3).
<table>
<thead>
<tr>
<th>Number</th>
<th>Buffer system</th>
<th>pH solution A</th>
<th>pH solution B</th>
<th>Fmoc-F₂ Soluble (3mg/ml)</th>
<th>Final conc.</th>
<th>Final pH</th>
<th>Self-assembly</th>
<th>Pass inversion (after 1 hour)</th>
<th>Solution clarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NaOH: 10 x PBS (10:1)</td>
<td>12.4</td>
<td>7.3</td>
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<td>1x PBS</td>
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<td>2.</td>
<td>NaOH: 6x PBS (10:1)</td>
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<td>7.3</td>
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<td>0.6 x PBS</td>
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<td>No</td>
<td>Clear</td>
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<td>3.</td>
<td>NaOH: 4x PBS (10:1)</td>
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<td>7.3</td>
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<td>0.4 x PBS</td>
<td>10.6</td>
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<td>No</td>
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<td>4.</td>
<td>NaOH: 4x PBS (10:2)</td>
<td>12.4</td>
<td>7.3</td>
<td>Yes</td>
<td>0.8 x PBS</td>
<td>8.6</td>
<td>Partial</td>
<td>No</td>
<td></td>
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<tr>
<td>5.</td>
<td>NaOH: 10 x PBS: 20% acetic acid (10: 1: 0.02)</td>
<td>12.4</td>
<td>5.2</td>
<td>Yes</td>
<td>1x PBS 0.004% (v/v) acetic acid</td>
<td>7.3</td>
<td>Partial</td>
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<td>6.</td>
<td>NaOH: 6x PBS: 20% acetic acid (10: 1: 0.02)</td>
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<td>4.52</td>
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<td>0.6 x PBS, 0.004% (v/v) acetic acid</td>
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<td>1 x PBS</td>
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<td>-</td>
<td>No</td>
<td>1 x PBS</td>
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</table>

NaOH : Phosphate based buffers
3.3.3 *SEM characterisation of Fmoc-F₂ Hydrogel nanostructure*

The SEM image analysis found the structure of Fmoc-F₂ hydrogels to be comprised of nanofibres with a mean diameter of 54.57 ± 6.67 (SD) nm (Figure 3.5) with a smooth and regular morphology with the appearance of β-sheet-like fibres within the structure of the larger nanofibres (Figure 3.6). No significant difference in nanofibre size or morphology was seen when using the buffer system in comparison to the acid/base method (p=0.082). A number of interconnecting fibres were also visible supporting the claims that the self-assembly process of fibre formation is through the interlinking of β-sheets (Figure 3.6 A & B). Nanofibres were seen to exist as both discreet and interlinking fibres of larger size. Although in a dry state, low magnification images found the nano-fibrous structure to have a number of pores of varying size ranging between 0.2 to 4µm in diameter (Figure 3.5).

*Figure 3.5 Low magnification SEM Image of Fmoc-F₂ hydrogel structure:* hydrogel samples were prepared and 25 µl drops were added to SEM stubs, samples were air dried for 24 hours. Samples were coated with 2 nm of palladium and imaged under high vacuum SEM (n=3).
Figure 3.6 High magnification SEM Images of Fmoc-F₂ hydrogel structure. A-discreet fibres, B- Interlinking fibres: hydrogel samples were prepared and 25 µl drops were added to SEM stubs, samples were air dried for 24 hours. Samples were coated with 2 nm of palladium and imaged under high vacuum SEM (n=3).
3.3.4 Circular dichroism analysis

The results of CD analysis of the assembled Fmoc-F$_2$ hydrogel (Figure 3.7) revealed a significant CD shift peak between 220-240 and UV absorption curve from 260-280 nm. Furthermore, the presence of peaks between 180-220 nm, with the first peak appearing in the positive range and the second appearing in the negative range, indicated a positive Cotton effect. These values are supported by HT values below 600 indicating no significant absorbance effecting the data output.

![Figure 3.7 Fmoc-F$_2$ hydrogel circular dichroism analyses. CD- circular dichroism, HT- High tension voltage value (relative to sample absorbance) CD experiments were performed using 3 mg/ml pre-prepared Fmoc-F$_2$ hydrogel. Hydrogel was analysed using a 15 µl quartz glass-well slide with readings taken from 180-320 nm wavelength. The polarisation of light was recorded and was plotted against wavelength (n=3).](image)
3.3.5 Rheological analysis of produced hydrogels

The findings of the shear stress sweep analysis (Figure 3.8) showed a significant effect of hydrogel dilution on hydrogel shear modulus ($G^*$). 1.5 mg/ml Fmoc-F$_2$ was found to have the greatest $G^*$ values with a peak shear modulus of 7.7 Pa. Conversely, the highest concentration of hydrogel (3 mg/ml) was found to have a lower shear modulus of 4.5 Pa. However, 3 mg/ml concentrations were also found to have a downward step-like trend in response to the increasing shear stress unlike that of the 1.5 mg/ml hydrogel. While, lower concentrations of hydrogel of 0.5 and 1 mg/ml had a significantly lower shear modulus in response to increasing shear stress.

From these results of frequency sweep analysis (Figure 3.9) 0.2 Pa was chosen as the steady state point of the hydrogel and 0.5, 1 and 1.5 mg/ml hydrogels were tested using a frequency sweep analysis with a static shear stress of 0.2 Pa. Samples of 1.5 and 1 mg/ml were found to have an similar behaviour both showing matching $G'$ and $G''$, demonstrating viscoelastic behaviour. In the case of the 0.5 mg/ml solutions, the respective hydrogels demonstrated similar viscoelastic properties, the storage modulus was significantly lower than that of 1 and 1.5 mg/ml samples and viscosity ($G''$) was found to be higher than that of elasticity. However, with increasing frequency 0.5 mg/ml hydrogels were seen to revert their properties with $G'$ increasing crossing over with $G''$ at 2 Hz.
Figure 3.8 Dynamic shear stress sweep rheology analysis of Fmoc-F₂ hydrogel: Hydrogel samples were prepared at 3, 1.5, 1 and 0.5 mg/ml conc. 500 µl samples were tested for response to increasing shear stress from 0 to 20 Pa. Samples were found to have a stress profile dependent on concentration with 1.5 mg/ml presenting with the greatest shear stress properties. (n=3)
**Figure 3.9 Dynamic Frequency sweep rheology analysis of Fmoc-F2 hydrogel:** Hydrogel samples were prepared at 1.5, 1 and 0.5 mg/ml conc. in DMEM culture medium supplemented with 10 % FBS. 500 µl samples were tested for response to increasing shear frequency from 0.1 to 10 Hz with a static shear stress of 0.2 Pa (steady state from increase shear stress experiment). (n=3)
3.4 Discussion

SEM and CD analyses were performed in order to greater understand the mechanisms of Fmoc-F$_2$ peptide assembly. The findings of this study not only provide evidence of the self-assembly structure and properties of Fmoc-F$_2$ hydrogel as reported in current literature (Tang et al., 2009), but also offer new insights about the mechanisms and preparation conditions controlling their formation. Results from hydrogel preparation found transparent hydrogels were formed following the neutralisation of the Fmoc-F$_2$ peptide solution when prepared at 3 mg/ml concentrations. Hydrogel were found to be capable of repeatable self-assembly and positive inversion test after 45 minutes. The transparent nature of Fmoc-F$_2$ hydrogels is highly beneficial to 3D cellular culture study and tissue engineering applications as they are suitable for in vitro microscopic analysis of cellular growth. Furthermore, the ability of Fmoc-F$_2$ hydrogel to reversibly self-assemble provides an additional benefit to tissue engineering applications where injectability is desirable. As the hydrogel can be formed and then disrupted and mixed with living cells and subsequently re-set to form a hydrogel/cell construct in situ.

Confocal imaging was performed to study the self-assembly process and structures under physiological conditions i.e. pH neutral and buffered salt solution (PBS solution). The results of confocal imaging (Figure 3.4) revealed the formation of UV-Fluorescent fibres that appear to change with size and number with time. The formation of peptide aggregates was seen to act as initiation points for the self-assembly, reducing in apparent size with time as fibres formed. The confocal analysis also suggests that acid-base micro domains may induce the formation of peptide spherical aggregates, from which the fibres are gradually formed as pH equilibration throughout the medium is reached. The effect of acid-base domain
formation makes the reproducible production of hydrogels from this Fmoc-F$_2$ remarkably difficult, as measurements using pH meters cannot detect acid microdomains trapped into the hydrogel aggregates. Therefore, during the preparation of hydrogels using a drop-wise addition of acid, the operator cannot accurately measure the pH of the hydrogel. While this method is reported in many publications relating to Fmoc-F$_2$ as an acceptable method for preparation (Smith et al., 2007, Yan, 2010), significant variations in hydrogel formation were identified in these preliminary experiments. This issue has also been investigated in recent publications comparing variation in material properties of Fmoc-F$_2$ hydrogels (Raeburn et al., 2012). As a result, the development and testing of a series of buffer systems was performed to find a reproducible method for the preparation of Fmoc-F$_2$ based hydrogel. The findings from these studies found only buffer system six was suitable (see Table 3.2), as the addition of too great a concentration of phosphate salts in the end working solution, whilst sufficiently buffering the solution, would cause the peptide to precipitate. Moreover, due to the theoretical pK$_a$ of the COOH functional group (pK$_a$ = 3.5), Fmoc-F$_2$ exerts an acidic effect on the solution. Therefore, the buffer solutions detailed in Table 3.1 was used as a guide for preparations. However, as the presence of Fmoc-F$_2$ at higher concentrations would have an increasing acidic effect on the pH of the solution, therefore the balance of peptide and buffer concentration is important as a drop in pH could lead to the potential precipitation of nanofibre aggregates if the pH was to fall below 6.2 (Tang et al., 2009). However, buffer system 6 was found to reproducibly form Fmoc-F$_2$ hydrogels facilitating rapid assembly of the hydrogel passing the inversion test in less than 20 minutes. This finding shows a remarkable improvement in the assembly
times found from drop-wise method preparations and those reported in the literature, being in up to 1 hour (Raeburn et al., 2012, Smith et al., 2007, Yan, 2010).

SEM analysis of the Fmoc-F$_2$ hydrogel nano-structure, found the hydrogel to be formed from a dense network of nanofibres with a mean diameter of 54.57 nm (Figure 3.5). Similar in size to the nanostructure fibres of type II collagen found in cartilage and the intervertebral disc of approximately 40 to 50 nm in diameter (Mayne and Burgeson, 1987). The hydrogel nanofibres were found to be smooth and regular with inter-linking and merging of different fibres (Figure 3.5). While the network appeared to be dense in dried form, pores in the network were seen to vary in size, ranging from 0.2 to 4 µm (see Figure 3.5). However, this finding is not likely to be representative of the pore size in the hydrated gel, as the results from confocal analysis show a less dense matrix than that of the SEM when assembled (Figure 3.4 B). In addition, SEM analysis also found nanofibres to be formed of small subunit fibres similar in appearance to β-sheets (see figure 3.6 B). These findings support those of the current literature, that present evidence of monomer assembly to form β-sheets which stack through π-π interactions to form larger fibres (Tang et al., 2009).

In support of SEM analysis, CD analysis was performed in order to assess the molecular assembly mechanism involved in the formation of Fmoc-F$_2$ based hydrogels. The results of CD analysis revealed the presence of a negative Cotton effect between 180-220 nm. Cotton effect is a phenomena cause by the characteristic shift in the relative absorption of left and right polarised UV-light to each extreme of negative and positive with changes in the UV-wave-length during CD analysis (Elie1 and Wilen, 1994). The presence of a negative Cotton effect is indicative of β-sheet assembly structures (Elie1 and Wilen, 1994) and supports the findings from SEM image analysis and the claims of previous publications (Jayawarna et al., 2009,
Smith et al., 2007). Furthermore, the presence of a peak between 220-240 has been proposed to also indicate β-sheet assembly (Smith et al., 2007). In summary, the findings of this study, while confirming the findings of current literature that postulate that Fmoc-F2 hydrogels self-assembly to form β-sheets which in turn stack to form nanofibres through π-π interactions (Yan, 2010), provide evidences that the assembling process can be achieved at pH conditions compatible to clinical applications.

Rheological analysis of the Fmoc-F2 hydrogel was performed in order to study material properties of the produced hydrogel in response to both shear stress and frequency. The findings of the study were reported in $G'$ values (a measure of material elasticity) and $G''$ (a measure of viscosity) (Mezger, 2006). The results of rheological analysis by shear stress sweep found 1.5 mg/ml hydrogel to have the greatest peak shear modulus of 7.7 Pa. While 3 mg/ml was found to have a lower peak shear modulus of 4.5 Pa. Reason for this finding is unclear from the data, but presents a number interesting questions with regards to the materials concentration to modulus relationship. However, it may be argued that this finding may be result of the effect of hydrogel dilution in PBS providing a more pH stable hydrogel system.

Further to these findings, frequency sweep analysis revealed all of the produced hydrogels to present with viscoelastic behaviour. However, the most dilute hydrogel (0.5mg/ml) was found to possess a greater degree of viscosity ($G''$) over elasticity ($G'$). These findings not only are consistent with those of previous literature (Tang et al., 2009), but also clarify the apparent contradictory results published in a recent review paper (Raeburn et al., 2012) that highlight a wide range of variation of the reported rheological properties. Whereby the work of Jayawarna et al, 2006 reports typical values 500 Pa up to 20 KPa while the paper by Zhou et al, 2009 found the
range to of modulus to be between 2-10 KPa (Jayawarna et al, 2006)(Zhou et al, 2009). The wide range of differences in reported ranges may largely be due to difference in measurement methodology and hydrogel preparation. The present work shows how different preparation conditions can affect the rheological properties of the hydrogels and in particular emphasise the effect played by the initial monomer concentration.

3.5 Conclusions

Hydrogels were successfully formed from the Fmoc-F$_2$ peptide units in biocompatible setting conditions. Furthermore, the Fmoc-F$_2$ hydrogel was found to form from nucleation centres evolving into a complex nanofibre structure formed of interlocked β-sheets, the morphology and viscoelastic behaviour of which are dependent on parameters such as pH and peptide concentration. The combination of optimised preparation conditions with the properties of the final Fmoc-F$_2$ gel appeared to be suitable for the use of this biomaterial technology in the treatment of IVDD either through an injectable filler or through its combination with cells in a tissue engineering approach. Therefore, the following chapter aims to investigate the biocompatibility of Fmoc-F$_2$ hydrogel in order to assess its suitability for this type of clinical application.
Chapter 4: In vitro Biocompatibility

study of Fmoc-F₂ Peptide Hydrogels
4.1 Introduction

The \textit{in vitro} biocompatibility of Fmoc-F\textsubscript{2} nano-structured materials has been evaluated in the recent publication by (Jayawarna \textit{et al.}, 2009). The reported biocompatibility demonstrates specific cell type growth as Fmoc-F\textsubscript{2} based hydrogels appear to support only primary bovine chondrocytes and not other cell phenotypes. Furthermore, cell proliferation was seen in these studies suggesting that the chondrocytes used were likely to be dedifferentiated or fibroblast like in phenotype as previously discussed in section 1.2.2, yet interestingly they presented with a higher survival rate and proliferation than 3T3 fibroblasts (Jayawarna \textit{et al.}, 2009).

It is speculated that the overall poor cell substrate properties of this type of material prevents the adhesion of most cell types, which is likely to affect viability. However, as seen with carbon nanofibres, surface charge can also have a negative effect on cells as they induce cytotoxicity (Kolosnjaj \textit{et al.}, 2007). Considering the findings of this research it could be hypothesised that due to the hydrophobicity of Fmoc-F\textsubscript{2} fibres cell growth may be inhibited due to poor cell adhesion. Furthermore in 3D culture cell growth may also be inhibited by the blocking cell nutrient and ion transport due to hydrophobic nature of this material. This theory is supported by the findings of the publications by (Jayawarna \textit{et al.}, 2009, Zhou \textit{et al.}, 2009). These papers show that cellular growth on these types of hydrogels can be remarkably improved with the integration of specific integrins within the nanofibre structure. As reported, the simple addition of 50 % Fmoc-S in the nanofibre structure during assembly can change the surface charge of the nanofibre making the whole structure more hydrophilic, due to the presence of a reactive OH side group of the serine. In addition to this, the reduced rate of chondrocyte cell growth was demonstrated when
cells were cultured in 3D hydrogel opposed to 2D (Jayawarna et al., 2009) thus suggesting that entrapment may inhibit the transport of nutrients and ions into the cells. This explanation would provide a plausible reason why chondrocytes alone can be grown in the Fmoc-F$_2$ hydrogel. Indeed, due to the evolutionary development nature of this cell type, chondrocytes are well adapted to survive in environments with low nutrient and oxygen availability (Goldring, 2006). Due to the limited number of publications evaluating biocompatibility of this class of material (Zhou et al., 2009), it is important that further research is conducted to evaluate the risks and benefits of these materials and their potential degradation products.

4.1.1 Degradation products and potential toxicity

Currently, little is known about the degradation rate and products of Fmoc-F$_2$ hydrogels. Therefore, it is important that the potential degradation of the monomer structure is assessed in terms of in vitro and in vivo degradation. Peptide hydrolysis is a chemical reaction where water molecules or hydroxyls attacks the carbonyl group of an ester or amide bond giving rise to the addition of hydrogen breaking the linkage (see Figure 4.1).

![Figure 4.1 Chemical diagram of peptide bond hydrolysis](image)

In the case of Fmoc-F$_2$ monomers, both the amide and ester bonds would be subject to hydrolysis in an aqueous environment. This process would over time lead to the
production of free Phe monomers, dibenzofulvene (DBF) and 9-fluorenemethanol (9-flu) through the theoretical reaction shown in Figure 4.2. However, both DBF and 9-flu have a low solubility in water and may therefore form a precipitate if the rate of hydrolysis exceeded the maximum solubility of the degradation products.

