UNIVERSITY OF STRATHCLYDE

Department of Pure and Applied Chemistry

Development of a Peptide Hydrogel with Tuneable Stiffness to Support Culture of Functional β-Cells (MIN6 cells)

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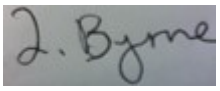
‘This thesis is the result of the author’s original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.’

Signed:  Date: 07/09/15

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Contents

Abstract

Pancreatic β-cells are responsible for production of insulin in response to an increase in glucose within the bloodstream. A failure of this process as in type I diabetes has devastating consequences for the body. The β-cells have also proven difficult to culture *in vitro*, exhibiting slow proliferation and loss of glucose-induced insulin secretion.

Peptide based hydrogels have previously been used successfully to support a wide range of cell types. In this study, peptide hydrogels that are formed by the co-assembly of two components, fluorenylmethoxycarbonyl-diphenylalanine (Fmoc-FF) and Fmoc-serine (Fmoc-S) were used. These were previously shown to support culture of bovine chondrocytes and human dermal fibroblasts but have yet to be tested with β-cells. The hydrogels have a tuneable stiffness, a factor demonstrated to be important for cell survival. A range of Fmoc-FF/S hydrogels of varying stiffness values were developed to test compatibility with the β-cells. The hydrogels were assessed in terms of cell viability and ability of the cells to retain their glucose sensitive insulin secretion.

First hydrogels of peptide concentrations 6 to 30 mM and stiffness values 0.5 to 23 kPa were generated. MIN6 cells cultured within these hydrogels in a 3D like manner showed the Fmoc-FF/S hydrogel of 25 mM peptide concentration and 14.4 kPa was able to sustain the greatest number of MIN6 cells ($5.5x10^4$) and showed the greatest glucose-induced insulin production at 15 ng/ml in low glucose (5 mM) and 24 ng/ml in high glucose (25 mM) conditions. The cells within this hydrogel not only retained their ability to secrete insulin in response to a change in glucose levels (5 to 25 mM) but were able to do so in a time appropriate manner, increasing from base level after 15 mins when glucose was increased from 5 to 25 mM, peaking at 90 mins and then returning to base level values when the glucose concentration was lowered again to 5 mM, similar to the physiological response, where β-cells respond depending on the concentration of glucose.

This work shows for the first time that the Fmoc-FF/S system is compatible with the β-cell type and the importance of tailoring matrix stiffness, already established to be an important factor in cell fate, viability and functionality in a range of other cell lines, in order

to best preserve the glucose-induced insulin secretion functionality of the MIN6 cells.

1. Introduction

1.1 The Pancreas and Insulin Production

The pancreas is a complex, glandular organ that possesses both endocrine (secretes directly into bloodstream) and exocrine (secretes into ducts) functions (Flay & Gorelick, 2004). It is located in the upper left abdomen, directly behind the stomach, with the head of the pancreas surrounded by the duodenum of the small intestine and the tail-end adjoining the spleen (Flay & Gorelick, 2004). The exocrine pancreas secretes enzyme containing-pancreatic fluid into the pancreatic duct, which enters into the duodenum to aid in digestion by further breaking down lipids and proteins. Breakdown of the digestive enzymes found in the pancreatic fluid can damage the organ, causing acute and chronic inflammation in a condition known as pancreatitis (Mitchell *et al.* 2003). The endocrine pancreas by contrast secretes hormones directly into the bloodstream. It contains many different cell types (α, β, δ, PP and ε) that cluster together to form discreet groups of cells known as the Islets of Langerhans, which are dispersed throughout the organ as seen in figure 1.

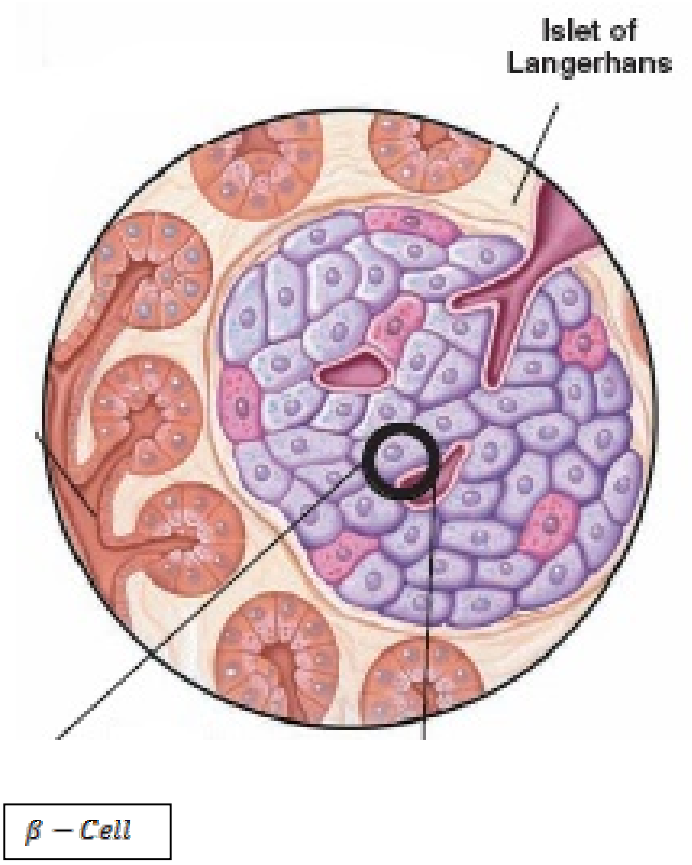


Figure 1: The islet of Langerhans, a cluster of the different cell types found in the endocrine pancreas, with the β-cell highlighted.