Figure 4.2 Theoretical degradation pathways of Fmoc-F₂
4.1.1.1 Phenylalanine toxicity and metabolism

Phenylalanine is an essential amino acid most commonly known for its use as a component in the artificial sweetener aspartame. Phenylalanine is classed as a hydrophobic amino acid due to its benzyl ring side group. While phenylalanine is generally considered to be “non-toxic”, it has been shown to generate cytotoxic effects at relatively high concentrations around >2 mM (330 mg/ml) (Schumacher et al., 2008). Whilst this toxicity could be a concern at higher concentrations, the total concentration of phenylalanine present in the produced Fmoc-F$_2$ hydrogels is within the range of 3-5 mg/ml which is approximately 100 times less than the minimum inhibitory concentration (Schumacher et al., 2008). In consideration of these findings it is unlikely that any cytotoxic effects that could be caused by Fmoc-F$_2$ based hydrogels would be a result of the release of the phenylalanine component of the hydrogel.

4.1.1.2 Dibenzofulvene and 9-fluorenemethanol Toxicity and Metabolism

The by-products from the hydrolysis of Fmoc-F$_2$, dibenzofulvene and 9-fluorenemethanol (9-flu) have yet to be tested for their in vitro and in vivo toxicity. However, from the current literature, similar organic chemicals based on the dibenzo-structure of 9-flu have been studied for their in vivo metabolic pathways of degradation (Faust, 1994). The mechanism of this degradation is thought to be the enzymatic hydrolysis of C=C bonds of the benzene rings driven by cytochrome P450, specifically CYP-1-A1 (Belitsky and Yakubovskaya, 2008, Faust, 1994). The hydrolysis by this pathway leads to an -OH group replacing the C=C bond, therefore increasing the solubility of the compound and allowing for renal clearance (Faust, 1994). In consideration of this research it is likely that any in vivo degradation of the
Fmoc- protecting group may be able to be processed and excreted through this pathway.

4.1.2 Chapter Aims

This chapter aims to investigate the biocompatibility and cytotoxicity potential of Fmoc-F$_2$ nano-structured hydrogels. These studies are necessary to assess and evaluate the feasibility of these materials for applications in regenerative medicine.

In particular, two main objectives have been pursued:

1. To investigate the effect of the hydrogels on cells when used as either 2D or 3D substrates on a range of cell types. This investigation took into account the adsorption of serum proteins on the gel fibres that could be related to cell behaviour.

2. To investigate the cytotoxicity of hydrogel leachate and the potential degradation products generated during the hydrolysis of the Fmoc-F$_2$ hydrogels.
4.2 Materials and methods

4.2.1 Fmoc-F$_2$ hydrogel serum protein adsorption study

Serum protein adsorption to Fmoc-F$_2$ hydrogel was assessed in order to evaluate specific protein interaction with the pre-set hydrogel. The studies were conducted using 3 x 1 ml volumes of pre-set hydrogel in a 6-well tissue culture plates (Corning). Hydrogel samples (n=3) were incubated for 24 hours at 37 °C, 5% CO$_2$ in 5 ml of F-12K growth medium with an osmolality of between 275 to 357 mOsm/kg (PAA) supplemented with 10 % (v/v) foetal bovine serum (FBS) (PAA). Following incubation, the growth medium was aspirated and the hydrogel samples were washed 3 times with 1 ml of phosphate buffered saline (PBS). To remove the adsorbed proteins, the wells were then treated with 200 µl of 10 % (v/v) SDS in PBS for 20 minutes. Samples were analysed using Bradford assay reagent to quantify total protein and SDS- polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.1.1 Bradford assay

Bradford assay for the quantification of total protein was performed using 6 x 1 ml of 1:4 Bradford’s assay reagent (BioRad)/de-ionised water. Samples of 10 µl were added to the pre-mixed assay reagent and protein UV absorbance was read at 595 nm. Concentrations were calculated from bovine serum albumin protein standards prepared using the same method with a range from 0.5 – 50 µg/ml (n=3).
4.2.1.2 SDS-PAGE

SDS-PAGE analysis was performed to identify specific serum protein adsorption to the hydrogel. SDS-PAGE gel was prepared by mixing the reaction reagents detailed in Table 4.1 in numerical order. Following mixing of the separating gel reagents, 3.5 ml of the polymerising acrylamide solution was transferred to an SDS-page gel cast (Bio-Rad) by pipette, the gel was covered with 1 ml of di-H$_2$O and left for 1 hour to set.

After the separating gel had set, excess water was removed and the stacking gel was prepared following the mixture detailed in Table 4.1. The stacking gel polymerising solution was then quickly transferred into the cast by pipette with care taken to exclude bubbles; a well comb was then inserted into the stacking gel to form the sample wells. The gel was allowed to set for 45 minutes. Following complete polymerisation the wells of the gel, 15 µl of the protein samples and broad molecular weight standards (Bio-Rad) were loaded into the wells and run at 100 mV in 500 ml of running buffer (25 mM Tris-HCl, 200 mM glycine and 0.1% SDS) for 40 minutes.
Reagent & Separating gel (10%) & Stacking gel (4%)  
---  
1. H₂O & 6.15 ml & 3.075 ml  
2. 1.5 M Tris-HCl, pH 8.8 & 3.75 ml & N/A  
3. 0.5 M Tris-HCl, pH 6.8 & N/A & 1.25 ml  
4. 20 % (w/v) SDS & 75 µl & 25 µl  
5. 30 % (w/v) acrylamide/bis-acrylamide & 5 ml & 650 µl  
6. 10 % (w/v) ammonium persulfate (APS) & 150 µl & 25 µl  
7. TEMED (Bio-Rad) & 15 µl & 15 µl  

*Table 4.1 SDS-PAGE gel formulations*

Following protein separation the SDS-PAGE gel was carefully removed and stained using Coomassie blue (Bio-Rad) for 1 hour. The gel was then washed with 1% acetic acid/ethanol until gel background was clear. The visible protein bands were then imaged using a photographic imager (Chemiimagener, Alpha Innotech).
4.2.2 2D Primary bovine chondrocyte biocompatibility study

4.2.2.1 Primary bovine chondrocyte isolation from metacarpal cartilage

12 Amputated bovine lower legs were obtained from Tottingworth abattoir within 4 hours of animal death. The legs were washed under running water and skinned using a single sterile scalpel. The hoof and lower phalangeal joint were then removed by lateral section of the supporting tendon and incision along the synovial cavity. Once removed, the remaining metacarpal phalangeal joint was sprayed and washed thoroughly with 70 % ethanol and transferred to a sterile flow cabinet. The synovial cavity was then exposed by cutting along the flexor tendon until exposing the cavity (Figure 4.3 A). Once exposed, sections of cartilage were cut laterally from the joint using a sterile scalpel (Figure 4.3 B) and transferred to a sterile centrifuge tube containing high glucose DMEM (with an osmolality of 324 -333 mOsm) supplemented with 0.1% (w/v) collagenase (Sigma, UK). The tissue sections were then incubated for 20 hours at 37 °C 5% CO₂ in order to digest the cartilage and recover the cells. Following incubation, the digest suspension was filtered through a 70 µm cell sieve (BD Bioscience, UK) and the collected cell suspension was centrifuged to collect isolated chondrocytes. Excess growth medium was aspirated and the cell pellet was then re-suspended in 1 ml of high glucose DMEM and counted using a haemocytometer. Viability of isolated cells typically varied between 55-80% following first 24 hour incubation of initial plating. Chondrocyte phenotype of isolated cells was confirmed by RT-PCR analysis of aggrecan expression.
4.2.3 RT-PCR gene expression analysis

RNA was extracted from isolated cell pellets (Section 4.2.2.1) using 100 µl of cell lysate buffer (RNeasy Kit, Qiagen). Cell lysate was then passed through RNA purification filter and washed with 3 x 250 µl 70% ethanol and eluted using the provided elution buffer (RNeasy Kit, Qiagen). Collected RNA samples were analysed using a nano-spectrophotometer (NanoDrop). The collected RNA solution was then reverse transcribed to create cDNA using Reverse transcriptase. Samples of 2 µg of purified RNA were mixed with 5 µg of oligo dT primer, 5 mM DNTPs and 15 units of AMV reverse transcriptase (BioRad) samples were incubated at 42 °C for 1 hour and then samples were melted at 95 °C for 5 minutes. Sample were then stored at -20 °C. RT-PCR was performed on cDNA samples using the primers detailed in Table 4.2 the reaction mixtures in Table 4.3. Samples were run for 40 cycles of 95 °C for 10 seconds, 60 °C for 25 seconds and 72 °C for 25 seconds and PCR amplification were monitored using fluorescence detection at 521 nm.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence</th>
<th>Pubmed. ID.</th>
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<td>β-actin Forward</td>
<td>CGA GCA TTC CCA AAG TTC TAC</td>
<td>AY141970-1349</td>
</tr>
<tr>
<td>β-actin Reverse</td>
<td>TTC CTG TAA CAA TGC ATT TCG</td>
<td>AY141970-1454</td>
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<tr>
<td>Aggrecan Forward</td>
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<td>U76615 - 4836</td>
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<tr>
<td>Aggrecan Reverse</td>
<td>GCT TGC TCC TCC ACT AAT GTC</td>
<td>U76615 - 4941</td>
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*Table 4.2* Bovine primer sequences

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</tr>
<tr>
<td>Sample</td>
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</tr>
<tr>
<td>H₂O</td>
<td>6.5</td>
</tr>
<tr>
<td>Sybr Green</td>
<td>10</td>
</tr>
</tbody>
</table>

*Table 4.3 RT-PCR Reaction mixture*
4.2.3.1 Preliminary biocompatibility studies

Prepared Fmoc-F$_2$ hydrogel (2 x 5 ml) was assembled using the method detailed in Section 3.2.3 and then disrupted by repeated pipette aspiration. Once in a liquid-like form, 20 µl of Fmoc-F$_2$ hydrogel were then transferred to the wells of a 96-well plate and incubated at 37 °C for 4 hours to allow re-assembly. The hydrogel coated wells and the relative controls where pre-equilibrated with 100 µl of high glucose DMEM + 10% FBS for 24 hour prior to cell seeding. Primary bovine chondrocytes (PBC) were then seeded at a density of 10,000 cells per cm$^2$ onto the pre-set hydrogels and tissue culture plastic controls were then incubated at 37 °C 5 % CO$_2$ for 24 hours. 10,000 cells per cm$^2$ was used in order to allow sufficient surface area of cell proliferation over study time course. In the case of the 3D culture, samples were prepared by disrupting the preformed hydrogel and mixing 10,000 cells per 100 µl of hydrogel. Hydrogels and controls were then incubated at 37 °C 5 % CO$_2$ for 24 hours with the addition of 1 ml of growth medium to each well after 2 hours. After 24 hours incubation cell viability was assessed using the Calcein-AM staining, MTS assay, Toluidine blue staining and lactate dehydrogenase (LDH) Assay. For calcein-AM treatment cells were stained using 2 µM calcein-AM 1mM stock in DMSO (Sigma, UK) prepared in the experimental culture medium to assess cell viability and morphology. Cells were incubated for a further 20 minutes and imaged using a Nikon eclipse te2000-u microscope. Florescence was achieved using a UV light source and images were acquired using 20 and 40 times magnification optics with a cannon 5D mounted camera.
4.2.4  *LDH assay*

The LDH assay, detects the levels of the enzyme LDH in a given solution. The LDH reading of a cell growth medium is relative to cell death during studied incubation time. As LDH is abundant inside all cells when the cell membrane integrity is lost during cell death the LDH is released. This LDH is detected through its reaction with tetrazolium salt to form the photo-detectable formazan product. The conversion is measured using a spectrophotometer allowing for an accurate quantification of LDH. Growth medium was aspirated from hydrogel containing wells and hydrogel samples were treated with 100 µl of lysis buffer (Promega, UK) and incubated for 30 minutes. 50 µl of the aspirated growth mediums and positive control lysis buffers was added to the wells of a 96 well plate and mixed with 50 µl of substrate solution. The plate was then incubated at room temperature protected from light for 30 minutes. After 30 minutes incubation, 50 µl of stop solution was added to each of the experimental wells. The sample absorbance was then read at 490 nm using a Biotek 96-well plate spectrophotometer. Background medium readings were subtracted from all experimental readings. The results were plotted as relative assay absorbance and percentage cell death of relevant control.

4.2.5  *MTS assay*

The MTS assay measures mitochondrial activity of viable cells by the measurement of the chemical reduction of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) in the presence of NADH and ATP. The reduction is measured by the light absorbance of
the end product of formazan at 490 nm wave length (Barltrop and Owen, 1991). The MTS assay was performed by the addition of 20 µl of MTS solution (Promega, UK) to six of the experimental wells per sample and incubated at 37 °C 5 % CO₂ for 2 hours. Following incubation, sample absorbance was measured at 490 nm using BioTek 96 well plate reader.

4.2.6 *Toluidine blue staining*

Toluidine blue O is a non-specific proteoglycan stain used in the histological assessment of tissues containing high levels of sulphated ECM proteins (Rosenberg, 1971). The staining was used to assess, in a qualitative manner, the production of ECM containing proteoglycans during cell culture. Following culture, cells were washed once with PBS and fixed with 3.7 % formaldehyde for 15 minutes. Following sample fixation, cells were washed with 1 x PBS and stained with 50 µl of 1mg/ml Toluidine blue O (Sigma, UK) in PBS for 5 minutes. The cells were then washed three times with PBS and imaged by light microscopy (Axiovert-25, Carl-Zeiss, Germany).

4.2.7 *Calcein-AM staining*

Calcein- AM is a chemical derivative of calcein that is a fluorophore capable of excitation in the visible spectrum using UV light once activated through breakage of it ester link. The breaking of the ester linkage occurs in metabolically active cells by intracellular esterases. Thereby staining the gross structure of living cells.

Calcein-AM staining was perform buy supplementing the culture medium with 2µM/L of Calcein-AM (Sigma, UK) in DMSO. Cells were then incubated in sufficient volume to cover growth surface and incubated for 20 minutes at 37 °C, 5%
CO₂, 95% atmospheric air. Cells were then imaged using a Nikon eclipse te2000-u microscope, Nikon.

4.2.1 *In vitro Fmoc-F₂ hydrogel leachate and degradation product cytotoxicity studies*

Hydrogel leachate were prepared by incubating 100 µl of 3 mg/ml Fmoc-F₂ hydrogel samples in 1 ml of DMEM + 10 % FBS for 24 hours at 37 °C, 5 % CO₂. Following incubation growth media were aspirated and either filtered through a 0.2 µm sterile filter to remove large gel fragments or were used without filtration. A cell density of 20,000 cells per cm² 3T3 cells were seeded in to the wells of a 96-well tissue culture plate and incubated in 50 µl of growth medium for 4 hours. 3T3 cells were selected as a standard cellular model for cytotoxicity as degradation products and leachates *in vivo* will affect not only chondrocytes as many other cells of the body that may also come in contact. Cell density was selected based on previous laboratory SOPs in order to provide sufficient growth space to allow proliferation over the study time course. Following incubation, growth medium was aspirated and replaced with 100 µl of control medium, filtered leachate or unfiltered leachate. Cells were then incubated for a further 24 hours at 37 °C, 5 % CO₂. Following incubation cells were stained with calcein-AM and viability analysis was tested using LDH and MTS assays.

Possible Fmoc-F₂ hydrolysis products outline in Section 4.1.1 were prepared in serial dilutions in DMEM growth medium supplemented with 10 % FBS. Dose ranges were prepared to include the maximum calculated possible release from 3 mg/ml Fmoc-F₂ hydrogel assuming 100 % hydrolysis within 24 hours (75 µg/ml – 9-flu and 82 µg/ml - phenylalanine). 3T3 mouse fibroblast cells were cultured in DMEM
growth medium supplemented with 10 % FBS until 80% confluent and passaged using 5 ml of 1 x trypsin (Sigma, UK) and collected by centrifugation at 300 x g for 5 minutes. Cells were counted using a haemocytometer and seeded at a density of 20,000 cells/cm² into the required wells of a 96-well plate. The cells were incubated for 4 hours at 37 °C 5% CO₂. After 4 hours of incubation, cells were washed and treated with experimental growth medium within the range of degradation product doses detailed in Table 4.4. The cells were then incubated at 37 °C 5% CO₂ for 48 hours. Following incubation cell viability was assessed using MTS assay and live cell imaging using Calcein-AM staining (section 4.2.4).

<table>
<thead>
<tr>
<th>Degradation product</th>
<th>Dose conc. (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(High-low)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1280 640 320 160 80 40 20 10 5 1</td>
</tr>
<tr>
<td>9-Chloroacetic acid</td>
<td>1250 625 312.5 156.25 78.12 39 19.53 9.76 4.38 2.44 1.22</td>
</tr>
</tbody>
</table>

*Table 4.4 Concentrations of degradation products*
4.2.2  *Statistical analysis*

All data sets that were found to be normally distributed were analysed using two-tailed Tukey’s t-test analysis unless otherwise stated in text. Significance was determined using a significance level of 0.05 ie. Values greater 0.05 were not considered to be statistically significant. All statistical analysis was performed using PRISM. Where multiple comparisons where required two-way ANOVA was used with post-hoc Turkey’s t-test analysis.
Results

4.2.3 Serum protein adsorption study

The results of the serum protein adsorption study showed a significantly lower concentration of total serum proteins bound to the hydrogel in comparison to TCP after 1 hour incubation (\(p<0.05\)) (Figure 4.4). The total protein concentration found in the hydrogel after 1 hour incubation was 0.87 µg/ml, a large reduction when compared to the TCP control which was found to have a 10 times greater protein concentration bound to its surface (10.37 µg/ml). However, after 24 hours the hydrogel had a significantly higher adsorption of protein (15 µg/ml). This finding could indicate that the serum proteins have higher affinity for the hydrogel nanostructure than that for TCP. However, this is not necessarily the case as the exposed surface area of the fibres is bound to be much higher than the TCP making these values not truly comparable.

![Figure 4.4](serum_protein_adsorption.png)

**Figure 4.4** Serum protein adsorption to Fmoc-F₂ hydrogel – Bradford assay protein conc. readings of total protein adsorbed to Fmoc-F₂ hydrogel at 1 and 24 hours incubation. Comparison was performed with 24 hour TCP control (\(*P<0.05\)) (n=3)
The specific serum proteins adsorbed to the surface of each material were compared using SDS-page separation. The results of the separation shown in Figure 4.5 indicate the clear differences in the types of proteins adsorbing on the two surfaces. Fmoc-F₂ hydrogel adsorbed only albumin at 67 kDa after 1 hour incubation, while control TCP has adsorbed the full range of serum proteins. While the only clearly visible protein band on Fmoc-F₂ hydrogel at 1 hour incubation is in the molecular weight range of albumin (Putnam, 1984), there were also very slightly stained bands indicating the trace adsorption of proteins of higher molecular weight. These were observed during the visual inspection of the SDS-PAGE, but not well captured in the recorded images. Conversely, following 24 hour incubation of the hydrogel with serum, there was a large increase in a wide range of serum proteins. Transferrin and heavy Immunoglobulin (IG) chains were also identified as clearly distinct bands (Figure 4.5). Densitometry analysis of albumin found there to be significantly less albumin bound to Fmoc-F₂ hydrogels after 1 hour incubation when compared to TCP control samples (p=0.00051).
Figure 4.5 SDS-PAGE gel image of serum protein adsorption to Fmoc-F₂ hydrogel

Hydrogel samples (n=3) were incubated for 24 hours at 37 °C, 5% CO₂ in 5 ml of F-12K growth medium (PAA) supplemented with 10 % (v/v) foetal bovine serum (FBS) (PAA). To remove the adsorbed proteins, the wells were then treated with 200 µl of 10 % (v/v) SDS in PBS for 20 minutes. Samples were analysed using Bradford assay reagent to quantify total protein and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Identified proteins: Transferrin – 78 KDa, Albumin – 67 KDa, Ig heavy chains – 57 KDa.
Figure 4.6 Albumin adsorption to Fmoc-F$_2$ hydrogel – albumin densitometry reading from SDS-PAGE separation of bound serum proteins. TCP samples were found to significantly higher levels of albumin bound at 1 hour than the Fmoc-F$_2$ hydrogel ($p=0.00052$). 24 hour samples were not found to be significantly different between material groups ($p=0.32$) ($n=3$).
4.2.4 RT-PCR analysis of extracted primary bovine chondrocytes

The results from RT-PCR gene expression analysis revealed the positive amplification of aggecan cDNA in the isolated PBC cells (Figure 4.7). Ct value for aggecan were found to be 17.42, ± 0.31 (SD) while the β-actin amplification had a Ct value of 19.10, ± 0.23 (SD) Statistical analysis using tukey’s t-test found there to be a significantly greater aggecan expression than β-actin in the isolated cell population (p= 0.00051).

Figure 4.7 RT-PCR amplification cycle plot of aggecan and β-actin expression in isolated PBCs. Samples were run for 40 cycles of 95 °C for 10 seconds, 60 °C for 25 seconds and 72 °C for 25 seconds and PCR amplification were monitored using fluorescence detection at 521 nm. aggecan Ct value: 17.42, ± 0.31 (SD) β-actin Ct value of 19.10, ± 0.23 (SD) Statistical analysis using tukey’s t-test found there to be a significantly greater aggecan expression than β-actin in the isolated cell population (p= 0.00051). blue and purple represent aggrecan cDNA amplification, yellow and red represent β-actin cDNA amplification (n=3).
4.2.5 2D primary bovine chondrocyte biocompatibility study

The results of the hydrogel biocompatibility study and in particular the Calcein-AM, showed that PBCs are viable when cultured on the Fmoc-F₂ hydrogel both in 2D and 3D conditions (Figure 4.8, Figure 4.9). However, cells cultured on hydrogels preconditioned with serum-enriched medium for 1 hour showed reduced cell size (0.009×10)(Figure 4.8-B & 4.9). Furthermore, a significantly greater cell death was detected by LDH assay (Figure 4.10). The cells treated grown on the 24 hour treated hydrogels demonstrated a significant difference in cell morphology between cells cultured in 3D and 2D. Cells cultured in 3D presented a spherical morphology and were significantly smaller size by surface area (p=0.001×5) (2.2 μm² ± 0.6 SD). While cells grown in 2D had a fibroblastic spindle like morphology similar to that of PBC cells grown on tissue culture plastic with a surface area of 13.8 μm² ± 5.4 SD (Figure 4.8).
Figure 4.8 Calcein-AM staining of PBC cells cultured on Fmoc-F₂ hydrogel (x20 magnification) - A-control PBC (TCP) 1 hour pre-treatment, B-2D PBC culture hydrogels – 1 hour medium incubation, C-2D PBC culture hydrogels – 24 hour medium incubation, D - 3D PBC culture in Fmoc-F₂ hydrogel. Cells were cultured for 24 hours at 37 °C, 5% CO₂ (n=3)
**Figure 4.9** Chondrocyte cell size cultured in 2d and 3D Fmoc-F₂ hydrogel: PBC cells were cultured on Tissue culture plastic (TCP), or 2D hydrogel treated for 1 hour or 24 hours with DMEM supplemented with 10% FBS and in 3D in 1 hour pre-treated Fmoc-F₂ hydrogel. Cell measurements of surface area were measured using Image J software, a total of 21 cells per sample in randomly acquired fields were measured and averaged ±SD (n=3). (*=p<0.05)
Quantitative assessment of cell viability for the 1 and 24 hour pre-treatments were performed by LDH assay and the findings supported the calcein-AM staining data, with no significant difference in cell death found between cells cultured on TCP control or Fmoc-F₂ hydrogel after 24 hour pre-treatment by T-test analysis (p=0.093) (Figure 4.10). However, cells cultured on hydrogels pre-treated for just 1 hour had a significantly higher percentage cell death at 7.33 % ± 0.184 (Figure 4.10).

![Figure 4.10 PBC viability on Fmoc-F₂ hydrogel - LDH cell viability assay - 24 hours incubation Bovine chondrocytes cells were seeded at 10,000 cells per cm² and incubated for 4 hours. Cells were treated with 24 hour leachate medium both filtered and unfiltered and incubated for 24 hours at 37 °C 5% CO₂, 95% atmospheric air. Cells supernatants were then tested using the LDH assay and levels were compared to 100 % lysis controls and background media (n=3) statistical analysis was performed using tukey’s t-test analysis with control for comparison (*p= 0.0018)
4.2.6 Hydrogel leachate cytotoxicity study

The findings of the leachate cytotoxicity studies revealed no observed effect on cell morphology (Figure 4.11) or viability in comparison to TCP control (Figure 4.112 and 4.113) (filtered – p=0.22, unfiltered – p= 0.109). LDH assay reading showed no significant release above that of the untreated controls and readings for both filtered and unfiltered leachate were below 5 % cell death value. Cell morphology in all samples was found to be a mixture of spread and spherical cells indicating a mixed phenotype population. Analysis of cell morphology found there to be no significant difference between mean cell sizes between samples when compared to control (filtered – p=0.96, unfiltered – p= 0.87).

**Figure 4.11 24 hour hydrogel Leachate PBC biocompatibility Calcein-AM staining (x10 magnification) -**

- **A**-control medium,
- **B**-Filtered leachate,
- **C**- leachate.

Bovine chondrocytes cells were seeded at 10,000 cells per cm² and incubated for 4 hours. Cells were treated with 24 hour leachate medium both filtered and unfiltered and incubated for 24 hours at 37 °C 5% CO₂, 95% atmospheric air. Cells were stain with 2µM calcein-am solution and imaged using fluorescence microscopy. (n=3)
Figure 4.12 24 hour PBC hydrogel leachate LDH cytotoxicity assay- Bovine chondrocytes cells were seeded at 10,000 cells per cm\(^2\) and incubated for 4 hours. Cells were treated with 24 hour leachate medium both filtered and unfiltered and incubated for 24 hours at 37 °C 5% CO\(_2\), 95% atmospheric air. Cell culture media was aspirated and tested using LDH assay, reading were recorded by spectrophotometer readings using a 96 well plate reader. No statistical difference was found between control and leachate samples (filtered – p=0.22, unfiltered – p=0.109) (n=3)

MTS assay results revealed a small reduction in cellular metabolic activity, an indicator of cell growth, in cells contacting unfiltered supernatants (Figure 4.13). However, this reduction was not found to be significant by T-Test analysis when compared with control (filtered – p=0.66, unfiltered – p=0.52). Additional observations during culture revealed the presence of gel small fragments in the unfiltered leachate samples at 0 hours incubation. Gel fragments were not visible after 24 hours, suggesting possible further degradation of the fragments and/or their consumption through cell metabolism.
Figure 4.13 24 hour PBC hydrogel leachate MTS viability assay. Bovine chondrocytes cells were seeded at 10,000 cells per cm$^2$ and incubated for 4 hours. Cells were treated with 24 hour leachate medium both filtered and unfiltered and incubated for 24 hours at 37 °C 5% CO$_2$, 95% atmospheric air. Cells were treated with 10% v/v MTS assay solution and incubated for 2 hours. Mitochondrial activity was recorded by spectrophotometer readings using a 96 well plate reader. Statistical analysis samples V control found no significance by tukey’s t-test analysis (filtered – $p=0.66$, unfiltered – $p=0.52$) (n=3).
4.2.7 Degradation component in vitro cytotoxicity studies

The results of the degradation product studies revealed a strong dose-response toxicity of 9-flu at concentrations exceeding 78.1 µg/ml (Figure 4.14). A reduction in cell spreading was seen at concentrations greater than 78.1 µg/ml, with a clear reduction in observed cell adhesion at concentrations above 625 µg/ml (Figure 4.15). The formations of crystals at the two highest concentrations were observed which may have contributed to changes in cell adhesion (Figure 4.15 – image C). However, no cytotoxic effect was seen at 78.1 µg/ml or lower concentrations, with significant toxicity at 156.3 µg/ml (p=<0.05).

Figure 4.14 Dose toxicity curve of degradation product 9-flu. 3t3 mouse fibroblast cells were seeded at 10,000 cells per cm² and incubated for 4 hours. Cells were treated with a range of 9-flu concentrations ranging from 0-1250 µg/ml and incubated for 24 hours at 37 °C 5% CO₂, 95% atmospheric air. Cells were treated with 10% v/v MTS assay solution and incubated for 1 hour. Mitochondrial activity was recorded by spectrophotometer readings using a 96 well plate reader. (n=3)
Figure 4.15 Light microscopy of 3T3 murine fibroblast morphology 9-flu dose curve, (x20 magnification) A)-control, B)-78.1 µg/ml, and C) 1250 µg/ml. Cells were treated with a range of 9-flu concentrations ranging from 0-1250 µg/ml and incubated for 24 hours at 37 °C 5% CO₂, 95% atmospheric air. (n=3)

As reported in literature, the concentration range of phenylalanine used in this study was found to have no cytotoxic effect on the 3T3 cells. Conversely, the presence of elevated levels of phenylalanine was found to increase significantly the metabolic activity of the cells in doses above 80µg/ml (Figure 4.16) (p=0.033).

Figure 4.16 Dose toxicity curve of degradation product Phe with 3T3 cells. Cells were treated with a range of Phe concentrations ranging from 0-1280 µg/ml and incubated for 24 hours at 37 °C 5% CO₂, 95% atmospheric air. Cells were treated with 10% v/v MTS assay solution and incubated for 2 hours. Mitochondrial activity was recorded by spectrophotometer readings using a 96 well plate reader. (n=3)
4.3 Discussion

The findings of the serum protein adsorption study revealed a tendency of the microfiber surface to minimise total protein adsorption. In addition, the analysis of the protein species adsorbing with the Fmoc-F2 nanofibre surface showed a prevalent albumin adsorption after 1 hour pre-treatment. The adsorption of albumin to a biomaterial surface has been identified in numerous publications to actively block cell adhesion to a given material surface (Curtis and Forrester, 1984). However, 24 hour pre-treatment of hydrogels revealed a time dependent increase in total protein adsorption that involved other types of proteins mainly of high molecular weight, the overall pattern being more similar to that found on TCP. The results show that there is a strong link between cell morphology and serum protein adsorption to the material. The findings from these studies may be explained by the Vroman effect, in which different serum proteins bind to the surface of materials in a time dependant manner. The findings of the work by Vroman et al, 1980 found albumin commonly binds to materials at short time exposure to serum. However, other proteins such has fibronectin bind after longer incubations, fibronectin known to facilitate cellular adhesion (Vroman et al, 1980). This effect as demonstrated by the results of this chapter could like play a critical role in the cell biocompatibility of the material and govern the time related pre-treatment effect observed. The findings support the ability of Fmoc-F2 hydrogels to allow the adhesion and growth of chondrocyte phenotypes as reported by (Jayawarna et al., 2009) this ability may be linked to specific integrin/bioligand interactions. In the paper by Jayawarna et al., 2009, PBCs were found to adhere and grow in 2D culture studies, whereas 3T3 fibroblast cells were unable to attach and grow on the hydrogel (Jayawarna et al., 2009). Previous studies found that 3T3 fibroblasts required fibronectin-material interaction for
functional cell adhesion (Horbett and Schway, 1988). From the results of the protein adsorption study only albumin was found to adsorb to the hydrogel after 1 hour. These findings therefore provide a possible explanation for the poor substrate properties that result in limited adhesion and growth of fibroblasts in this study.

The results of the 2D PBC biocompatibility studies revealed good cell adhesion with a cell morphology similar to that of chondrocytes grown on control TCP. While this finding was not reported in the recently published abstract by Smith et al. (2011), the previous publication by (Jayawarna et al., 2009) reported cell spreading consistent with normal TCP culture. However, the spreading of the PBC on these surfaces also suggested a process of de-differentiation of the cells that are known to have a spherical morphology when in a differentiated state (Benya and Shaffer, 1982). This was indeed achieved when PBCs were entrapped in the self-assembled hydrogels suggesting the suitability of these gels to act as chondrocyte carriers in cartilage tissue engineering. The results of the cell surface area analysis reveal a significantly lower surface area in cells growth on the hydrogel after 1 hour pre-treatment and cell growth in 3D after 24 hours treatment. This suggests that cells may have reverted to the differentiated phenotype in response to the poor cell substrate properties of the Fmoc-F\textsubscript{2} hydrogel.

From the findings of both 2D and 3D studies on Fmoc-F\textsubscript{2} hydrogels it was shown that there was good biocompatibility in both conditions. However, the cell-biomaterial interaction was closely linked to the adsorption of proteins. Indeed, on hydrogel pre-treated with medium for just 1 hour prior to seeding where prevalent albumin adsorption occurs, reduced cell morphology and adhesion was observed by calcein-AM staining (Figure 4.8). Furthermore, the results of LDH assay found a significant increase in cell death with just 1 hour hydrogel pre-treatments. Although
this level was within the acceptable ISO cytotoxicity guideline limit of 30\% (International-Standards, 2009), the findings show no significant cytotoxicity once the hydrogel has been pre-treated for 24 hours (Figure 4.9). These data would suggest that the later adsorption of serum proteins ensures substrate conditions suitable for cell growth and survival. Given the biodegradable nature of the hydrogels, leachate studies were conducted in order to ascertain if any short-term leachate products could cause potential toxicity to cells. The investigation of hydrogel leachate cytotoxicity revealed no significant effect on cell morphology, viability or metabolic activity (Figures 4.11, 4.12 and 4.13). This result demonstrated the biocompatibility of Fmoc-F\textsubscript{2} hydrogel release products that occur during short-term culture. Furthermore, the presence of small fragments of hydrogel observed at early incubation times where not visible after 24 hour culture, thus suggesting that the hydrogel fragments may have been metabolised by the cells or an enzymatic product released by the cells. This observation suggests that Fmoc-F\textsubscript{2} hydrogel may be bio-resorbed over time without harmful effect to cells. This property may be highly beneficial to both in vitr\textit{o} and in viv\textit{o} concepts, allowing for the cells to replace the hydrogel matrix with their own native ECM overtime. Furthermore an important factor to consideration is that of the culture medium effect, as osmotic pressure has been found to significantly affect ECM molecule production (Negoro \textit{et al}, 2008) and cell volume (Bush & Hall, 2001). If osmolarity of too high or two low this will significantly affect cell behaviour. The rational for the use of DMEM in these studies was to keep consistence with existing literature for comparison with Fmoc-F\textsubscript{2} hydrogel biocompatibility studies. DMEM used has an osmolarity of 330-350 mOsm, greater than that of cartilage, however, the study by Negoro \textit{et al}, 2008 found optimal conditions of aggrecan synthesis to be at 370 mOsm (Negoro \textit{et al},
Furthermore the work of Bush et al, 2005 found there was no significant effect of osmolarity on cell viability (Bush et al, 2005).

Theoretical degradation product cytotoxicity was investigated in order to determine if any potential long-term degradation by-products would pose any potential cytotoxic effects on general cell types, such as fibroblasts. The theoretical long-term degradation products of gel hydrolysis, phenylalanine and 9-flu, where tested in using a dose response curve method. The results found no cytotoxicity of phenylalanine by MTS assay within the release range (Figure 4.15). Furthermore, phenylalanine was found to significantly increase cell proliferation which could be potentially beneficial to the tissue formation. However, significant cytotoxicity was found with 9-flu at concentrations above 156 µg/ml (Figure 4.14). However, the maximum release value of the hydrogel was calculated to be 75 µg/ml assuming 100% degraded over a 24 hour period. Therefore a risk of toxicity from this potential release product is not likely to occur in vitro or in vivo.