β-cells are the most abundant cell type in the Islet of Langerhans, making up 65-80% of the total mass. These β-cells are responsible for the production of the hormone insulin, which is key to the regulation of glucose levels in the body (Bonner-Weir, 2004). Other cells types found within the endocrine pancreas include α-cells, which make up 15-20% of the mass of the Islet of Langerhans, and secrete the hormone glucagon, an antagonist to insulin, which acts on the liver to increase glucose levels in the body when glucose levels are low. δ-cells contribute 3-10% of the total mass of Islet of Langerhans, and produce the hormone somatostatin that acts to inhibit the release of other hormones including insulin and glucagon. PP cells represent 3-5% of the mass of Islet of Langerhans and produce pancreatic polypeptide, which regulates secretions of both the exocrine and endocrine pancreas (Stefan *et al.* 1982). ε-cells can also be found in the Islet of Langerhans in small quantities, making up less than 1% of the total mass. ε-cells secrete the hormone ghrelin,

more commonly associated with the stomach and small intestine and acts on brain cells to induce the sensation of hunger (Andralojc *et al*. 2009).

Tumors can occur in the endocrine pancreas and represent 2-10% of all pancreatic cancers (Sliwinska-Mosson *et al,* 2014). Insulinomas, tumors of the islet cells are the most common form of endocrine pancreatic cancer, charcterised by increased production of insulin and so can be accompanied by hypoglycaemia (low glucose levels). Fortunetely, 90% of these tumors are benign and can be easily treated with somatostatin produced by δ- islet cells (Sliwinska-Mosson *et al*, 2014). Other forms of cancer that can occur in the pancreas includes pancreatic adenocarcinomas (Ryan *et al*, 2014). Approximately 85% of all pancreatic cancers are adenocarcinomas with this form of pancreatic cancer being the 8th and 9th leading cause of cancer death in men and women respectively. Furthermore, it has a 90% fatality rate, largely due to the disease not being easily diagnosable until an advanced state or post-metasisis (spreading of the cancer to other organs) and resistance to most forms of available therapies (Ryan *et al*, 2014).

Glucose regulation is an important homeostatic process to ensure that blood sugar is maintained within tolerable limits and is controlled by the endocrine pancreas. When glucose levels in the bloodstream rise, β-cells of the islet of Langerhans upregulate expression of the glucose transporter, GLUT2. Glucose then binds to the receptor and enters into the β-cell. Once inside the β-cell, glucose is phosphorylated into glucose-6-phosphate and metabolised through glycolysis, triggering the closure of cellular membrane potassium channels. The positive charge inside the β-cell causes depolarisation and opening of the calcium channels, allowing an increase in the intracellular Ca^{2+} ion concentration. This sharp increase in Ca^{2+} levels stimulates the release of insulin, which is then free to act on other cells (Newgard & McGarry, 1995). This process is depicted in figure 2.

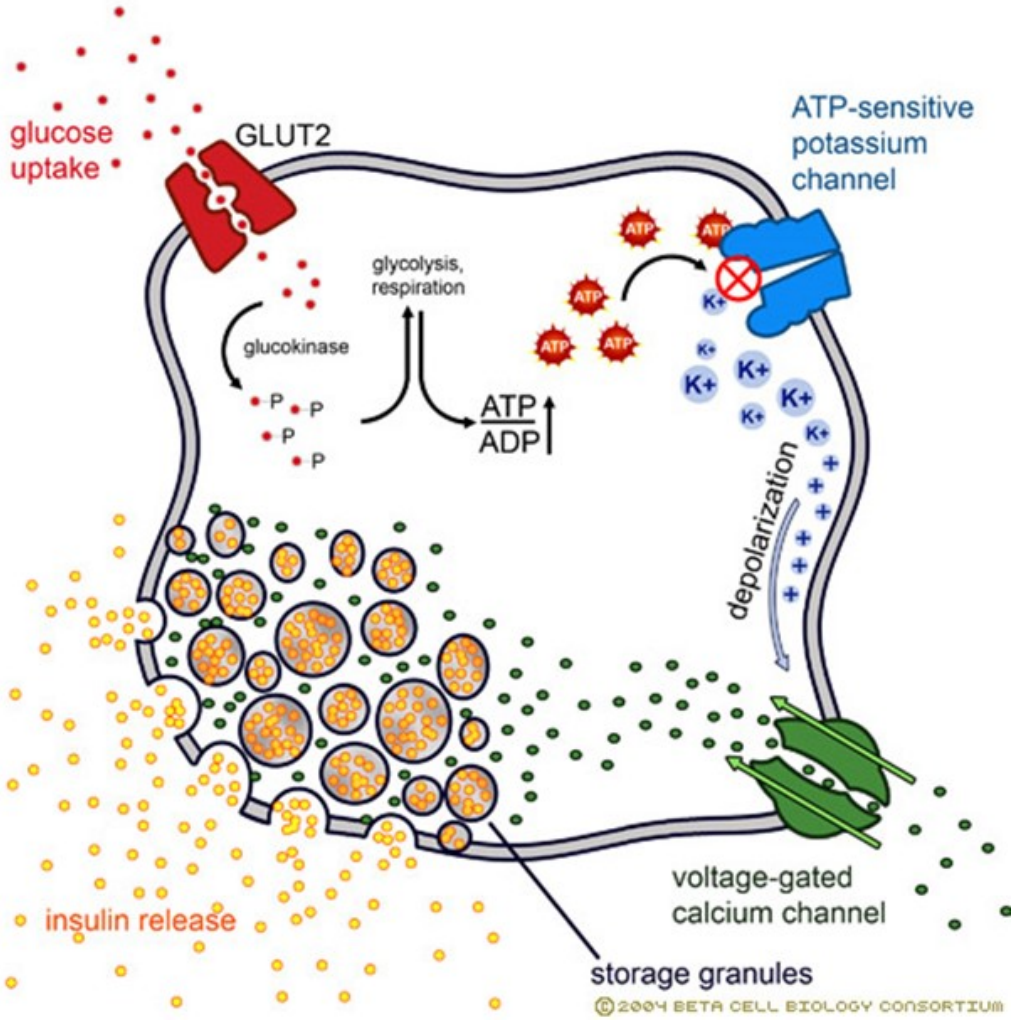


Figure 2: Transport of glucose into the β-cell and its susbsequent metabolism, triggering the release of insulin (Beta Cell Biology Consortium, 2015).

To facilitate the transport of glucose from the bloodstream into other cells, insulin binds to insulin receptors on the target cells' surface. This binding by insulin to the receptors enables the cell to uptake glucose by promoting expression of glucose transporters in the plasma membrane of the cell (Pang & Shafer, 1983). For example, the glucose transporter GLUT4 is upregulated by insulin in skeletal, fat and cardiac cells (Bryant *et al* 2002), GLUT3 is upregulated in brain, sperm and white blood cells (Simpson *et al*, 2008) and the GLUT1 receptor in red blood and endothial cells (Olsen & Pessin, 1996). GLUT2 is also expressed by hepatic, small intestine and renal cells (Efrat, 1997). As well as its role in promoting glucose uptake into cells, insulin has many physiological effects beyond that of providing energy for cell metabolism, in particular on the liver, which acts in conjunction with the pancreas to balance blood glucose levels. Other actions of insulin include; the storage of

glucose in the form of glycogen in the liver which can be converted back into glucose when levels are low, increased levels of lipid synthesis and inhibition of lipolysis, protein synthesis,increased potassium uptake (Benziane & Chibalin, 2008), and decreased sodium excretion by the kidneys (Gupta *et al* 1992).

1.2 Pancreatic Extracellular Matrix

In general, the extracellular matrix (ECM) is composed largely of proteins and polysaccharides, many of which are secreted by the cells with which it interacts, to provide a boundary and mechanical support for surrounding cells (Michel *et al.* 2010). It plays an important role in the survival and function of most cell types, for example through cell-cell communication and cell adhesion (Abedin & King, 2010). Different tissues and cell types throughout the body interact with unique combinations of ECM components e.g. different protein profiles. The communication and adhesive functions of the ECM are mediated through protein receptors on the cell membrane, e.g. cell-specific associated integrins that recognise peptide signalling sequences within the components of the ECM. An example is the peptide-derived signal sequence arginine-glycine-aspartic acid (RGD, using the one letter amino acid code in figure 4), contained within many of the proteins that are found in the ECM. This detection of the RGD signal sequence by the cell associated integrins promotes adhesion of the cell to the ECM [Pierschbacher and Ruoslahti, 1984]. Cell-integrin interaction with peptide sequences within the ECM components transmits cellular signals, resulting in focal adhesion, further integrins aggregating to the ECM binding site, initiating cascades that can effect genetic expression (Boudreau & Jones, 1999). This allows for regulation of processes such as adhesion, proliferation (cell growth), differentiation (cell specialisation) and cell survival [Larsen *et al.*2006].

β-cells of the pancreas are reliant upon the ECM for their survival as well as their ability to secrete insulin [Wang and Rosenberg, 1999]. The importance of this interaction between the β-cell and the ECM for survival is demonstrated by the levels of apoptosis that have been observed when these cells are removed from their pancreatic environment and grown in monolayers. For example, in one study a total of 60% apoptosis (in particular, anoikis of the adherent cells) was observed after two days of isolation of the pancreatic cells from the ECM [Rosenberg *et al.* 1999]. The pancreatic ECM is rich in the glycoprotein fibronectin, which displays the RGD sequence capable of binding integrins associated with the islets

cells. This not only allows for the adhesion of such cell types but also reduces the amount of apoptosis in mature pancreatic cells [Pinkse *et al.* 2006]. Another component abundant in the pancreatic ECM is the protein collagen, with type IV in particular being associated with the islet cells. Integrins displayed by these islet cells bind to collagen through non-RGD binding domains, such as GFOGER and GAOGER (where O represents hydroxyproline) [Farndale 2003]. This interaction has been shown to increase survival of the islet cells [Pinkse *et al.* 2006] as well as increasing insulin secretion by the β-islet cells [Weber *et al.* 2008]. The glycoprotein laminin is also highly dispersed throughout the pancreatic ECM. Laminins have been shown to affect adhesion and migration of many cells types and are found in many different isoforms (Ishikawa, et al., 2014). Laminin proteins also bind islet-associated integrins via non-RGD binding sites including YIGSR, PDSGR, IKLII and IKVAV. Studies have demonstrated the ability of laminins to improve secretion of insulin by the β-cells [Weber *et al.* 2007] as well as increase survival of pancreatic islet cells *in vitro* [Pinkse *et al.* 2006]. The structure of the laminin protein is shown in figure 3(B).