4.4 Conclusions

In conclusion, the prepared Fmoc-F₂ hydrogel has been found to possess a good degree of biocompatibility with relevant cell types. While the degradation products of hydrolysis are not toxic at the concentrations relevant to the hydrogel preparation, 9-flu was found to have a significantly toxic effect at higher concentrations, a finding not yet observed or reported in literature. However, from the findings of biocompatibility from there is sufficient evidence to warrant the further study and investigation of this materials ability to support cell growth and tissue formation for regenerative medicine applications.
Chapter 5: *In vitro* investigation of IGF-1 peptide for the treatment of IVDD
5.1 Introduction

The role of biological factors in the progression of IVDD has been shown from literature to be central to the degeneration of the NP (Zhao, 2007). As a result, in order to control and treat IVDD, the disruption of the biological pathway of degeneration is an essential in order to treat this condition. IGF-1 plays an important role in cartilaginous tissue homeostasis particularly in the maintenance of the ECM (Firth and Baxter, 2002). However, its use as a therapeutic agent for the treatment of IVDD is likely to be ineffective, due to the up-regulation of IGF-BPs (Zhou et al., 2009) that at high levels inhibit the activity of IGF-1 (Hwa et al., 1999). Therefore, as previously discussed in Chapter 1, IGF-1 regulatory proteins might be therapeutically bypassed by the use of the IGF-1 C active region site of the peptide (sequence GYGSSSRRAPQT). In this chapter, this sequence was identified and synthesised to provide a bioactive molecule able to stimulate tissue regeneration in combination with self-assembly Fmoc-F₂ hydrogels. Indeed, the bioactivity of this peptide does not appear to have yet been reported in literature. It is hypothesised that the active sequence may elicit a similar response to that of the native IGF-1 protein, in the up-regulation of ECM components such as collagen type II and aggrecan. Noticeably, IGF-1 has been found to have a proliferative effect not only on chondrocytes but also on other cell types such as the human mesenchymal stem cells (Huang et al., 2012). The ability of the IGF-1 protein to elicit a proliferative response in various cell types provides a useful manner of measuring the efficacy of the produced sIGF-1 peptide. Increase in cellular proliferation has a direct link to cell metabolic activity through increased cellular respiration (Cory et al., 1991).
5.1.1 Cell sources and potential use in IVD regeneration

In evaluating the efficacy of the produced sIGF-1 peptide, a cell model that will reflect the clinical situation should be used in order to test different factors. In the case of IVDD, the cells of specific interest are chondrocyte NP cells. Although human IVD chondrocytes would provide a good model for the study of regenerative medicine strategies, obtaining human NP chondrocytes in quantities sufficient for clinical application is extremely difficult. In consideration of this it could be argued that different cell sources should be considered for harvesting for their use in intervertebral disc regeneration. As a result, many research groups are currently focusing on the use of host derived stem cells or autologous chondrocytes that can be harvested without causing donor’s morbidity. Currently, there has been a lot of research focusing on the expansion and differentiation of human MSC’s into chondrocyte-like cells. While there has been some success, namely the work of Xu et al (2007), the issue of controllable cell expansion in order to obtain a sufficient number of cells for tissue engineering or injectable delivery is still one of the current limitations (Xu et al., 2007). However, while autologous chondrocytes are used in clinical research, the expansion of these cells is well documented to result in the significant loss of chondrocytic phenotypic expression (Brodkin et al., 2004). However, from a number of studies elements of de-differentiation, with regards to expression can be achieved with 3D culture of cells in a number of different materials (Benya and Shaffer, 1982, Talukdar et al., 2011). While, this work demonstrates that, given correct environmental cues, chondrocytes have the potential to revert to their native cell expression, this is a rather difficult process to be triggered. Namely, full restoration of native chondrocyte phenotype both in terms of expression and cellular morphology has yet to be demonstrated (Benya and Shaffer,
1982). While the highlighted studies utilise biomaterials to create a 3D environment in which it is hoped cells will elicit the desired expression of specific markers, the findings of the study by Acosta et al., (2006) illustrate the importance of the biological and chemical cues which fundamentally affect marker expression regardless of the ECM environment in which cells are integrated. Indeed, the cellular environment of the NP of a healthy IVD is arguably perfect for the regeneration of its nucleus pulposus, as they have been shown to be unable to prevent the phenotypic changes which occur during degeneration (Zhao, 2007). As discussed previously in Section 1.2.2, these changes have been found to be mainly influenced by the release of inflammatory mediators which in turn drive the up-regulation of factors deleterious to phenotypic expression and ECM regulation (Le Maitre et al., 2005a).

In consideration of these findings that emerge from a review of the literature, it is the investigator’s view that future tissue engineering and regenerative medicine approaches will require a combination of biocues in addition to the availability of a suitable synthetic biomaterial. For this reason, the identification of the sIGF-1 peptide sequence and its testing as a synthetic analogue capable of eliciting a biological response in clinically-relevant cell types is an important step forward to make Fmoc-F$_2$ hydrogels suitable as injectable scaffolds for *in vivo* IVD regeneration.
5.1.2 Chapter aims

This chapter aims to investigate the potential biological effects of the produced sIGF-1 peptide on chondrocytes and human MSCs. This aim is to be achieved through a range of proliferative studies to measure the possible responses in terms of GAG production and cell metabolism.

Specific aims

1. Study the effect of the sIGF-1 peptide on PBC proliferation
2. Study the effect of the sIGF-1 peptide on PBC phenotypic expression
3. Study the effect of the sIGF-1 peptide on hMSC proliferation
4. Study the effect of the sIGF-1 peptide on hMSC phenotypic expression
5.2 Materials and methods

5.2.1 *In vitro study of IGF-1 peptide bioactivity in primary bovine chondrocytes*

P1 Primary bovine chondrocytes isolated using the method described in Section 4.2.2 were seeded at a cell density of 10,000 cells per cm$^2$ in 96 well TC plates (Corning, US) and incubated for 4 hours in DMEM culture medium supplemented with 10% FBS at 37 °C, 5% CO$_2$ and 95% atmospheric air. Following 4 hour incubation, the culture medium was changed and supplemented with a serial dilution of IGF-1 peptide ranging from 0 to 1000 ng/ml. Doses were added to appropriate wells and cells were culture for an additional incubation time of either 24 hours or 48 hours. Experiments were performed in 8 wells per treatment variable and repeated to obtain n=3. Following incubation cells were either stained with 2 μM of calcein-AM solution or cell medium was aspirated and replaced with control medium supplemented with 10% (v/v) MTS solution and incubated for a further 2 hours at 37 °C, 5% CO$_2$ and 95% atmospheric air. Samples stained with calcein-AM solution (see section 4.2.3) were imaged using fluorescence microscopy, using a UV light source with x 20 and x 40 magnification optics (Nikon eclipse te2000-u microscope, Nikon). Following MTS incubation experimental plates were read using a spectrophotometer plate reader (Biotek, UK) at 490 nm. Readings were expressed as relative absorbance (ABS).
5.2.2 Synthetic IGF-1 peptide effect on chondrocyte GAG production

P1 primary bovine chondrocytes were seeded at a cell density of 50,000 cells per cm² in 24 well plates and incubated for 24 hours at 37 °C, 5% CO₂ and 95% atmospheric air in high glucose DMEM supplemented with 10% FBS with or without the addition of 1000 ng/ml of the synthesised IGF-1 peptide. Following incubation cell medium was aspirated and cells were passaged using 200 µl of trypsin and incubated at 37 °C, 5% CO₂ and 95% atmospheric air for 10 minutes, cell suspension was then neutralised with 400 µl of culture medium and collected by centrifugation at 500G for 5 minutes. Cells were then counted using a haemocytometer to obtain relative cell numbers of GAG assay comparison. Dimethyl-methylene Blue (DMB) (GAG assay) solution was prepared using the protocol reported in Table 5.1 (Barbosa et al., 2003). Aliquots of DMB solution (250 µl) were transferred to the wells of a 96-well plate. Cell supernatant samples (40 µl) were then added to the wells containing DMB solution. Solution absorbance was read for absorbance at 590 nm using a micro-plate reader (BioTek, UK). Absorbance values were converted to concentration values using a stand curve slope-intercept equation (y=mx+c) (See appendix 8.2)
<table>
<thead>
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<th>Reagent</th>
<th>Quantity</th>
</tr>
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<tr>
<td>Distilled water</td>
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</tr>
<tr>
<td>1,9-Dimethyl-methylene Blue (DMB)</td>
<td>0.016 g</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Sodium formate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Formic acid</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

**Table 5.1 GAG assay (DMB Solution) preparation**

5.2.3 *In vitro study of IGF-1 peptide bioactivity in primary human stem cells*

P3 Primary Human bone marrow derived mesenchymal Stem cells’s (hMSC) (Lonza, UK) were seeded at a cell density of 7,000 cells per cm² in 96-well TC plates (Corning, UK) and incubated for 4 hours in Lonza Stem cell medium supplemented with chemically-defined human serum (Lonza, UK) at 37 °C, 5% CO₂ and 95% atmospheric air. Following 4 hour incubation culture medium was changed and Lonza medium supplemented with serial dilution doses of IGF-1 peptide ranging from 0 to 200 ng/ml was added to appropriate wells and the cells were incubated at 37 °C, 5% CO₂ and 95% atmospheric air for a further 24 or 48 hours. Experiments were performed in 8 wells per treatment variable and repeated to obtain n=3. Following incubation at the different time points, cell medium was aspirated and replaced with control medium supplemented with 10% (v/v) MTS solution and incubated for a further 2 hours at 37 °C, 5% CO₂ and 95% atmospheric air. Following MTS incubation experimental plates were read using a spectrophotometer plate reader (BioTek, UK) at 490 nm, readings were expressed as relative absorbance values minus background controls (ABS). Cell samples were also stained using HPI
for cell counting and immunologically stained using antibodies against Stro-1 and Nanog proteins.

5.2.4 *Immunocytochemistry*

Stem cell phenotype was confirmed by immuno-staining of typical markers of multipotency, Nanog and STRO-1. Nanog is a transcription regulator expressed in pluripotent stem cells (Chang *et al.*, 2009). Cells were fixed with 200 µl of 3.7 % (v/v) formaldehyde for 15 minutes and then washed 3 times with 1 ml of 1 % (v/v) BSA in PBS. Cells were then incubated in 200 µl of 1% BSA in PBS as blocking solution for 2 hours at room temperature. Following incubation, solutions were aspirated from cells. Primary antibodies for Stro-1 (Mouse Anti-Human Stro-1 IgG, Sigma UK) and Nanog (Mouse Anti-Human Nanog IgG, Sigma UK) were re-suspended in 1% BSA in PBS at 1:200, and 1:100 dilutions respectively and 200 µl of each were added to the pre-treated cell samples. The cells were incubated for 4 hours at room temperature. Following incubation, samples were washed 3 times with 1 ml of 1 % BSA in PBS and treated with Rabit anti-mouse IgG secondary antibody conjugated with FITC (green) (Stro-1) and Goat anti-mouse IgG secondary antibody conjugated with Rhodamine (red) (Nanog) (Sigma, UK) for 4 hours at room temperature. Samples were then washed 3 times with 1 ml of 1% BSA in PBS and imaged using an eclipse te2000-u, Nikon florescence microscope.
5.2.5 *Statistical analysis*

All data sets that were found to be normally distributed were analysed using two-tailed Tukey’s t-test analysis unless otherwise stated in text. Significance was determined using a significance level of 0.05 ie. Values greater 0.05 were not considered to be statistically significant. All statistical analysis was performed using PRISM. Where multiple comparisons where required two-way ANOVA was used with post-hoc Turkey’s t-test analysis.
5.3 Results

5.3.1 IGF-1 peptide effect on primary bovine chondrocyte growth

The dose curve effect of the sIGF-1 peptide over 24 hours on PBC cell metabolism revealed a positive effect on cell proliferation with all doses greater than 25 ng/ml producing a significant increase in cell proliferation when compared to the untreated control (25 ng/ml - p=0.0001). While 12.5 ng/ml was found to have a greater mean value than that the control but was not statistically significant by T-test analysis (p=0.64) The optimum dose was found to be 100 ng/ml producing a 17.14 % ± 5.4 (p=0.00014) increase in cell proliferation. While, 500 ng/ml was found to elicit the greatest increase in cell proliferation by 19.24 % ± 2.3 (p=0.008) increase. Cell morphology imaging was achieved through calcein-AM staining and showed no significant change in cell size by surface area with the addition of 1000 ng/ml of sIGF-1 peptide (p=0.962). This finding suggest that the mixed cell population of different phenotypes has not shifted as a result of IGF-1 treatment (Figure 5.2). Cell morphology in all treatments and controls were found to consist of a mixture of spherical, partially spread and spindle-like cells with a fibroblastic cell morphology, consistent with the early stages of PBC 2D culture de-differentiation. While cell morphology was not notably changed, cells treated with IGF-1 peptide were seen in greater numbers consistent with that of the findings from MTS proliferation assay results (Figure 5.1).
Figure 5.1 Effect of IGF-1 peptide on Primary bovine chondrocyte proliferation (24h) – PBC cell were seeded at 10,000 cell per cm\(^2\) in 96 well plates and incubated for 24h at 37 °C, 5 % CO\(_2\) & atmospheric air in high glucose DMEM supplemented with 10% FBS with a dose range of 0-1000 ng/ml of synthetic IGF-1 peptide. Following cell incubation cells were incubated for 2 hours in 10% MTS solution, absorbance was recorded at 590 nm using a micro plate reader. (*= p<0.05)(n=3)

Figure 5.2 Effect of IGF-1 peptide on Primary bovine chondrocyte morphology – PBC cell were seeded at 10,000 cell per cm\(^2\) in 96 well plates and incubated for 24h at 37 °C, 5 % CO\(_2\) & atmospheric air in high glucose DMEM supplemented with 10% FBS with and without and addition of 1000 ng/ml synthetic IGF-1 peptide. Following incubation cells were stained with 2 µM of calcein-AM and imaged by fluorescence microscope. A-Control (TCP), B-1000 ng/ml synthetic IGF-1 peptide. (n=3) no significant size difference was found between treatments by t-test analysis (p=0.962)
The dose curve effect of the sIGF-1 peptide over 48 hours on PBC cell metabolism revealed a positive effect on cell proliferation with all doses greater than 12.5 ng/ml producing a significant increase in cell proliferation similar to that of 24 hours, with 100 ng/ml being the optimum dose eliciting an 18.85 % increase in cell proliferation.

Figure 5.3 Dose effect of IGF-1 peptide on PBC proliferation (48h) – PBC cell were seeded at 10,000 cell per cm² in 96 well plates and incubated for 48h at 37 °C, 5 % CO₂ & atmospheric air in high glucose DMEM supplemented with 10% FBS with a dose range of 0-1000 ng/ml of synthetic IGF-1 peptide. Following cell incubation cells were incubated for 2 hours in 10% MTS solution, absorbance was read at 590 nm using a micro plate reader. (*= p<0.05) (n=3)
The results of the GAG production study found there to be a significant increase in Medium GAG concentration with the addition of 1000 ng/ml of sIGF-1 peptide. GAG production was found to be 23.16 µg/ml per 100,000 cells for untreated cell samples, while cells treated with 1000 ng/ml of sIGF-1 peptide produced an average of 30.11 µg/ml of GAG per 100,000 cells, a 30% increase in GAG production (Figure 5.4)

*Figure 5.4 Effect of IGF-1 peptide on PBC GAG production (24h) – PBC cell were seeded at 50,000 cell per cm² in 96 well plates and incubated for 24h at 37 °C, 5 % CO₂ & atmospheric air in high glucose DMEM supplemented with 10% FBS with and without and addition of 1000 ng/ml Synthetic IGF-1 peptide. (= p<0.05)(n=3)*
5.3.2 *IGF-1 peptide effect on human mesenchymal stem cell growth*

The findings from the sIGF-1 peptide dose response study on hMSCs revealed a significant increase in hMSC proliferation with the addition of all experimental doses tested. Similar to the findings of PBC dose-response study, the optimum dose was found to be 100 ng/ml. However, the response curve was found to have a different trend with a greater response at lower doses. With 12.5 ng/ml eliciting a 41.3% increase. Whereas the 100 ng/ml produced a 59.8 % increase in cell proliferation (Figure 5.5).

*Figure 5.5 Dose effect of IGF-1 peptide on hMSC proliferation (5 days) – PBC cell were seeded at 7,000 cell per cm² in 96 well plates and incubated for 5 days at 37 °C, 5 % CO₂ & atmospheric air in high glucose DMEM supplemented with 10% FBS supplemented with a dose range of 0-200 ng/ml of synthetic IGF-1 peptide. Following cell incubation cells were incubated for 2 hours in 10% MTS solution/culture medium, absorbance was read at 590 nm using a micro plate reader. (*= p<0.05) (n=3)*
Results of HPI cell viability staining revealed cells to be viable and in greater numbers with the treatments of both hIGF-1 and sIGF-1 peptide after 5 days. HPI staining found no necrotic cells in any of the experimental samples. From HPI staining cell counts performed and the data has been expressed in Figure 5.6. The results of statistical analysis by T-test comparison with controls found both the IGF-1 and sIGF-1 peptide to significantly increase cell proliferation after 5 days. However, no significant difference was seen at 24 hours culture time. Direct comparison between treatments revealed no significant difference between the full hIGF-1 protein and the sIGF-1 peptide in proliferative effect at equal molarity (Figure 5.7).