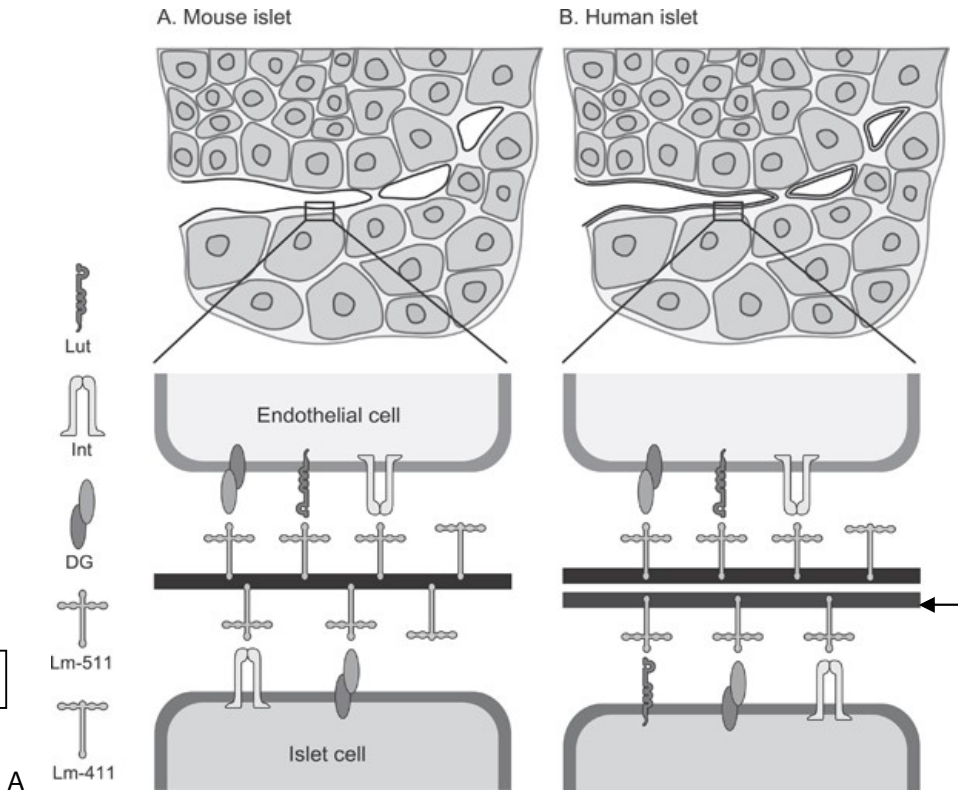
A study assessed the role different ECM proteins play in the survival and function of β-cells by investigating how the proteins collagen type I, IV, fibronectin and laminin improve islet cell survival and function *in vitro* in terms of adhesion, viability and functionality (Weber *et al.* 2008). To ascertain the effect of the different proteins on the islet cells, the proteins were coated onto a solid surface, where islet cells were then cultured in a 3D gel-matrix. The study found that use of both types of collagen increased adhesion of the cells, while fibronectin was important for retaining insulin releasing capabilities (Weber *et al.* 2008). Another study (Bogdani *et al.* 2014) has shown that changes to the composition of the ECM of islet cells may contribute to the pathogenesis of type 1 diabetes by allowing immune and inflammatory cells to enter the islet cells and cause dysfunction. Therefore maininting the microenvironment typical of healthy islet cells may be critical to maintaining healthy and functional cells in culture (Bogdani *et al.* 2014).

1.3 Pancreatic Basement Membrane

Another important feature within the pancreatic environment is the islet cells' interaction with the basement membrane (BM), a sheet-like component of the ECM [Otonkoski *et al*. 2008]. The BM of the human islet cell is particularly distinct from other cell types in the fact that it is double layered, surrounding both the supplying capillaries and the islets

themselves. The double layer provides the islet cells with their own, separate layer of the basement membrane rather than one, single layer shared with the endothelial cells of the blood vessels as is typical for other cell types (figure 3A) [Otonkoski *et al*. 2008].

The islet cells' BM has its own, unique laminin profile different from that of the endothelial cells, containing only laminin (lm)-511 [Virtanen *et al*. 2008]. Lm-511 contains 1 β, 1γ and 5α chains and is a trimeric protein found in the basal lamina portion of the BM, that promotes adhesion and helps maintain the phenotype of the cells that are bound through its integrin binding site. To further demonstrate the independence of the pancreatic cells' BM from that of the endothelial cells', it also lacks laminin-411 (1β, 1γ, 4α) found in the layer contacting the endothelial cells [Virtanen *et al*. 2008]. Laminin-511 has been shown to be relevant to the insulin producing capabilities of the β-cells [Kragl and Lammert 2010], by enhancing insulin release when the β-cell is exposed to glucose via increased mitochondrial activity and the upregulation of the glucose-phosphorylating enzyme glucokinase, which acts to promote insulin release by aiding in the metabolism of glucose within the β-cell [Johansson 2009]. The terminal ends of the laminin α-chain contain heparin binding domains, which influences the self-assembly of the laminin proteins [Yurchenco *et al.* 1990]. Heparin binding is also thought to affect the structure of the islet cells' BM by changing the conformation of the laminins to which it is bound and so plays an important role in the regulation of insulin secretion. The structure of the laminin protein is shown in figure 3B.



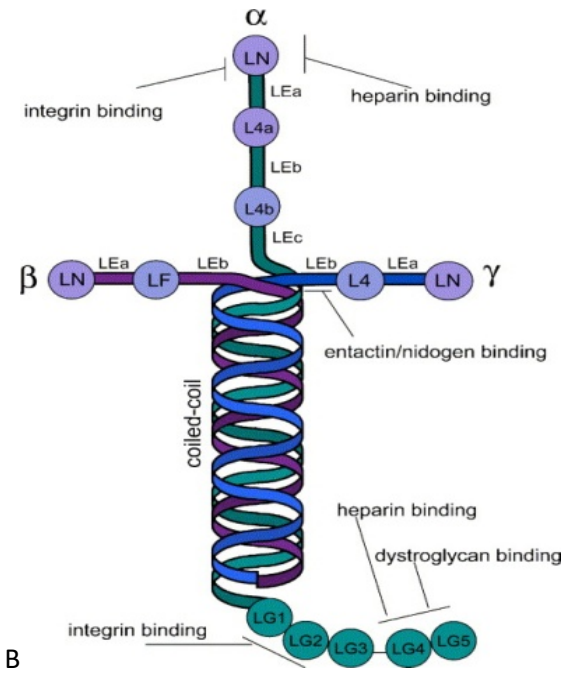


Figure 3: A, Comparison between the mouse and human islet cell, where the unique double membrane in the human pancreatic islet cell can be observed. It also highlights the interaction and relationship between pancreatic cells, basement membrane and

endothelial cells of the blood vessels, as well as the laminins discussed above. LM = laminin, Int = Integrins, Lut = lutheran Source: Otonkoski *et al.* 2008. B, Structure of the laminin protein, including each of its chains. The number of specific α, β and γ chains depends on the isoform of the protein. The heparin and integrin binding sites are highlighted. Source: Nguyet and Senior, 2006. LG = laminin globular domain, LN = Lamin N-terminal domain

1.4 Diabetes Mellitus

Diabetes mellitus is an extremely devastating disease characterised by glycaemic dysfunction (Zimmet *et al.* 2013). Globally, 382 million people were diagnosed with diabetes in 2013, with an estimated rise to 592 million by 2035 (Guariguata, *et al*. 2014). The highest prevalence of the disease was found in the Pacific island nation of Nauru, with 34% of the country affected (Zimmet *et al.* 2013). The incidence of diabetes is increasing across the world, causing a huge burden on many health care systems, particularly in low to middle income countries. In 2010 the worldwide cost of diabetes was $376 billion, 12% of the world's total health care expenditure and is expected to cost $472 billion by 2030. (Zimmet *et al.* 2013).

Diabetes mellitus falls under two distinct classifications: type 1 and type 2. Type 2 diabetes typically has an onset later in life; however there was a five-fold increase in the prevalence of the disease between 1995 and 2007 in those aged 15 and younger (Zimmet *et al.* 2013). Type 2 is defined by reduced levels and tissue resistance of insulin, as well as β-cell dysfunction. Although there are genetic susceptibilities [Ahlqvist *et al.* 2011], lifestyle factors such as obesity can initiate the disease process. This form of the disease can be treated with oral drugs such as metformin, thiazolidinediones, sulfonylureas etc. (Krentz *et al* 2008) and a reduction in body weight [Forbes and Wylie-Rosett, 2009]. However, progressive degeneration of β-cells can occur and so those with type 2 diabetes may eventually require insulin injections [Turner *et al.* 1997].

Type 1 diabetes mellitus on the other hand is characterised by the autoimmune destruction of insulin producing pancreatic β-cells, leading to a failure to maintain blood glucose levels [Montanucci *et al.* 2011]. Scandinavian populations exhibit the highest incidence of type 1, for example with a diagnosis rate of more than 60/100 000/year in Finland and 47/100 000/year in Sweden (Tuomilehto, 2013). The destruction of the β-cells is mediated by T-

cells, lymphatic cells derived from the thymus that normally fight off infections. The loss of the β-cells leaves the pancreas unable to produce insulin. Those at the highest genetic risk of developing diabetes have susceptible mutations in their Human Leukocyte Antigen (HLA) class II genes, the human major histocompatibility complex, whose molecules are involved in presenting antigenic proteins to T-cells. In particular two key genes are involved; one is HLA-DR that codes for a ligand in the T-cell receptor and the other is HLA-DQ. The protein coded for by the latter gene is involved in presenting antigens to T-cells, specifically in presenting self-antigens to regulatory T-cells that ensure the immune system does not recognise self-antigens as foreign and therefore eliminate the body's own cells [Nepom and Kwok, 1998]. The failure of these proteins to regulate normal T-cell responses to the β-cells can lead to the onset of diabetes. Environmental triggers of the disease are not yet fully understood but the onset of type 1 diabetes has been linked to viral and microbial infections (Regnell & Lernmark, 2013) as well as obesity and vitamin D deficiency (Munger *et al*, 2013).

The lack of insulin production that results from the destruction of the pancreatic β-cells has devastating physiological and pathological effects. For example, in the short term glucose uptake by the liver and muscles is prevented, leading to a build-up of toxic glucose levels in the bloodstream, further impacted by the release of glucose from the liver, a process normally suppressed by the insulin hormone. The result of glucose unavailability for metabolism is alternative energy sources such as fatty acids being used for cell survival. This leads to an overproduction of ketones, a breakdown product of fatty acids, and ultimately culminating in ketoacidosis, a clinical emergency state due to the electrolyte imbalance, dehydration, low blood pressure and cerebral oedema that is associated with the process [Kitabchi *et al.* 2006].

Although diabetes type 1 can be successfully treated with insulin injections it continues to cause long-term health issues for patients. These include macro/microangiopathy; damage to the blood vessels, which increases likelihood of cardiovascular disease as well as leading to retinopathy (damage to the retina) and nephropathy (kidney damage) [GSEDNu 2006]. Neuropathy (nerve damage) also occurs due to microangiopathy as well as direct damage to the nerves [Lluch *et al*. 1998]. Despite a compliance with insulin injections, some patients still fail to regulate blood sugar levels, known as brittle diabetes and suffer multiple hypoglycaemic (low blood sugar) episodes [Shapiro J *et al.* 2000]. As a result, a treatment

that would effectively reverse the disease process is highly sought and is an intensive area of research.

1.5 Islet-Cell Transplantation

Direct transplantation of pancreatic islet cells has been explored as means of treating diabetes type 1 but so far has failed to yield widespread success due to a lack of available donors and poor cell survival *in vivo*. The failure can be attributed to the inability of the islet cells to vascularise and reform a connection to the ECM [Chow *et al.* 2010].

Currently this form of treatment is limited to patients without consistent glycaemic control and those already undergoing renal transplantation due to the immunosuppressants required [Serup *et al.* 2001]. These immunosuppressants are potent drugs that suppress the immune system in order to prevent rejection and it has been shown that these drugs themselves have limited the success of islet cell transplantation. This limitation is due to damage caused to the β-cells within the islet clusters and promotion of insulin resistance in peripheral tissues [Shapiro J *et al.* 2000]. To date, 44% of participants in the Collaborative Islet Transplant Registry were non-dependent on insulin three years post-treatment. Despite continual improvement in the procedure and success of islet-cell transplants, many challenges still exist such as the continued poor availability of donor pancreata, low yield of pure islet cells during isolation and the difficulty in the culture of these cells (Bruni *et al*, 2014).