Figure 5.6 Effect of synthetic IGF-1 peptide of hMSC viability (5 days). A-control, B-IGF-1 peptide, C-IGF-1full protein. P3 hMSC’s were seeded at a density of 7,000 cells per cm² and incubated with human mesenchymal stem cell medium (Lonza) medium supplement with either 13.2 nM of synthetic IGF-1 peptide or human IGF-1 protein. Cells were incubated for 5 days at 37°C, 5 % CO₂ & atmospheric air. Following incubation cell were stained with 10µM HPI solution in culture medium, cells were imaged by fluorescence microscopy x 20 magnification.
Figure 5.7 Effect of synthetic IGF-1 peptide on hMSC growth (5 days). P3 hMSC’s were seeded at a density of 7,000 cells per cm$^2$ and incubated with human mesenchymal stem cell medium (Lonza), medium was supplemented with either 13.2 nM of synthetic IGF-1 peptide or human IGF-1 protein. Cells were incubated for 5 days at 37°C, 5% CO$_2$ & atmospheric air. Following incubation cell were stained with 10µM HPI solution in culture medium, cells were imaged by florescence microscopy and cells were counted using imaged random field of view numeration and expressed as mean number of viable cells per field (*=p<0.05) (n=3).
5.3.3 Stem cell morphology and immuno-staining results

From the results of IGF-1 treatment, cells were stained with H &E and calcein-AM for morphological analysis of the cells. Results found cells in treatment groups to have a greater degree of spreading with cells spread over a larger surface area when treated with both sIGF-1 peptide and hIGF-1 (Figure 5.8). However, while the majority cells presented with a greater spreading, a small number of cells in the populations were also of a similar size or smaller to that of the control. Finding that population morphology was not completely homogenous. The findings of immuno-staining for progenitor cell markers Stro-1 and Nanog found positive staining for both markers in control and treatments groups (Figure 5.9). However, no differences in Stro-1 and Nanog staining were observed between controls and treatments (Figure 5.9).
Figure 5.8 Effect of synthetic IGF-1 peptide of hMSC cell morphology (5 days). P3 hMSC’s were seeded at a density of 7,000 cells per cm$^2$ and incubated with human mesenchymal stem cell medium (Lonza), medium was supplemented with either 13.2 nM of synthetic IGF-1 peptide or human IGF-1 protein. Cells were incubated for 5 days at 37°C, 5 % CO$_2$ & atmospheric air. Haematoxylin & eosin stain - left, Calcein AM right. A&B –Control, C&D-IGF-1 peptide, E&F-IGF-1. (n=3)
**Figure 5.9 Effect of synthetic IGF-1 peptide of hMSC phenotypic markers.** P3 hMSC’s were seeded at a density of 7,000 cells per cm$^2$ and incubated with human mesenchymal stem cell medium (Lonza), medium was supplemented with either 13.2 nM of synthetic IGF-1 peptide or human IGF-1 protein. Cells were incubated for 5 days at 37°C, 5 % CO$_2$ & atmospheric air. HMSC treated with produced IGF-1 peptide. Stro-1 immunostaining - left, Nanog immunostaining - right. A&B –Control, C&D-IGF-1 peptide, E&G-IGF-1 (n=3).
5.4 Discussion

The study of the effects of the sIGF-1 peptide on PBC and hMSC cell types was performed in order to assess the biological effect that this peptide has on the growth and phenotype of cell types relevant to cartilaginous tissue regeneration. From these studies PBC cells were tested in a dose curve response study finding a significant dose curve relationship effect on cell proliferation over 24 and 48 hour treatments (Figures 5.2, 5.4 & 5.5). Furthermore, cell morphology and viability was not found to be affected by the treatment with sIGF-1 peptide (Figure 5.2). PBC Cells were found to be spread with a spindle-like morphology. However, some spherical cells were observed suggesting the population of cells to be mixed in phenotype (Figure 5.3). From comparison, no significant differences were identified between the control and treatment groups in cell population morphology thus suggesting that the IGF-1 has not had a significant effect on the phenotype of the cells. As discussed previously in section 1.2.2, the switch in cellular morphology from spherical to spread in an indication of a switch for differentiated to de-differentiated phenotype and expression. Indicating that the IGF-1 peptide has not been able to revert phenotype. While, the sIGF-1 peptide was found to increase cell proliferation it was also found to significantly increase GAG production at a treatment dose of 1000 ng/ml (Figure 5.6). While this treatment dose was found to have a significant increase on GAG production over 24 hours, it could be argued that from the results of the dose curve response studies that lower doses may be capable of eliciting a similar response, as the results of the dose response curve found significant effect on cell proliferation/metabolism with treatments from 25 ng/ml to 1000 ng/ml. The optimum dose of sIGF-1 peptide was found to be 100 ng/ml, as it elicited a 15% increase in cell proliferation while higher doses were not found to elicit a
significantly greater increase in proliferation. In comparison with the results of the dose curve effect of sIGF-1 on hMSC cells, the proliferative effect of sIGF-1 was found to be much greater and more effective at lower doses (Figure 5.7). The results showed that the lowest tested dose of 12.5 ng/ml was effective, stimulating a 41.3% increase over the 5 day study. In addition, 100 ng/ml was found to elicit a 59.8 % increase in cell proliferation by MTS assay. A significantly greater increase in proliferation in comparison to that of PBC cells which showed only small increase in cell proliferation of 17.14% at 100 ng/ml treatments. While it may be argued that the difference in percentage cell growth could be a result of the cell study duration (24 & 48 hours and 5 days for PBC and hMSC cells respectively) these incubation periods were selected based on the approximate doubling time of the respective cell types. In addition to these findings, analysis of cell marker expression found the sIGF-1 peptide not to effect hMSC expression of multipotency markers Stro-1 and Nanog (Figure 5.12). This finding shows that although effecting proliferation over short time periods, the sIGF-1 peptide does not affect the differentiation of hMSC expression over short duration of exposure. In conclusion, the sIGF-1 peptide is capable of eliciting a bioactive response in both hMSC and bovine chondrocytes, while eliciting a significant increase in GAG production in chondrocyte cells. These findings demonstrate the potential of this bioactive peptide for the treatment of IVDD as a therapeutic agent for the stimulation of tissue repair.
Chapter 6: In vitro 3D cartilaginous tissue engineering in bioactive self-assembly peptide hydrogels
6.1 Introduction

The development of in vitro cell culture systems has significantly changed since the early work of Carrel and Burrow (1911). However, despite the significant changes, methods first used to culture cells are still applied today (Carrel and Burrow, 1911). While cartilage tissue engineering has progressed considerably, the expansion of primary chondrocytes in 2D culture systems has been shown to have many limitations, primarily due to the rapid loss of cell phenotype (Brodkin et al., 2004). While the development of 3D scaffolds for tissue engineering have facilitated a degree of phenotypic restoration (Benya and Shaffer, 1982), the ability for cell expansion is limited within these systems. When combined with the concern that full restoration of phenotypic expression of native cartilage ECM components has not yet been achieved, the limited in vitro expansion of differentiated chondrocytes raises questions of the clinical feasibility of cell expansion in 3D scaffolds. It could be argued that currently biomaterials are required to tackle both the issue of cell expansion while controlling expression. Moreover, there are also various factors that are known to effect the phenotypic expression of chondrocyte cells, such as cell density, medium composition, availability of growth factors, cell anchorage proteins and ECM composition (Acosta et al., 2006, Arner and Pratta, 1989, Benya and Shaffer, 1982, Boubriak et al., 2009, Brodkin et al., 2004). Therefore, it could be argued that mimicking the native environment of cartilage or the NP would provide the best phenotypic outcome for the implanted cells. In consideration of this, it is the view of the author that the parameters of any tissue engineering experimentation should aim to closely mimic the environment of a target tissue with regards to both the native cell density and the soluble factors that affect cell phenotype.
6.1.1 *Nucleus pulposus cellular models and tissue engineering*

The NP chondrocyte cells of the human IVD, as previously discussed in Chapter 5 Section 5.2 would be considered as an ideal model of *in vitro* studies for NP tissue engineering. However, due to the limited availability of NP chondrocytes, the use of articular chondrocytes is considered a suitable alternative (Lee C, R *et al.*, 2007). In order to model the environment of the NP, the options available are to either use the native cell density of the NP or that of the relevant cell model. The rationale for the use of chondrocytes at this density is twofold: (i) chondrocytes in their native cartilage tissues largely regulate their own expression and metabolism due to the absence of blood supply to the tissue (Zhao, 2007). In consideration of this, the concentration of growth factors such as IGF-1 or TGF-β present in a given culture system is largely dependent on the relative cell density within the system and the volume and content of the tissue fluid. Considering the effects of these molecules on cellular production of ECM components, as previously discussed in Chapter 1, it would seem logical that any strategy for tissue engineering or *in vivo* regeneration, should aim to populate a scaffold with a cell density equal or greater than that of the native density, thereby providing sufficient levels of endogenously produced growth factors to stimulate ECM formation. (ii) The number of cells implanted inevitability determines the final cell density of the tissue if successfully formed. Given the relative slow cell proliferation rates of chondrocytes in 3D culture systems, the seeding of chondrocytes at cell densities lower than that of the native cartilage may not be sufficient to both proliferate to occupy the given volume while also laying down dense ECM within a time frame compatible with the degradation rate of the implanted scaffold.
While, the current trend in tissue engineering for the treatment of IVDD aims to produce cartilage tissue in vivo for the replacement of the NP. In aiming to achieve the in vivo generation of cartilaginous tissue it is important that a review of the structure and composition of native cartilage tissues is first performed, to provide comparative data to assist in the interpretation of the findings of any experimental studies.

6.1.2 Cartilage tissue structure and composition

Cartilaginous tissues such as the NP are formed of a highly specialised ECM, which as previously discussed in Chapter 1, is formed primarily of collagen type II and aggrecan (Whatley and Wen, 2012). The structure and morphological nature of both the chondrocyte cells and the cartilage tissue as a whole, are similar to that of articular cartilage, however, there are a number of distinct differences, namely the cellular density and collagen architecture (Buckwalter et al., 1979). The NP has a lower cell density than cartilage and it possesses banded collagen ring like structures that increase in size from the central region to the interface with the annulus fibrosus (Buckwalter, 1982, Buckwalter et al., 1979). The histological investigation of the cartilaginous tissues is commonly performed by a number of different histological staining methods, including haematoxylin and eosin (H & E) and safranin-O (Figure 6.1) (Rosenberg, 1971). The use of histological investigation is extremely important in the evaluation of tissue growth and formation as it allows the qualitative assessment of tissue composition, morphology and cellular phenotype. This is specifically true for the investigation of cartilaginous tissues, were significant changes in the cell morphology from spherical to spindle-like can be indicative of fibroblastic expression (Benya and Shaffer, 1982). While there are
many different methods for studying tissue composition the use of histological stains is the most common.

Figure 6.1 Safranin-O staining of bovine articular cartilage, SZ – surface zone, MZ-middle zone, OCJ – Osteochondral juncture. Adapted from (Amin et al., 2008)

Safranin-O is a commonly used non-specific proteoglycan stain that binds strongly with the sulphated GAG chains of aggrecan (Rosenberg, 1971). While the stain allows for the identification of the presence of GAGs, the safranin-O cannot identify the specific proteoglycan produced or accurately quantify the GAG concentration. However, GAG content of cartilage tissues can be accurately measured with the use of the DMB assay (Barbosa et al., 2003). GAG concentrations in human articular cartilage tissues have been found to be approximately 418 µg/mg of dry mass (Rogers et al., 2006). While, the production of GAGs is a strong indication of a chondrocytic phenotype, the identification of the specific proteoglycans produced is important to truly understand the cellular phenotype. This is commonly achieved using gene expression analysis and/or immuno-histochemistry. However, less
complex methods, such as cellular morphology assessment can also give a good
indication of cellular phenotype (Brodkin et al., 2004). NP chondrocytes in healthy
IVD samples are typically spherical, similar to that of articular cartilage
chondrocytes (Figure 6.1). With increasing depth an increase in cell size is observed,
while a decrease in cell number in seen the closer to the CEP (Buckwalter, 1982).
While there are many similarities between articular cartilage and the NP, the
structural alignment of cells in pillar-like arrangements is observed in articular
cartilage (Gray, 1918); however this phenomenon is not seen in NP tissue. While
there is currently no scientific evidence for the cause of this difference, one possible
explanation may be due to differences in directional loading of the two tissues.
Within cartilage there are significant differences in cellular expression between the
different zones of the cartilage. Superficial zone cells typically have a lower
expression of proteoglycan and aggrecan than the cells of the middle and deep zones.
Furthermore, superficial zone cells produce a greater amount of collagen II fibres
which are arranged in parallel alignment with the joint surface (Aydelotte &
Kuettner, 1988). Superficial zones cells also secrete of proteoglycan 4 that isn’t
found in middle or deep zone cells (Schumacher et al., 1994). Proteoglycan 4 is
essential in maintaining lubrication of the cartilage surface (Schumacher et al.,
1994). Conversely, cells of the deep and middle zone produce higher levels of
aggrecan while producing a collagen matrix with larger fibres than that of the
superficial zone cells (Poole et al., 1982).

In the study and assessment of 3D engineering scaffolds the use of histological
methods to investigate cell physiology and tissue composition are essential, as the
formation of a functional tissue should be examined for the presence of the required
attributes of a given target tissue e.g. positive safranin-O staining in cartilaginous
tissue/biomaterial constructs. Furthermore, the gene expression of ECM components such as collagens I and II, and aggrecan are also essential indicators of cellular phenotype which ultimately determine the structure of a formed tissue. In the NP and articular cartilage, the ratio of collagen II : collagen I expression is 80:1. While the expression of aggrecan is approximately 10 times greater than that of collagen I (Boutell et al., 2000). The restoration of this expression ratio is essential to both the concept of in vitro cartilaginous tissue engineering, and to the development of a regenerative medicine treatment for IVDD.

6.1.3 Chapter aims

This chapter aims to study the interaction of PBC cells with 3D Fmoc-F₂ hydrogel constructs formulated with or without the sIGF-1 peptide. The studies aim to firstly investigate the feasibility of acellular Fmoc-F₂ hydrogel injection for IVD NP regeneration by studying the materials ability to support chondrocyte migration and population. Secondly, this chapter also aims to investigate the feasibility of Fmoc-F₂ hydrogels as 3D tissue engineering scaffolds for the in vitro formation of cartilaginous tissue, while also testing the feasibility of cell loaded delivery for the treatment of IVDD.

Specific Aims

1. To study the 3D interaction of PBC cells with Fmoc-F₂ hydrogel
2. To assess PBC cellular migration through Fmoc-F₂ hydrogels
3. To study the effects of 3D culture on PBC cells in Fmoc-F₂ hydrogel constructs and their ability to form cartilaginous tissue in vitro
4. To study the PBC cell gene expression of ECM components in 3D Fmoc-F₂ hydrogel constructs.
6.2 Materials and methods

6.2.1 sIGF-1 peptide integration and release study

sIGF-1 peptide was prepared with Fmoc-F2 hydrogel using the method outlined in Chapter 3. The hydrogels were prepared with the addition of 1 µg/ml of sIGF-1 peptide and cast in 500 µl discs in 24 well plate wells. The hydrogels were incubated in 1 ml of PBS at 37 ºC, 5% CO₂, 95 % atmospheric air for intervals of 10, 20, 30, 60 and 120 minutes. PBS samples were then analysed using HPLC with 10 µl injections following the protocol detailed in Chapter 2.2. HPLC peak areas were recorded and converted to a concentration using sIGF-1 peptide standard curve (see appendix 8.3). Concentrations were plotted in a graph of concentration vs. time.

6.2.2 3D In vitro PBC cell migration and proliferation study

P6 PBC cells were selected as previously discussed in section 1.2.2, due to their dedifferentiated phenotype. The rational for this selection was to test the ability of the hydrogel to revert the cell phenotype to a differentiated phenotype. This would serve the purpose also of testing the clinical feasibility of in vitro expansion of a patients cells to then be re-differentiated and a tissue construct to be formed for implantation. (P6) were detached from incubation flasks using 5 ml of trypsin (Sigma, UK) over a 5 minute incubation at 37 ºC, 5% CO₂, 95 % atmospheric air. The harvested cells were incubated in 10 ml of high glucose DMEM + 10% FBS (Fisher, UK) and collected by centrifugation at 500 G for 5 minutes. Cell pellets were re-suspended in 1 ml of culture medium and counted using a haemocytometer. Cells were seeded at a density of 1,000,000 cells/construct on to the surface of 500 µl hydrogel discs precast in 24 well plates with and without the addition of sIGF-1 peptide at 1µg/ml. The hydrogel was incubated 37 ºC, 5% CO₂, 95 % atmospheric
air in 1 ml of high glucose DMEM + 10% FBS culture medium with daily medium changes. Experimental sets were stopped at 1, 3 and 6 day intervals and constructs were lysed with LDH lysis buffer (Promega, UK) to obtain total cell LDH readings (see appendix 8.1). Samples not treated with lysis buffer were stained using 2 µM calcein-AM 1mM stock in DMSO (Sigma, UK) prepared in the experimental culture medium to assess cell viability and morphology. Cells were incubated for a further 20 minutes and imaged using fluorescence microscopy. Following surface imaging, the hydrogels were then fixed for 45 minutes using 1 ml of 3.7 % formaldehyde per disc. The hydrogels were then serially bisected perpendicular to the uppermost disc using a microtome blade to create small hydrogel sections of approximately 2-3 mm in thickness. The hydrogel sections were vertically orientated on a glass slides and the cells were imaged using fluorescence microscopy in order to obtain a cross sectional view of the hydrogel surface and lower regions to assess cell migration.  

Cell migration was measured in ImageJ from the seeding surface to the furthest cell in each image (n=3).