1.6 Proliferation of β-Cells

As one of the major limitations to the success of islet cell transplantations described in section 1.5 is low availability of the islet cells it has been attempted to first allow proliferation of donor cells *in vitro* in order to increase cell numbers for transplant (Lechner *et al*. 2005). It is also important to study islet cells *in vitro* in order to better understand the cells' biological functions and the role the islet cells play in diseases such as diabetes [Skelin *et al* 2010]. However, pancreatic cells have proven difficult to study *in vitro* due to their slow proliferation rates, loss of morphology and glucose-induced insulin secretion function when removed from their natural environment [Gallego-Perez *et al.* 2012]. They also

exhibit high levels of apoptosis once isolated from the pancreas (Rosenberg *et al* 1999). Given the crucial role of the ECM and the influence of the mechanical environment on β-cell viability, encapsulation and growth of islet cells within biological and synthetic matrices has been attempted to preserve β-cell viability and functionality *in vitro* e.g. the co-culturing of islet cells with the growth factor hormone prolactin on a laminin-111 based matrix [Labriola *et al.* 2007]. Both the prolactin hormone and the laminin protein have been shown to be important for normal cell development in the pancreas. The study showed this approach to be successful, with the pancreatic cells being able to not only proliferate but retain insulin releasing capabilities in response to glucose, a feature often lost in culture [Gallego-Perez *et al.* 2012]. However this method was only conducted with the cells cultured in 2D and requires an exogenous source of the hormone [Labriola *et al.* 2007]. Another study developed a polycaprolactone-gelatin (PCL) matrix, a polyester based gel that provided a solid structure while allowing for binding of pancreatic cells. Advantages of this technique are that it allows the pancreatic cells to properly cluster and anchor in a 3D manner, promoting long-term survival *in vitro.* It is also biodegradable, so has the capacity to be developed further for *in vivo* applications (Gallego-Perez *et al*, 2012).

1.7 Matrix Stiffness

As was described in section 1.2, the ECM plays a vital role in the survival and functionality of cells *in vivo*. As cells that are cultured *in vitro* are no longer able to interact with the ECM, they rely upon interactions with the matrix in which they are grown. Therefore it is important that the microenvironment of the cells in culture closely resembles that of the native ECM (Mammoto *et al*, 2008). One of the most vital roles played by the ECM is mechanical signalling, which can affect cell morphology, adhesion and proliferation etc. (Discher *et al*, 2005) and so matrix stiffness is an important aspect in matrix design. The important role of matrix stiffness was clearly demonstrated in a study that involved controlling the stiffness modulus of a collagen-1 coated polyacrylamide matrix in which mesenchymal stem cells were cultured, with cells grown in different matrices of varying stiffness differentiating along different lineages due to mechanical interactions between the cells and the matrix (Engler *et al*, 2006). For example stem cells that were cultured on a soft matrix differentiated into neuron-like cells, while those cultured within a rigid matrix became osteogenic-like cells (Engler *et al*, 2006). Further investigations of the effect matrix

elasticity has on a range of different cell types has been conducted. An example is a study on the importance matrix stiffness has in relation to the cell morphology of cardiac myocytes (McCain *et al.* 2014). In this study, myocytes were cultured in polyacrylamide matrices that represented elasticities typical of both healty (13 kPa) and pathological (90 kPa) myocardium, with the myocytes cultured on stiffer matrices having wider morphologies, possibly contributing to the disease state (McCain *et al.* 2014). Stiffness of the ECM has also been shown to be important in cancer development (Taddei *et al*, 2013). These studies show the diverse effects the stiffness of the ECM can have on a range of cell types and the consequences of changes in the mechanical properties, from directing stem cell fate (Engler *et al*, 2006) to participating in disease processess such as heart disease (McCain *et al.* 2014) and cancer (Taddei *et al*, 2013). The signifigance of the effects of ECM stiffness in a myriad of biological processes highlights the importance of controlling the mechanical properties of the matrices used for cell culture to maintain the natural environmental niche of the cell under study. The stiffness modulus values for many organs/tissues are already defined (Collier *et al*, 2010); however this is still to be established for pancreatic tissue. Studies on the effect of matrix stiffness specifically on the islet cells have yet to fully establish the relative importance of this feature and its implications for islet cells survival, viability and function (Raza *et al*, 2013).

1.8 Utilisation of Stem Cells

Stem cells are undifferentiated cells that have the capacity to specialise into a number of cell types and so play a vital biological role in tissue generation. The number of cell types an individual stem cell has the capacity to differentiate into depends greatly on its origin. Embryonic stem cells, derived from the developing embryo, are described as pluripotent as they have the ability to become any cell type dependent on a multitude of genetic, environmental and biochemical cues [Thomson *et al.* 1998]. Stem cells can also be derived from adult tissue and are mostly described as multipotent as there are a limited number of cell types into which they can differentiate. An example are mesenchymal stem cells, which are found in the adult bone marrow and are known to have the capacity to become chondrocytes of cartilage, osteoblasts of bone or adipocytes of fat [Short *et al.* 2003]. Other adult-derived stem cells include induced-pluripotent stem cells, where adult cells can

be programmed to take on embryonic-like stem cell characteristics by the addition of key transcription factors [Takahashi and Yamanaka, 2006].

Researchers have recognised the great potential of these stem cells in the area of regenerative medicine and so have exploited them in the possible treatment of diabetes [Thomson *et al.* 1998]. In one study, mouse embryonic stem cells (EC) were transfected with the human insulin gene via a plasmid vector and then cultured in the presence of glucose before being implanted into mice [Soria *et al.* 2000]. This resulted in 60% of the diabetic induced animals returning to normal glucose control, with the other 40% remaining hyperglycaemic (high glucose levels) 12 weeks after transplantation. However, the mice that had received the transplant of EC-derived-β-cells took much longer for their insulin levels to return to normal after exposure to glucose than did the non-diabetic mice (210 min opposed to 120 min). Although a promising method, issues such as possible rejection and tumour formation still exist thus this area needs further study before becoming a viable method of treating diabetes [Soria *et al.* 2000].

Other studies have utilized bone-marrow derived adult stem cells in the regeneration of insulin-secreting cells. In one such study stem cells from the bone marrow of adult mice were injected directly into irradiated mice in order to replace the cells which were lost in the radiation process, ensuring only stem-cell derived cells were present. Once in the pancreas these cells were able to differentiate into β-like cells, exhibiting the characteristics of normal β-cells, e.g. they were able to secrete insulin in response to glucose and expressed expected transcription factors. This method by-passed the need for complex biochemical and genetic signals normally required for differentiation to occur by allowing this process to proceed biologically. Although this approach was successful, the differentiation occurred only *in vivo* thus other studies were needed to establish the mechanisms that govern the route from adult bone marrow stem cell to pancreatic cell [Ianus *et al,* 2003]. To examine further how transplanted stem cells can reverse the diabetic state embryonic stem cells were differentiated *in vitro* into insulin producing cells and labelled (Ren *et al*, 2010) before transplantation into mice. After one week insulin positive cells were detected in the spleen and were able to maintain glycaemic control (Ren *et al*, 2014).

1.9 MIN6 Cells

As previously described in sections 1.2 to 1.6 pancreatic islet cells have proven to be difficult to study *in vitro* due to large volumes of apoptotic cells when removed from the pancreatic environment (Rosenberg *et al*, 1999), as well as slow proliferation rates and loss of glucose sensitive insulin secretory function [Gallego-Perez *et al.* 2012]. Primary human β-cells are also difficult to ascertain due to limited availability of pancreatic tissue as well as difficulty in isolating the cell type [Skelin *et al* 2010] As a result many different approaches have been taken to improve the ability of islet cells to thrive *in vitro* by developing model systems, utilising a number of different cell types to represent the islet cells, particularly the β-cells crucial for the insulin production function sought to be maintained (Nichols *et al*, 2014). Many different cell lines are available that can be used to represent the β-cell in model systems. One of the earliest cell lines made available were rat insulinoma cells (RIN) derived from pancreatic tumours that secrete insulin. These cells maintain their ability to secrete insulin *in vitro* but lose their sensitivity to glucose [Halban *et al* 1983]. Another tumour cell line that is available is INS-1, which retain the capacity to secrete insulin in response to varying glucose levels. However the total insulin content of INS-1 cells is 80% less than native β-cells. They also require mercaptoethanol for proliferation, a toxic chemical that can cause protein denaturation [Asfari *et al* 1992].

An alternate cell line however are Murine-Derived insulinoma (MIN6) cells, which have the same morphology and physiology as normal β-cells but are much more tolerant of *ex vivo* laboratory conditions. These MIN6 cell lines, isolated from insulinomas in transgenic mice, have shown great potential in retaining insulin producing capability in culture when exposed to glucose. For example, it was shown in 2D cell culture that MIN6 cells were capable of producing a marked increase in insulin release when they were exposed to 25mmol/l glucose than when exposed to 5mmol/l glucose, similar to that of healthy β-cells, which release insulin following detection of an increase in glucose concentration [Miyazaki *et al.* 1990]. This is comparable to that of the normal physiological situation, where blood glucose concentrations are typically 4 - 5.9mmol/l pre-prandial (before meals) and rise to around 7.8mmol/l up to 2 hours after eating, inducing insulin secretion [International Diabetes Federation, 2007]. Furthermore, it was demonstrated that glucose transport, phosphorylation and utilization was also comparable to that of normal islet cells, thus MIN6

cells provide a good model for evaluating islet cell behaviour. Another feature that makes this cell line desirable as a comparative of normal beta cells is the high expression in these cells of GLUT 2, a glucose transport receptor found in both the pancreas and liver when the cells are exposed to glucose. It plays a key role in glucose sensing and thus glucose-induced insulin secretion [Efrat 1997]. In conjunction to this the cells exhibit low expression of the GLUT 1 transport receptor when exposed to glucose, the expression of which is high only when glucose levels are low [Olson and Pessin 1996). Again, all of which is similar to normal pancreatic beta cells [Ishihara *et al*. 1993].

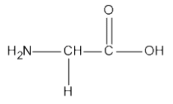
1.10 Hydrogel Matrices

In recent times, biomolecular materials e.g. hydrogels, that have the capacity to replicate the natural environment of cells have become an intensive area of research in cell culture and tissue regeneration (Cosson *et al*, 2015). The materials that make up these systems are composed of molecular polymeric or nanofibrous networks that span the sample and immobilise the aqueous phase, thus all hydrogels have in common a high water content (typically >90%) while in the bulk they behave as solids (Seliktar, 2012). The success of such materials in directing stem cell differentiation [Dawson *et al.* 2007] and sustaining cells in culture [Montanucci *et al.* 2011] leads to the promise of using such technologies to sustain functional pancreatic cells, which may in the future lead to the development of *in vivo* treatment of diabetes. There are many different examples of these systems, each with varying successes and limitations**.** Many different compounds can act as the basis for these hydrogels including but not limited to; the polyether, polyethylene glycol (PEG) (Weber *et al*, 2008), natural proteins such as collagen and laminin (Zhang *et al*,2012), as well synthetic peptides (Zhang) and peptides functionalised with non-peptidic moieties to induce self-assembly properties- such as aliphatic peptide amphiphiles (Lao & Tong, 2011) and aromatic peptide conjugates (Jayawarna *et al*, 2009).