6.2.3 3D cartilaginous tissue engineering in bioactive Fmoc-F$_2$ hydrogel

Fmoc-F$_2$ based hydrogels were prepared using the method detailed in Chapter 3 with or without the addition of 1µg/ml of sIGF-1 peptide. PBCs (P1) isolated using the method detailed in Chapter 4.2, were prepared in cell suspensions of 3 x 10$^7$ cells per ml in DMEM + 10% (v/v) FBS. The cell suspensions where then mixed in a 1:1 v/v ratio with prepared hydrogels and set in 500 µl discs using a 24 well plate, thus creating discs with a cell density of 1.5 x 10$^7$ cells per ml equal to the cell density in human cartilage and approximately 3 times that of the human NP (Maroudas et al.,
1975, Stockwell, 1971). The cell constructs were incubated at 37 °C, 5% CO₂, 95 % atmospheric air for 2 hours to allow the hydrogels to set. Following setting, 2 ml of growth medium was added onto the surface of the hydrogel disc. Experimental plates were then incubated at 37 °C, 5% CO₂, 95 % atmospheric air for intervals of 1, 3, and 6 days with daily growth medium changes. Tissue constructs were imaged using an inverted Nikon light microscope with a cannon 5D SLR camera at 1, 3 and 6 days. Cell constructs were measured at timed stop points using callipers to record changes in construct diameter. Constructs were divided into analysis groups for gene expression analysis, cell counting and histological assessment. Growth medium was analysed for GAG content using DMB assay.

6.2.4  Histological preparation of samples

Following 6 days incubation, the tissue constructs were fixed in 10 ml of 3.7 % (v/v) formaldeyde in PBS at 4 °C for 24 hours, following fixation, tissues were dehydrated using 3 x 5 minute incubations in increasing ethanol concentration of 25, 50, 75, 95 and 100 % (v/v). Samples were incubated in Histoclear (Fisher Scientific, UK) 2 for 3 x 3 minute incubations. Tissues were then submerged in liquid paraffin wax and incubated for 4 hours at 62 °C. Following incubation, tissue samples were orientated in histology casts and set in paraffin wax overnight on a cool plate at -4 °C. Embedded tissues were then sectioned using a microtome (Leica Biosystems, UK), cutting sections to a thickness of 5 µm. Sections were mounted on to poly- L-lysine coated glass slides (Fisher Scientific, UK) and air dried overnight. Sections were then stained with Safranin-O or H & E.

6.2.5  Haematoxylin & eosin staining
Prepared tissue sections were incubated for a total of 9 minutes in excess Histoclear to remove paraffin wax from the tissue. Sections were then rehydrated in 3 minute incubations repeated in triplicate in decreasing ethanol concentrations 95, 75, 50, 25 % (v/v) and finally in deionised water. Following rehydration, tissue sections were then stained with excess Harris’s haematoxylin solution (Sigma, UK) for 5 minutes. Sections were then transferred to running tap water and washed for 3 minutes. Sections were then counter stained using excess eosin solution (Sigma, UK) for 2 minutes. Sections were then washed in 3 changes of de-ionised water for 2 minutes. Samples were dehydrated rapidly through increasing ethanol concentrations of 25, 50, 75, 95 and 100 % (v/v) for 2 minutes each. Stained sections were then mounted with DPX (Sigma, UK) and sealed with a glass cover slip. Sections were then imaged using an inverted Nikon light microscope with a cannon 5D SLR camera.

6.2.6 Safranin-O staining

Prepared tissue sections were incubated for a total of 9 minutes in excess Histoclear 2 to remove paraffin wax from seconded tissue. Sections were then rehydrated in 3 minute incubations repeated in triplicate in decreasing ethanol concentrations 95, 75, 50, 25 % (v/v) and deionised water. Following rehydration tissue sections were then stained with excess Harris’s haematoxylin solution (Sigma, UK) for 5 minutes. Sections were then washed in de-ionised water 3 times and counter stained with 0.01 % (w/v) Fast Green for 2 minutes. Sections were submerged for 15-20 seconds in 1% acetic acid and stained with 1 mg/ml Safranin-O (Fisher Scientific, UK) in de-ionised water for 5 minutes. Sections were then transferred to running tap water and washed for 3 minutes. Following staining samples were dehydrated rapidly through increasing ethanol concentrations of 25, 50, 75, 95 and 100 % (v/v) for 2 minutes each. Stained sections were then mounted with DPX (Sigma, UK) and sealed with a
glass cover slip. Sections were then imaged using a Nikon light microscope with a cannon 5D SLR camera.

6.2.7 Tissue digestion and cell collection

Cells were liberated from the newly formed tissue by digestion in 1 ml of 1 mg/ml collagenase solution for 4 hours at 37 °C, 5 % CO₂, 95% atmospheric air. Following digestion the cells were collected by centrifugation at 300 G for 5 minutes and counted using a haemocytometer. Cell pellets were then snap frozen in liquid nitrogen and stored at -80 °C for RT-PCR analysis. Digestion supernatant was stored at -80 °C for GAG content analysis

6.2.8 GAG content analysis

Dimethyl-methylene Blue (DMB; GAG assay) solution was prepared using the recipe in Table 5.1 (Barbosa et al., 2003). 250 µl of the DMB solution was transferred to an experimental well of a 96 well plate. Cell supernatant or tissue digest samples (40 µl) were then added to the wells containing DMB solution. Solution absorbance was read at 590 nm using a micro-plate reader (Biotek, UK). Absorbance values were converted to concentration values using a GAG standard solutions to create standard curve using slope-intercept equation (y=mx+c) (see appendix - Figure 8.2).
6.2.9 *RT-PCR gene expression analysis*

RNA was extracted from isolated cell pellets (Section 4.2.2.1) using 100 µl of cell lysate buffer (RNeasy Kit, Qiagen). Cell lysate was then passed through RNA purification filter and washed with 3 x 250 µl 70 % (v/v) ethanol and eluted using the provided elution buffer (RNeasy Kit, Qiagen). Collected RNA samples were analysed using a nano-spectrophotometer (NanoDrop). The collected RNA solution was then reverse transcribed to create cDNA using reverse transcriptase. 2 µg of purified RNA was mixed with 5 µg of oligo dT primer, 5 mM DNTPs and 15 units of AMV reverse transcriptase (BioRad) samples were incubated at 42 °C for 1 hour and then samples were melted at 95 °C for 5 minutes. Sample were then stored at -20 °C. RT-PCR was performed on cDNA samples using the primers detailed in Table 6.1 the reaction mixtures in Table 6.2. Samples were run for 40 cycles of 95 °C for 10 seconds, 60 °C for 25 seconds and 72 °C for 25 seconds and PCR amplification was monitored using fluorescence detection at 521 nm. The relative ratio of expression was calculated using the $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001). The conditions of the PCR reaction were selected based on previous literature for primer amplification and standard laboratory protocols (Brodkin *et al.*, 2004).
### Table 6.1 Bovine ECM Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 50–30</th>
<th>PubMed ID</th>
</tr>
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<tbody>
<tr>
<td><strong>Aggrecan</strong></td>
<td>Forward CAC CAC AGC AGG TGA ACT AGA 4836</td>
<td>U76615</td>
</tr>
<tr>
<td></td>
<td>Reverse GCT TGC TCC TCC ACT AAT GTC 4941</td>
<td></td>
</tr>
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<tr>
<td><strong>Collagen type II</strong></td>
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<td>X02420</td>
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<td>AY141970</td>
</tr>
<tr>
<td><strong>β-Actin</strong></td>
<td>Forward CGA GCA TTC CCA AAG TTC TAC 1349</td>
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</tr>
<tr>
<td></td>
<td>Reverse TTC CTG TAA CAA TGC ATT TCG 1454</td>
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Table 6.2 RT-PCR thermocycler reaction mixture

<table>
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<td>Forward primer</td>
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</tr>
<tr>
<td>Reverse primer</td>
<td>2.5</td>
</tr>
<tr>
<td>Sample</td>
<td>2.5</td>
</tr>
<tr>
<td>H₂O</td>
<td>6.5</td>
</tr>
<tr>
<td>Sybr Green</td>
<td>10</td>
</tr>
</tbody>
</table>

4.2.2 Statistical analysis

All data sets that were found to be normally distributed were analysed using two-tailed Tukey’s t-test analysis unless otherwise stated in text. Significance was determined using a significance level of 0.05 ie. Values greater 0.05 were not considered to be statistically significant. All statistical analysis was performed using PRISM. Where multiple comparisons where required two-way ANOVA was used with post-hoc Turkey’s t-test analysis.
6.3 Results

6.3.1 sIGF-1 peptide release from Fmoc-F_2

Results from sIGF-1 peptide release studies (Figure 6.2) found release of the peptide from the hydrogel constructs to occur rapidly over 60 minutes reaching an equilibrium with the buffered medium (200% of the volume of the construct). The release profile was seen to occur in a concentration gradient type fashion, with a rapid initial release slowing over time as equilibrium point is reached.

![Graph](image)

*Figure 6.2* sIGF-1 peptide release profile from Fmoc-F_2 hydrogel constructs: Preformed 500 µl hydrogels were prepared with the addition of 1µg/ml of sIGF-1 peptide, hydrogels were set and incubated in 1 ml of PBS for 2 hours at 37 °C, 5 % CO_2 & atmospheric air. PBS samples were taken at times interval and analysed using UV-HPLC for sIGF-1 peptide concentration against standard curve of concentration (n=3).
6.3.2 3D cell migration study

Light microscope imaging of 3D cell migration studies showed that a high density of cells remained on the seeding surface of the hydrogel disc after 6 days in culture. All cell samples presented with a circular morphology on the seeding surface with the available surface growth area being completely saturated at 6 days (Figure 6.3 S). However, below the seeding surface cells were found to have migrated through the uppermost area of the hydrogel but were found at a much lower density to that of the seeding surface. Upper regions below that of the immediate surface were found to have a lower number of cells present but with large channels of cells which appeared to have migrated through fishers in the hydrogel shown in Figure 6.3. No observable difference in cell morphology or number was seen between samples loaded with sIGF-1 peptide and those without. Approximate middle to lower regions of discs we found to be free of cells see Figure 6.3.
Figure 6.3 Light microscope imaging of PBC cell migration: PBCs seeded on to the surface of preformed Fmoc-F₂ hydrogel discs and culture for 6 days at 37 °C, 5% CO₂ & atmospheric air in high glucose DMEM + 10% FBS. Cells were imaged live using an inverted Nikon light microscope. S: Seeding surface, U: Upper regions, L: Lower regions. (eclipse te2000-u, Nikon) Arrows indicate columns of cells that were observed to grow through in channels within the hydrogel.
The results of cell viability assessment and migration using calcein-AM staining revealed cells to be viable and to have a complete coverage of the seeding surface. No difference was observed between sIGF-1 peptide loaded hydrogel and untreated hydrogels. Cell morphology appeared spherical similar to those observed by light microscope. Cross-sectional analysis of cells fixed within the hydrogel revealed migration from the seeding surface into the bulk of the hydrogel with cells becoming more dispersed and fewer in number with increasing depth from the surface. Cells appeared spherical and stained positive with calcein (Figure 6.5) and were found to have migrated up to a mean depth from the surface of 581 µm ± 23 (SD). No significant difference in cell migration was seen between experimental samples with or without sIGF-1 (p=0.072).

**Figure 6.4 Calcein viability staining of cell seeding surface:** PBCs were seeded on to the surface of preformed Fmoc-F$_2$ hydrogel discs and culture for 6 days at 37 °C, 5 % CO$_2$ & atmospheric air in high glucose DMEM + 10% FBS. Cell medium was aspirated and cells were stained using 2 µM Calcein-AM solution and incubated for a further 20 minutes. Hydrogel constructs were imaged using UV-fluorescence microscopy (eclipse te2000-u, Nikon).
Figure 6.5 Calcein viability staining of hydrogel cross-section. PBCs were seeded on to the surface of preformed Fmoc-F₂ hydrogel discs and cultured for 6 days at 37 °C, 5 % CO₂ & atmospheric air in high glucose DMEM + 10% FBS. Cell medium was aspirated and cells were stained using 2 μM Calcein-AM solution and incubated for a further 20 minutes. Hydrogels were then fixed for 45 minutes in 3.7 % formaldehyde and gel constructs were cut by hand into 1-2 mm cross-sections using a microtome blade. Cross-sections were vertically orientated on glass slides and imaged using UV-fluorescence microscopy (eclipse te2000-u, Nikon). Cell migration was measured in image j from the seeding surface to the furthest cell in each image (n=3)
From the results of LDH quantification of total cell number, cell growth was plotted as a function of time over the culture period (Figure 6.6). There was found to be a significant growth of cells in both conditions, with cell doubling time for approximately 6 days. No significant difference between the control and sIGF-1 loaded hydrogels were found (p=0.059). However, cell culture in the untreated hydrogel had a significant lag in growth from day 1 to day 3, but with greater growth from day 3 to day 6. Conversely, hydrogels treated with IGF-1 peptide showed significant growth from 1-3 days, but only a small amount of growth from day 3 to 6. However, at day 6 both treatments were found not to have significantly different cell numbers per cm$^3$.

**Figure 6.6 PBC growth in Fmoc-F$_2$ hydrogel migration cell culture model.** PBC cells were seeded on to the surface of preformed Fmoc-F$_2$ hydrogel discs and cultured for 6 days at 37 °C, 5 % CO$_2$ & atmospheric air in high glucose DMEM + 10% FBS. Following cell incubation intervals samples were lysed and LDH concentration was converted using slope of the line equation from the produced R value from the standard curve. One-way ANOVA with post-hoc Tukey’s analysis was performed to determine statistical significance between data sets (*=p<0.05). (n=3)
6.3.3 3D tissue engineering studies

The 3D culture of PBCs within Fmoc-F₂ hydrogel revealed a number of significant changes in both the hydrogel structure and its content over time. The hydrogel/cell constructs seeded at the native density of bovine articular cartilage were found to significantly contract over the 72 hours of culture when compared with the cell free control (48h - p= 0.0021, 72h- p=0.00020), followed by a phase of no change over the preceding 3 days of study duration (Figure 6.7 and 6.8). While cell free hydrogel controls were not found to significantly change in size or shape during the experimental period (day 1 v 6 control p= 0.101). Furthermore following incubations time points of 3 day or more hydrogel constructs were found to be self-supporting and stable under pipette force disruption, while cell free control hydrogels were capable of disassembly by pipette force. From light microscope assessment a daily internals, cells were observed to have an apparent spherical morphology at day 1 and 2 typical of that of the native cartilage. However, time points from 3 to 6 days, were unable to be obtained due to increase in tissue density disrupting light passing through the construct (Figure 6.9) light microscope examination at day 3 found there to be a significant formation of fibres similar to that of collagen matrix (Figure 6.9)
**Figure 6.7 Photographic images of PBC seeded Fmoc-F₂ hydrogel constructs.** Hydrogel were prepared at 3mg/ml concentration and mixed in a 1:1 ratio with prepared cell suspensions, creating a hydrogel construct with an end cell density of $15 \times 10^6$ cells per ml. Constructs were incubated in high glucose DMEM + 10% FBS at 37 °C 5% CO₂, atmospheric air. A- 24 hours incubation, B- 72 hours incubation.

**Figure 6.8 3D hydrogel construct size in culture.** Hydrogels were prepared at 3mg /ml concentration and mixed in a 1:1 ratio with prepared cell suspensions, creating a hydrogel construct with an end cell density of $15 \times 10^6$ cells per ml. Constructs were incubated in high glucose DMEM + 10% FBS at 37 °C 5% CO₂, atmospheric air. Tukeys t-test analysis was performed on samples at each incubation time in comparison with the cell free control (* = p<0.05). (n=3)
Figure 6.9 Light microscope cell imaging of 3D Fmoc-F₂ hydrogel constructs. Hydrogels were prepared at 3 mg/ml concentration and mixed in a 1:1 ratio with prepared cell suspensions, creating a hydrogel construct with an end cell density of $15 \times 10^6$ cells per ml. Constructs were incubated in high glucose DMEM + 10 % FBS at 37 °C 5% CO₂, atmospheric air for up to 6 days. Light microscope images were taken of the constructs at daily intervals. Images were acquired using a 20x dry objective lens.

The results of cell counts performed on cells liberated from constructs, found there to be a small, but significant, increase in cell number in both treatment groups from day 1 to 3. The increase in cell population from day 1 to 6 was found to be 53.34 % ± 13.9 and 61.33 % ± 8.6 for Fmoc-F₂ and Fmoc-F₂ + sIGF-1 peptide constructs respectively. However no significant difference was found by $t$-test analysis between the two treatments groups as p values between treatment groups were found to be greater than 0.05. Cell growth from 3 to 6 days was found to be significant for IGF-1 loaded constructs, but not for Fmoc-F₂ only (Figure 6.10), however, $t$-test analysis between treatment groups revealed no significant difference at 6 days. This finding shows that there is significant proliferation of cells within Fmoc-F₂ hydrogels.
Figure 6.10 PBC proliferation in 3D Fmoc-F2 hydrogel constructs. Hydrogel were prepared at 3mg/ml concentration and mixed in a 1:1 ratio with prepared cell suspensions, creating a hydrogel construct with an end cell density of 15 x10^6 cells per ml. Constructs were incubated in high glucose DMEM + 10% FBS at 37 °C 5% CO₂ for 1, 3 and 6 day periods. Following incubation, cells were isolated using collagenase treatment for 4 hours. Liberated cells were collected by centrifugation and counted using a haemocytometer. Statistical analysis was performed using a one-way ANOVA with post-hoc Tukeys t-test analysis. (n=3)

6.3.4 Histological analysis of 3D tissue constructs

Histological staining of tissue sections with H&E (Figure 6.11) revealed cells to have a circular morphology, with collagen deposition surrounding the cell in the form of a pericellular matrix (Figure 6.11, arrows). No apparent difference was seen in the distribution of cells or cell morphology between hydrogel with and without the addition of sIGF-1 peptide. Deeper regions of the tissues however, were found to have a greater degree of eosin staining for collagen (Figure 6.11, C and D).
Figure 6.11 H&E stained histological sections of 3D Fmoc-F₂ tissue constructs. 5µm thick sections were cut from paraffin wax embedded sections of the tissue formed after 6 days culture. Sections were stained with H&E stains to stain the tissue structure. Sections were then dehydrated and mounted with DPX. Sample images were acquired using a 40x magnification oil emersion objective lens using a Nikon eclipse te2000-u light microscope (Nikon). A&B- Fmoc-F₂ hydrogel construct and Fmoc-F₂ hydrogel construct + sIGF-1 respectively, outer tissue edge (media contact surfaces). C and D- Fmoc-F₂ hydrogel construct and Fmoc-F₂ hydrogel construct + sIGF-1 respectively, central tissue area. (n=3)

Histological assessment of samples stained with Safranin-O (Figure 6.12) revealed the tissue formed after 6 days culture to positively stain for the presence of proteoglycan (Figure 6.1). While no difference was observed between the two treatment groups, staining intensity was found to be greater in central regions with a lower intensity seen in the outer regions. Cell cytoplasm was also found to be positively stained for proteoglycan indicating cellular production proteoglycan was at high levels at 6 days.
Figure 6.12 Safranin-O stained histological sections of 3D Fmoc-F$_2$ tissue constructs. 5µm thick sections were cut from paraffin wax embedded sections of the tissue formed after 6 days culture. Sections were stained with Safranin-O. Sections were then dehydrated and mounted with DPX. Sample images were acquired using a 40x magnification objective lens using a Nikon eclipse te2000-u light microscope (Nikon) A&B- Fmoc-F$_2$ hydrogel construct and Fmoc-F$_2$ hydrogel construct + sIGF-1 respectively, outer tissue edge (media contact surfaces). C and D- Fmoc-F$_2$ hydrogel construct and Fmoc-F$_2$ hydrogel construct + sIGF-1 respectively, central tissue area. (n=3)
Analysis of matrix bound GAG content found there to be a time dependent increase in bound GAG content in both of the formulated hydrogel constructs. How t-test analysis found there to be no significant difference between the two hydrogels.

**Figure 6.13 Matrix bound GAG concentration in 3D constructs.** Hydrogel were prepared at 3mg/ml concentration and mixed in a 1:1 ratio with prepared cell suspensions, creating a hydrogel construct with an end cell density of 15x10^6 cells per ml. Constructs were incubated in high glucose DMEM + 10% FBS at 37 °C 5% CO₂, for 1, 3 and 6 day periods. Following incubation, cells were isolated from newly formed tissues using 1 mg/ml collagenase treatment for 4 hours. Liberated cells were collected by centrifugation and digested ECM supernatant was analysed using the DMB assay for GAG content quantification. GAG assay results were normalised to cell number (per 10 x10^4 cells). One-way ANOVA with post-hoc Tukey’s analysis was performed to determine statistical relevance between data sets.
Gene expression analysis of ECM components collagen I, II and aggrecan were analysed using qRT-PCR, to assess the affect of 3D culture in Fmoc-F₂ based hydrogels on PBC expression and phenotype. The results of raw analysis found gene application of all ECM components in PBCs cultured in 3D to have a lower Cycle threshold (Ct) than that of β- actin. While cells cultured in 2D were found to have Ct values greater than that of β-actin. From the raw Ct readings the gene expression for each treatment was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Cells cultured in 3D Fmoc-F₂ hydrogels with and without 1μg/ml of sIGF-1 peptide, were found to have a significantly greater expression of collagen I with a 2.2 fold increase in expression compared to that of cells cultured in 2D (Figure 6.14). However, collagen I expression was not found to be significantly different between constructs with and without sIGF-1 peptide (Figure 6.14). Expression of collagen II was also found to be >100 fold significantly increased in cells cultured in 3D Fmoc-F₂ hydrogel samples in comparison to 2D culture. However, in samples treated with 1μg /ml of sIGF-1 peptide, the level of collagen II expression was significantly lower, showing the addition of the sIGF-1 peptide reduces collagen II expression. Although this level was still found to be 80 times greater than that of 2D culture. Analysis of aggrecan expression revealed a significant 20 fold increase in expression in Fmoc-F₂ hydrogels while hydrogel treated with sIGF-1 peptide was also found to have a greater expression compared to control no significant increase in expression of aggrecan was found (Figure 6.14 C).
Figure 6.14 Fmoc-F₂ hydrogel 3D PBC culture effects on ECM component gene expression. P2 PBCs were prepared at a cell density of 15x10⁶ cells per ml in Fmoc-F₂ hydrogel constructs. The constructs were incubated for 6 days in high glucose DMEM supplemented with 10% FBS. Following 6 days incubation cells were isolated by collagenase and RNA samples were extracted and purified and reversed transcribed into cDNA. cDNA samples were then amplified using a qRT-PCR thermo-cycler (Rotor-gene Q, Qiagen). Ct values obtained were normalised to the reference gene Ct and expressed as a ratio of 2D expression using 2^ΔΔct equation ± SD. One-way ANOVA with post-hoc Tukey’s analysis was performed to determine statistical relevance between data sets. (n=3)
Relative expression ratios of all genes examined were analysed and found to show significant differences between the ratios of expression of collagen I, II and aggrecan (Figure 6.15). Collagen II showed the greatest expression followed by aggrecan and collagen I. The ratio of collagen I to collagen II was found to be 1:80, while aggrecan had a ratio to collagen I of 1:20 (Col-I : Agg).

![Gene expression relative to P3 2D TCP culture](image)

**Figure 6.15 3D PBC culture relative gene expression of ECM components.** P2 PBCs were prepared at a cell density of 15x10^6 cells per ml in Fmoc-F2 hydrogel constructs. The constructs were incubated for 6 days in high glucose DMEM supplemented with 10% FBS. Following 6 days incubation cells were isolated from the newly formed tissue and RNA samples were extracted and purified and reversed transcribed into cDNA. cDNA samples were then amplified using a qRT-PCR thermo-cycler (Rotor-gene Q, Qiagen). Ct values obtained were normalised to the reference gene Ct and expressed as a ratio of 2D expression using 2^-ΔΔct equation ± SD. One-way ANOVA with post-hoc Tukey’s analysis was performed to find statistical significance between the genes of interest (* p = < 0.05 by post hoc analysis for comparison with other genes). (n=3)
Figure 6.16 Ratio of Collagen II: Collagen I gene expression in 3D Fmoc-F₂ hydrogel constructs. P2 PBC cells were prepared at a cell density of 15 x10⁶ cells per ml in Fmoc-F₂ hydrogel constructs. The constructs were incubated for 6 days in high glucose DMEM supplemented with 10 % FBS. Following 6 days incubation cells were isolated from the newly formed tissue and RNA samples were extracted and purified and reversed transcribed into cDNA. cDNA samples were then amplified using a qRT-PCR thermo-cycler (Rotor-gene Q, Qiagen). Ct values obtained were normalised to reference gene Ct and expressed as a ratio of 2D expression using 2^-ΔΔct equation ± SD. (* p = < 0.05 by post hoc Tukey’s t-test analysis for comparison with 2D control, ** p = < 0.05 by post hoc analysis for comparison with both control and additional treatment groups) (n=3)
6.4 Discussion

The results of sIGF-1 peptide release from Fmoc-F₂ hydrogels (Figure 6.2) found there to be a rapid release from the hydrogel over a period of 20 minutes, reaching equilibrium after 30 minutes. The released sIGF-1 peptide concentration (283 ng/ml) equates to 84.9% of (1 µg/ml) peptide due to the total volume being 1.5 ml (0.5 ml hydrogel, 1 ml PBS) therefore the hydrogel diffusion rate is approximately 30 minutes to reach equilibrium in a cell and protein free hydrogel. While this release is rapid, it is important to consider that the release may be significantly different in the \textit{in vivo} environment as tissue hydration and liquid volume is far lower than that of a PBS solution model.

In an \textit{in vitro} cellular study, the released peptide would still be contained within the culture medium and as the results of the previous investigations in Chapter 5 revealed, the peptide can still elicit a significant effect on PBCs even at doses as low as 25 ng/ml. Therefore, with daily growth medium changes of 50 % volume the effective available peptide concentration will be serially reduced with each medium change. Considering this, an effective dose should be present in an \textit{in vitro} cell model up to 3 days of incubation. However, it is also important to consider that the addition of large numbers of cells and serum protein into the hydrogel during preparation may increase the peptide retention time. Although it could be argued that this is unlikely to extend retention beyond medium change intervals of 24 hours.

The 3D cell migration study was performed in order to assess the Fmoc-F₂ hydrogels ability to facilitate PBC cell migration, in order to validate the concept of acellular hydrogel injection. The results showed a significant in-growth/migration into the
hydrogel from the seeding surface over a period of 6 days. At central regions of the hydrogel, cells were found to have migrated through channels and cells were found to have grown through these channels in a non dispersive way, not evenly populating the hydrogel matrix (Figure 6.3, image U). Furthermore, lower regions of the hydrogel were not found to be populated with cells (Figure 6.3, Image L), suggesting 6 days may not be sufficient time for full migration. Cell morphology appeared to be circular in shape and was also found to be viable by intracellular calcein (Figure 6.4 and 6.5). While the cell morphology is an indication of cell phenotype, alone it is insufficient to ascertain whether the PBCs have a fibroblastic expression of a native chondrocytic expression. However, the spherical appearance of cells within the hydrogel is suggestive of a chondrocytic phenotype (Urban and Roberts, 1995). As with other cartilage tissue engineering research literature, it has been demonstrated that the restoration of chondrocytic morphology in the 3D culture environment is not only an effect of the 3D environment, but is also dependent on the material interaction with cells and culture conditions (Brona et al., 2011, Collin et al., 2011, Talukdar et al., 2011).

LDH assay was performed in order to assess cell proliferation over time. The results of the study found the enzyme to increase significantly over the 6 day period (Figure 6.6). However, the rate of proliferation was significantly lower than that typical of PBC cells when grown on 2D tissue culture plastic (Brodkin et al., 2004). In this study, the results were found to be similar to those of literature where PBC cells are cultured in Fmoc-F₂ hydrogel 3D, with cell doubling time of 6 days (Jayawarna et al., 2009). The reduced proliferation observed within the hydrogel scaffold may also be indicative of a switch in cellular phenotype (Boubriak et al., 2009); as PBC proliferation has been shown to be significantly lower in the native cartilage than
that of PBCs with a fibroblastic expression created by monolayer culture (Boubriak et al., 2009). In summary of the findings from the 3D cell migration study, PBCs seeded on to the surface of a preformed hydrogel were found to be capable of migrating through the Fmoc-F₂ hydrogel. However, the mechanism of this migration is not clear, and may be a result of cellular degradation of the hydrogel creating channels or simple due to gravitational effect. The cell distribution in central regions was found to be uneven, and lower regions were not populated with cells during the 6 day study. While the findings demonstrate that a cellular delivery of the hydrogel may be successful, in terms of cell migration and population, cell distribution may not be sufficient to form a homogeneous tissue.

Three dimensional cell culture studies were conducted in order to study the ability of the formulated hydrogels to support cartilaginous tissue formation in vitro, and to validate the possible use of cell loaded Fmoc-F₂ hydrogel as an injectable treatment of IVDD. The study utilised a high cell seeding density, that of human articular cartilage (1.5 x10⁷ cells/ml). The utilisation of this cell number was to strike a balance between the cell model (PBCs) and that of the cell density of the human NP. The results of the 3D tissue engineering studies revealed by immediate observation a rapid retraction in hydrogel/cell construct size over a period of 3 days. Forming a dense solid tissue that by physical examination was more robust and could be compressed using forceps rapidly recover its dimensions, unlike the cell free control hydrogel. By light microscope examination the reduction in size of the construct was associated with an observed reduction in visibly present hydrogel within the construct. Thus suggesting that cells rapidly removed and remodelled the surrounding 3D matrix, in turn cells after 2 days were seen to be connecting via translucent ECM-like matrix fibrils (Figure 6.9). Cell morphology was also observed
to appear spherical by light microscope up to 3 days. However, at time points greater than 3 days, given the density of the tissue it was impossible using light microscope to observe cell morphology (Figure 6.9). Quantification of cell number confirmed cell proliferation over 1, 3 and 6 day time points, with cell doubling time being 9.77 and 8.73 days for Fmoc-F$_2$ and Fmoc-F$_2$ + IGF-1 respectively. Although no statistically significant difference in cell proliferation was found between hydrogels with and without the addition of sIGF-1 peptide (Figure 6.6). While, IGF-1 was found to have a greater effect in 2D culture conditions (See chapter 5), the non significant result from the 3D studies may be a result of the rapid release of the IGF-1 peptide into the culture medium. Moreover the high number of cells contained with the hydrogel scaffold could also have lead to the metabolism of the peptide through intra and extracellular peptidases produced by the cells (Pasternak et al., 1987). In comparison with the existing literature, the cell proliferation rate is similar to those found in previous 3D culture studies in Fmoc-F$_2$ hydrogels. However, these studies investigated proliferation at significantly lower cell numbers (Jayawarna et al., 2009). The significantly slower proliferation rates in 3D culture studies compared to those in 2D, as previously discussed, is suggestive of a change in cellular phenotype (Benya and Shaffer, 1982, Boubriak et al., 2009).

The histological analysis of the tissue formed after 6 days incubation was conducted to investigate the ECM structure and possible content, as well as the cellular morphology. From the results of staining with H & E (Figure 6.11), formed tissues were found to be comprised of a high density of apparently spherical cells with deposition of colagenous ECM between cells. Higher intensity of staining was also observed in central regions of the formed tissues. While this finding indicates a high quantity of collagen deposition, it may be argued that the type of collagen produced
could be collagen I rather than collagen II. However, the result of gene expression analysis found that collagen II expression to be significantly greater than that of collagen I. Therefore, the findings of increasing collagen intensity toward central tissue regions this could perhaps as a result of lower oxygen or nutrient availability. In order to investigate the production of proteoglycan deposited into the ECM of the tissue, Safranin-O staining of sections was performed to qualitatively assess the presence of aggrecan. The histological staining with Safranin-O revealed no observable differences between hydrogel constructs with and without and addition of the sIGF-1 peptide (Figure 6.12). It may be argued that this could be as a result of the rapid release rate of the sIGF-1 peptide (Figure 6.2) or perhaps cell metabolism may have lead to the rapid degradation of the peptide. Alternatively, if cells were found to have a similar gene expression to that of native chondrocytes, it could be suggested that the synthetic sIGF-1 peptide may be competing with IGF-1 endogenously produced by the cells within the hydrogel construct and therefore its effect may be nullified.

In order to compare the formed tissue in terms of proteoglycan content, the GAG assay was performed to assess the concentration of ECM bound GAG. The results (Figure 6.13) showed a time dependent increase in total ECM bound GAG content in both Fmoc-F2 hydrogels with and without the addition sIGF-1 peptide. While no significant difference in GAG content was found between treatment groups, tissues were found to have a high content GAG after 6 days of culture, with GAG concentrations equivalent to 53.8 % of those found in human articular cartilage tissue (Rogers et al., 2006). This finding is supporting evidence that the tissues formed over the incubation period are cartilaginous in nature. The high GAG content although not equal to that of normal cartilaginous tissues, is remarkably high.
considering the short incubation of just 6 days. Considering this, it could be argued that it studied for longer durations, such as 12 or 21 days the GAG content of the formed tissue may well reach that of the native NP ECM.

The production of ECM components such as GAG can be detected using colorimetric assays the DMB assay. However, it may be argued that the GAG content measurements and histological staining methods are not sufficient to confirm that, while there are indeed GAGs present, these may not be bound to aggrecan but could perhaps be in a discreet form or bound to another proteoglycan, that is not desired in cartilage tissue engineering, such as versican (Zhao, 2007). In order to confirm the production of the relevant ECM components, the gene expression of cells within the newly formed tissues were analysed for the expression of collagen I, collagen II and aggrecan in order to assess the relative ratios of expression. The results found there to be a significant increase in the expression of ECM components collagen I, II and aggrecan in cells cultured in the 3D hydrogel system, when compared to 2D monolayer culture on tissue culture plastic. This effect may be a result of the significant reduction in cellular anchorage that is present in 2D culture but not in the 3D environment of the Fmoc-F₂ hydrogel constructs. As it has been shown from previous study that significant changes of cellular anchorage can effect chondrocyte expression of collagen II, with cells producing greater ECM expression in the absence of cellular anchorage (Benya and Shaffer, 1982, Tsai W and Wang, 2005). While the expression of all measured ECM components were up-regulated, collagen type II was found to have a 200 fold increase in expression in cells cultured within Fmoc-F₂ hydrogels, while Fmoc-F₂ hydrogels supplemented with sIGF-1 peptide were found to have a 100 fold increase in collagen II compared to the 2D control. This finding shows the sIGF-1 peptide to have an inhibitory effect on
collagen II expression. This effect on cellular expression may be a result of competitive inhibition, by which the sIGF-1 peptide competes with endogenously produced IGF-1 protein for the available receptors on the cell membranes. While the presence of the peptide will elicit a response the native IGF-1 protein may elicit a greater response from cells. While the sIGF-1 was found to reduce collagen II expression, it was found to have no significant effect on the expression of collagen I. Furthermore, although no statistically significant difference was found with regards to aggrecan expression, the sIGF-1 peptide did show a remarkably higher mean expression of aggrecan than that of cells from the tissues formed in untreated Fmoc-F₂ hydrogel constructs with a p value extremely close to statistical significance (p=0.0507). Therefore, it should be considered important that future work aims to expand upon this study to investigate the effect of the novel sIGF-1 peptide on aggrecan expression.

The comparison of the relative gene expression ratios were assessed and plotted for comparison of all ECM components (Figure 6.15) and the relative collagen II : collagen I ratio (Figure 6.16). The comparison of this data found the relative ratios of expression in 3D samples to be closely matched with the expression of ECM components in healthy native chondrocytes in bovine articular cartilage (Boutell et al., 2000), with collagen I being expressed at the lowest ratio, while collagen II was expressed at the greatest level, 80 times that of collagen I, with aggrecan at a ratio of approximately 10 times that of collagen I. This full restoration of collagen II:collagen I ratio and aggrecan to native cartilage expression levels have yet to be reported in literature and demonstrates that Fmoc-F₂ hydrogel as tissue engineering constructs have the potential to restore chondrocyte native expression following 2D
cellular expansion, while being able to be readily resorbed and remodelled by the cells which form the dense cartilaginous tissue.

In conclusion, the findings of these studies reveal the potential of Fmoc-F₂ self-assembly hydrogels in the *in vitro* engineering of cartilaginous tissues for regenerative medicine applications. The implications of these findings may offer new avenues for research and clinical treatments for not only IVDD, but other conditions affecting cartilaginous tissues, such as articular cartilage degeneration.
Chapter 7: Thesis conclusions and future work
7.1 Summary of findings & contributions to knowledge

The work contained within this thesis aimed to build upon the previous work lead by the research groups of Gazit, Ulijn, and Gough in the development and study of Fmoc-F$_2$ based hydrogels (Jayawarna et al., 2006, Jayawarna et al., 2009, Smith et al., 2007, Tang et al., 2009). However, with the specific aim of developing a novel bioactive hydrogel through the development and integration of the sIGF-1 peptide, to provide a bioactive hydrogel for application in the treatment of IVDD.

7.1.1 Peptide design and synthesis

The initiation of this work required first the chemical synthesis of the Fmoc-F$_2$ hydrogel and the design and synthesis of a bioactive molecule that in theory could disrupt the biological processes of IVDD while stimulating the regeneration. The design of the intended bioactive molecule was based on achieving the bioactivity of IGF-1 by synthesising the active site domain of the IGF-1 protein. The synthesis of the Fmoc-F$_2$ peptide was achieved through the development of a cost-effective conventional liquid synthesis method, achieving high purity following preparative HPLC purification. While, the synthesis of the designed sIGF-1 peptide (GYGSSRRAPQT) was performed using conventional solid phase peptide synthesis methods. The produced sIGF-1 peptide was obtained at a high purity following semi-preparative HPLC purification, and confirmed using micro time of flight mass spectroscopy. Following, the successful synthesis of the intended peptide sequences, the structures were modelled in order to simulate possible peptide interactions and structural conformation. The results of this analysis revealed the Fmoc-F$_2$ hydrogel acquired a stable low energy state when paired and assembled in a twisted helical chain like structure. These findings show that it is more energetically
favourable for the peptide to self-assemble than remain as discreet molecules. While
the analysis of the chemical structure of sIGF-1 peptide was found to orientate in a
twisted screw like formation, retaining the molecular shape it occupies within the
full IGF-1 protein. This finding provides novel information about the conformation
of the sIGF-1 peptide that has previously not been reported or studied in the
literature. Furthermore, these findings show that the sIGF-1 peptide structure can be
successfully produced using a simple solid phase peptide synthesis method,
achieving a high degree of purity and in a cost effective manner.

7.1.2 Fmoc-F₂ hydrogel preparation and material analysis

This work aimed to provide further supporting evidence of the previously published
work in the elucidation of the self-assembly mechanism of Fmoc-F₂ hydrogels
(Smith et al., 2007). SEM imaging found the hydrogel nanostructure to be formed of
a dense network of nanofibres of approximately 50 nm in diameter, comprised of
smaller interlocking β-sheets. This observation was confirmed by CD analysis,
showing the presence of a positive Cotton effect consistent with β-sheet assembly
(Eliel and Wilen, 1994). The findings of this work support the proposed assembly
mechanism in the available literature (Smith et al., 2007). Further work to
investigate the rheological properties of the produced hydrogels was also found to be
consistent with current literature (Raeburn et al., 2012, Tang et al., 2009). Finding
the produced Fmoc-F₂ hydrogels to be viscoelastic in nature. While there is
significant variation in the reported properties of this class of hydrogel (Raeburn et
al., 2012). The results correspond closely to the findings of publications of Tang et
al., 2009.
7.1.3 *In vitro biocompatibility study of Fmoc-F\textsubscript{2} peptide hydrogels*

This work set out to further the knowledge relating to the Fmoc-F\textsubscript{2} hydrogel biocompatibility, building on the previously published work, in order to greater understand the potential effects of this material on biological systems. This was achieved through the testing of the potential degradation by-products, providing novel cytotoxicity findings for the previously untested compound 9-flu. The results from the degradation product studies found there to be no significant toxicity from Phe. However, 9-flu was found to be toxic at higher doses through the spontaneous formation and growth of crystals under physiological conditions. Furthermore, the additional experimental 2D biocompatibility investigations, revealed the effect of serum protein treatment and preconditioning on cell spreading, adhesion and biocompatibility. While these findings support those of the published literature, the work documented in this thesis identifies novel properties of this material both in terms of chemical by-products and cellular response in both the 2D and 3D *in vitro* environment. Furthermore, the discovery of 9-flu’s potential toxicity through spontaneous crystal growth has not previously been reported in the literature and it is the view of the author that these findings stand as an important contribution to knowledge. Not only for the potential implications for future *in vivo* applications of Fmoc-F\textsubscript{2} hydrogels, but also the wider implications to human health through the routine use of 9-flu in areas of the chemical industry and medicine productions.

7.1.4 *In vitro investigation of IGF-1 peptide for the treatment of IVDD*

The development of the novel sIGF-1 peptide has previously not been reported in the literature, therefore this work aimed to test the efficacy of this peptide in cell models
relevant to regenerative medicine applications in the treatment of IVDD. To investigate the effectiveness of the sIGF-1 peptide on cell proliferation and metabolic activity tested the peptide in a dose response curve trial on both human MSC and PBC cells. The results found the peptide to significantly increase cell proliferation with a dose dependent response relationship in both cell types. Furthermore, investigation into GAG production found PBC cells stimulated with 1000 ng/ml of the sIGF-1 peptide had a significantly greater production of GAG than that of the untreated control, showing the significant effect of the peptide on cellular expression of proteoglycans essential to cartilage tissue formation. In addition, human MSC cells treated with the sIGF-1 peptide were found to retain their expression of pluripotency markers nanog and Stro-1, while significantly increasing in proliferation rate. These findings not only prove beneficial to potential MSC treatments for IVDD, but may also offer a potential cellular media additive to overcome the issue of stem cells expansion. The development, synthesis and biological testing of the sIGF-1 peptide have not been previously reported in the literature and stands as a novel drug discovery, which offers potential not only for the treatment of IVDD, but as a therapeutic agent that may be used in a wide variety of regenerative medicine treatments or in endocrine conditions such as Laron syndrome. Laron syndrome is a genetic condition effecting the structure of the IGF-1 receptor, leading to a reduced sensitivity to IGF-1 (Chernausek et al., 2007, Laron et al., 1966). This condition results is significantly reduced growth and development of children as well as resulting in dramatically reduced life expectancy (Ahmed, 2013, Chernausek et al., 2007). While, current treatment strategy relies on the regular injection of human recombinant IGF-1 for the duration of the patient’s life, this treatment is extremely expensive and requires twice daily injections (Chernausek et
al., 2007) and also frequently cause hypoglycaemia leading to seizures (Ahmed, 2013). Although the developed sIGF-1 peptide is also likely to require injection, as it may be digested if administered orally. The sIGF-1 peptide would be significantly cheaper to produce than the full human recombinant protein, as it can be produced using cost effective conventional peptide synthesis methods (Albericio, 2000). Furthermore, due to its more specific structure, the sIGF-1 peptide may not interact with insulin signalling to the same extent as the full recombinant protein and could therefore provide a potential treatment of Laron syndrome reducing the risks of seizures from loss of glycaemic control.

7.1.5 In vitro 3D cartilaginous tissue engineering in bioactive self-assembly peptide hydrogels

Further to current literature, tissue engineering studies were conducted to test the potential of Fmoc-F₂ hydrogels to support the formation of cartilaginous tissue in vitro. These studies examined the effect of the produced hydrogels on chondrocyte gene and protein expression within a cellular model reflective of current clinical feasibility. The findings of these studies not only explored the novel properties of Fmoc-F₂ hydrogel, but also discovered the potential of Fmoc-F₂ hydrogels to restore chondrocyte native phenotypic expression in vitro, a current major issue in the regeneration of cartilaginous tissue. Furthermore, the research achieved the rapid formation of cartilaginous tissue in just 6 days of in vitro culture. These findings not only demonstrate the remarkable potential of Fmoc-F₂ hydrogels for tissue engineering applications, but also provide a possible basis for the development of treatments not only of IVDD but other cartilage degenerative conditions such as OA.
Alongside the study of Fmoc-F₂ hydrogels, this PhD program developed a novel peptide based on the active site sequence on the human IGF-1 peptide structure. The findings of in vitro studies revealed the peptide to elicit a proliferative effect on both human MSCs and PBC cells. These findings show significant promise for the use of this sIGF-1 peptide as a potential therapeutic agent for IVDD or as a synthetic media supplement for stem cell culture, by increasing cellular proliferation while retaining cellular phenotype.

7.2 Envisaged Future research

From the experimental findings of the work of this thesis, a number future research project can be envisaged in order to further the development of fundamental research and in the development of future treatments of IVDD and other related conditions.

7.2.1 In vitro tissue engineering of human IVD nucleus pulposus

From the findings of significant cartilaginous tissue formation using a PBC cell model for tissue engineering. The use of human IVD chondrocytes within the developed cell model could be studied to investigate if human NP tissue can be formed. While these studies could use the developed protocol as conducted in the study detailed in this thesis, the future work could also study the tissue formation and development over extended culture periods. As discussed previously in section 1.2.4 mechanotransduction plays a key role in the regulation and formation of NP tissue and cartilaginous tissues, through the up-regulation of ECM components, aggrecan and collagen II (Larsson et al., 1991). In the development of the current model, mechanical stimulus could be studied in order to optimise the tissue formation. The
formation of new engineered NP tissue could be analysed for its ECM composition and structure, mechanical properties and cellular density in order to validate and compare the tissue formed with the natural NP. Furthermore, the model could be facilitated by the development of a bioreactor system in order to provide mechanical stimulation and nutrient flow dynamics of the NP. Further to this work, the study of MSC behaviour in the Fmoc-F₂ hydrogel system could be studied to ascertain cell compatibility and any phenotypic effect the hydrogel system may elicit. The development of stem cells as a cell source for the formation of NP tissue engineering may provide an alternative absence of suitable NP or chondrocyte cell source i.e donor or autologous cartilage. This work would stand as a proof of concept for the in vitro growth of replacement tissue for the treatment of IVDD. Furthermore, the formed tissues should be investigated for an in vivo study of Fmoc-F₂ hydrogel constructs in IVDD models.

7.2.2 In vivo study of Fmoc-F₂ hydrogel constructs in IVDD models

Following on from the research conducted, a number of in vivo studies are envisaged as potential future research. These would take the form of small animal models for the replacement of the NP, and could comprise both acellular and cellular delivery strategies, or focus on the implantation of cartilaginous tissue grown in vitro with the Fmoc-F₂ hydrogel system detailed in this thesis. The studies should in the view of the author include extensive histological investigation of recovered implanted tissue or hydrogel construct to investigate development and formation of ECM composition in addition to gene expression analysis of cell phenotype and ECM component production. Furthermore, explanted tissue should be assayed for inflammatory mediators such as IL-1 and TNF-α. This is proposed as the function and structure of implanted engineered tissue could be greatly affected in the long-
term if inflammatory mediators are unregulated. As previously discussed IL-1 and TNF-α up-regulation occurs in IVDD and drives a process of ECM remodelling and breakdown via MMP up-regulation.

7.2.3 *In vitro and in vivo study of sIGF-1 peptide for the treatment of IVDD and related conditions.*

While the successful testing of the sIGF-1 peptide revealed a significant cellular response in 2D culture in both human MSCs and PBC cells further work may be performed to study the specific effects of the peptide on the gene expression of ECM components in order to better understand the peptide effect on cellular phenotype. Furthermore, from the findings of the current literature on the effect of IGF-1, the full protein has been found to inhibit cellular expression and release of IL-1 which drives degradation. Therefore in consideration of this future study of the sIGF-1 peptide should also focus on testing the peptides ability to also inhibit this pathway. In addition other researchers have also found IGF-1 to regulate the effect of IL-1 in other cell types, potentially providing a wide variety of clinical conditions and diseases with which the sIGF-1 peptide may be utilised or studied as a therapeutic compound.

As previously discussed, the application of the developed sIGF-1 peptide outside of cartilaginous tissue regeneration may indeed prove useful in the study and treatment of Laron syndrome (Laron et al., 1966). In consideration of this, future development and study of this peptide could be extended to examine the growth promoting effects in genetic disorders affecting the regulation and functioning of IGF-1 pathway of which there are numerous developmental conditions.
7.3 Conclusions

In conclusion of the research findings of this thesis, the production, development and study of Fmoc-F₂ hydrogel for the treatment of IVDD has been achieved. This work included the chemical synthesis through liquid phase synthesis methods, successfully producing the desired peptide sequences at high purities. Preceding this work, a hydrogel was formulated from this peptide and studied for its structural nature and material properties. Subsequently, the hydrogel was investigated for its biological properties both in terms of cellular adhesion, protein interaction and biocompatibility. The results of these studies found the material to have good biocompatibility within internationally accepted standards (International-Standards, 2009). These studies enabled the *in vitro* study of large tissue engineering experiments to study the ability of the hydrogel to support the growth and formation of cartilaginous tissue *in vivo*. The findings of these studies revealed a remarkable and substantial tissue development over the duration of the study. Moreover, the cellular expression within the tissue was found to be similar to that of native health cartilage. This finding demonstrates not only the unique nature of this material, but also its novel ability to facilitate the full restoration of chondrocyte phenotype following a 2D cell expansion, a current and significant hurdle that has limited the progress of cartilaginous tissue repair and regeneration strategies for decades (add references). While this research stands as a proof of concept, it arguably requires significant further investment and research in order to translate the current findings into a clinical treatment for IVDD.
Chapter 8: Appendix
8.1 Supporting Materials and Results

8.1.1 Sample Calibration curves and raw graphs

8.1.1.1 LDH assay reading v cell number

Figure 8.1 Example LDH standard curve of mean cell number against assay absorbance 50 µl of the aspirated growth mediums and positive control lysis buffers was added to the wells of a 96 well plate and mixed with 50 µl of substrate solution. The plate was then incubated at room temperature protected from light for 30 minutes. After 30 minutes incubation, 50 µl of stop solution was added to each of the experimental wells. The sample absorbance was then read at 490 nm using a Biotek 96-well plate spectrophotometer.
8.1.1.2 GAG assay

![Graph showing GAG assay standard curve of GAG concentration against assay absorbance.](image)

\[ y = -0.0116x + 0.9076 \]
\[ R^2 = 0.9819 \]

**Figure 8.2 GAG assay standard curve of GAG concentration against assay absorbance.** Standards of known GAG concentrations were prepared in PBS. 40 µl of each standard were added to 250 µl of DMB solution. Samples were read using a biotek plate reader at 590 nm.
Figure 8.3 Standard curve of sIGF-1 peptide concentration at against HPLC peak area. Standards of known IGF-1 concentration were prepared in HPLC grade H$_2$O, 10 µl injections of standards and samples were run at a flow rate of 1 ml/min with a moving gradient of 100/0- 0/100 (water/acetonitrile) over 20 minutes. Sample detection by UV detector at 220 nm wave length.
Figure 8.4 Secondary antibody controls for immunocytochemistry (with dapi counterstain) Cells were fixed with 200 µl of 3.7 % (v/v) formaldehyde for 15 minutes and then washed 3 times with 1 ml of 1 % (v/v) BSA in PBS. Cells were then incubated in 200 µl of 1% BSA in PBS as blocking solution for 2 hours at room temperature. Following incubation, samples were washed 3 times with 1 ml of 1 % BSA in PBS and treated with Rabbit anti-mouse IgG secondary antibody conjugated with FITC (green) (Stro-1) or Goat anti-mouse IgG secondary antibody conjugated with Rhodamine (red) (Nanog). Left hand images = Rabbit anti-mouse IgG secondary antibody conjugated with FITC (green), Right hand Images = Goat anti-mouse IgG secondary antibody conjugated with Rhodamine (red) 

HMSC treated with produced IGF-1 peptide. Stro-1 immuno stain - left, Nanog immuno stain - right. A&B – Control, C&D-IGF-1 peptide, E&G-IGF-1 (n=3).
8.1.2 Gene expression raw amplification graphs

Samples were run for 40 cycles of 95°C for 10 seconds, 60°C for 25 seconds and 72°C for 25 seconds and PCR amplification was monitored using fluorescence detection at 521 nm. The relative ratio of expression was calculated using the $2^{-\Delta\Delta C_T}$ equation (Livak and Schmittgen, 2001). The conditions of the PCR reaction were selected based on previous literature for primer amplification and standard laboratory protocols (Brodkin et al., 2004).

Figure 8.3 Example raw data gene amplification cycles 3D and 2D samples. Samples were run for 40 cycles of 95°C for 10 seconds, 60°C for 25 seconds and 72°C for 25 seconds and PCR amplification was monitored using fluorescence detection at 521 nm. The relative ratio of expression was calculated using the $2^{-\Delta\Delta C_T}$ equation (Livak and Schmittgen, 2001). The conditions of the PCR reaction were selected based on previous literature for primer amplification and standard laboratory protocols (Brodkin et al., 2004).
Chapter 9: References


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