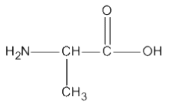
Self-assembling peptide based hydrogels have been used widely in the area of cell culture due to the ease of their formation and ability to be modified for desired characteristics such as hydrophobicity (Castillo Diaz et al, 2014), (Seliktar, 2012). These peptide hydrogels can then be used to represent the ECM as the proteins found within the membrane are

themselves composed of smaller building blocks: simpler peptide sequences that can be replicated in the design of the hydrogel. Peptide hydrogels are produced via the self-assembly of designed peptide building blocks with specific sequences. The sequences that make up these peptides are composed of amino acids (AA), the chemical building blocks for proteins coded for in DNA. Amide bonds form between the amide group of one amino acid and the carboxyl group of another to produce a peptide, with further extensions to form a polypeptide chain. By taking advantage of these sequences in the design of hydrogel scaffolds the natural functions of the incorporated peptides can also be incorporated into the hydrogel matrix and utilised for a number of purposes in the application of cell culture [Perez *et al.* 2011]. Amino acids are able to confer different properties to peptides and ultimately the materials into which they are incorporated due to differences in their side chains, such as adding aromaticity (e.g. histidine) or hydrophobicity (e.g. phenylalanine). It is the features of the side groups which are used to classify the amino acids, normally as follows: non-polar, aliphatic, aromatic, positively charged, negatively charged, and polar, uncharged (figure 4).

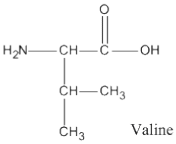
Non polar, aliphatic



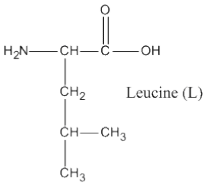
Glycine (G)



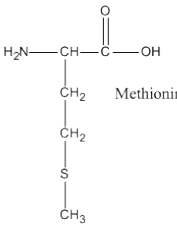
Alanine (A)



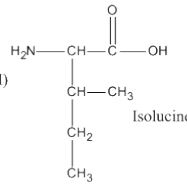
Valine (V)



Leucine (L)

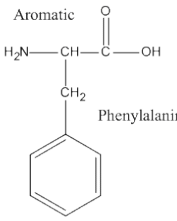


Methionine (M)

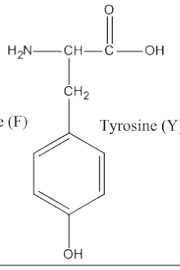


Isolucine (I)

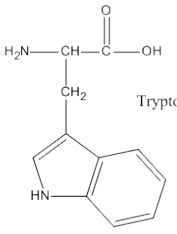
Aromatic



Phenylalanine (F)

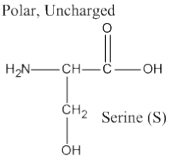


Tyrosine (Y)

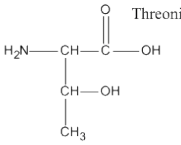


Tryptophan (W)

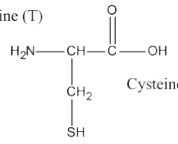
Polar, Uncharged



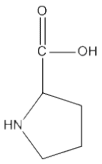
Serine (S)



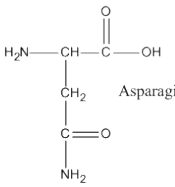
Threonine (T)



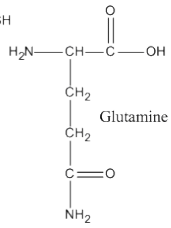
Cysteine (C)



Proline (P)

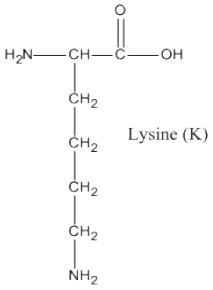


Asparagine (N)

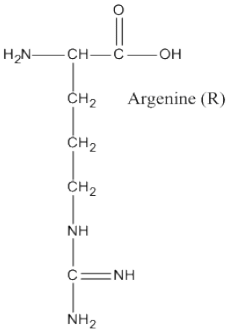


Glutamine (Q)

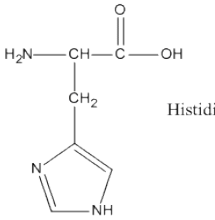
Positively Charged



Lysine (K)

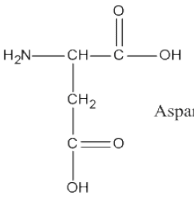


Argenine (R)

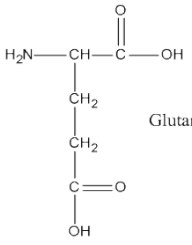


Histidine (H)

Negatively Charged



Aspartic Acid (D)



Glutamic Acid (E)

Figure 4: Structure of the 20 amino acids coded by DNA, classified by their R/side groups. One letter code for each is also given.

Once amino acids have covalently coupled to form peptides, these peptides can form more complex structures due to intramolecular H-bonds, resulting in the secondary structures of α-helices or β-sheets. The structure which the peptide ultimately takes is influenced by the amino acid sequence, with different amino acids having different propensities to form either the alpha helix or the β-sheet due to the structure of the amino acid itself and features of the side chain [Pace and Scholtz 1998].

Alpha helices, the structure of which was elucidated by Pauling in 1951 (shown in figure 5A), consist of twists of peptide chains, formed due to the hydrogen bonding between the amide N-H group and the carbonyl of another amide group, 4 residues further along the chain. The most common form is the right-handed helix, which has 3.6 residues per turn, meaning each amino acid equals a 100° turn. Less common is the left-handed form, which occurs when there are many glycine residues present in the helix. Alpha helices are tightly packed, with the amino acid side chains on the outside [Pauling *et al.* 1951]. Amino acids such as methionine, alanine, leucine and lysine have a tendency to form the α-helix structure, possibly due to the length of their side-chains reducing conformational entropy for interactions within the helical structure [Pace and Scholtz 1998].

β-sheets (shown in figure 5B) on the other hand are composed of peptide strands, forming an extensive backbone due to H-bonding between N-H and C=O groups in adjacent strands. The arrangements of these sheets can be parallel, anti-parallel or a mix of both. The anti-parallel arrangement is the most stable due to the N terminus of one strand being adjacent to the C-terminus of another, allowing for planar orientation of the H-bonds, their preferred state. In the parallel form, the N-termini align in the same direction, meaning that the H-bonds between the N and C-termini are non-planar [Pauling and Corey 1951]. Amino acids such as phenylalanine, proline and valine favour the β-sheet structure [Richardson and Richardson 2002].

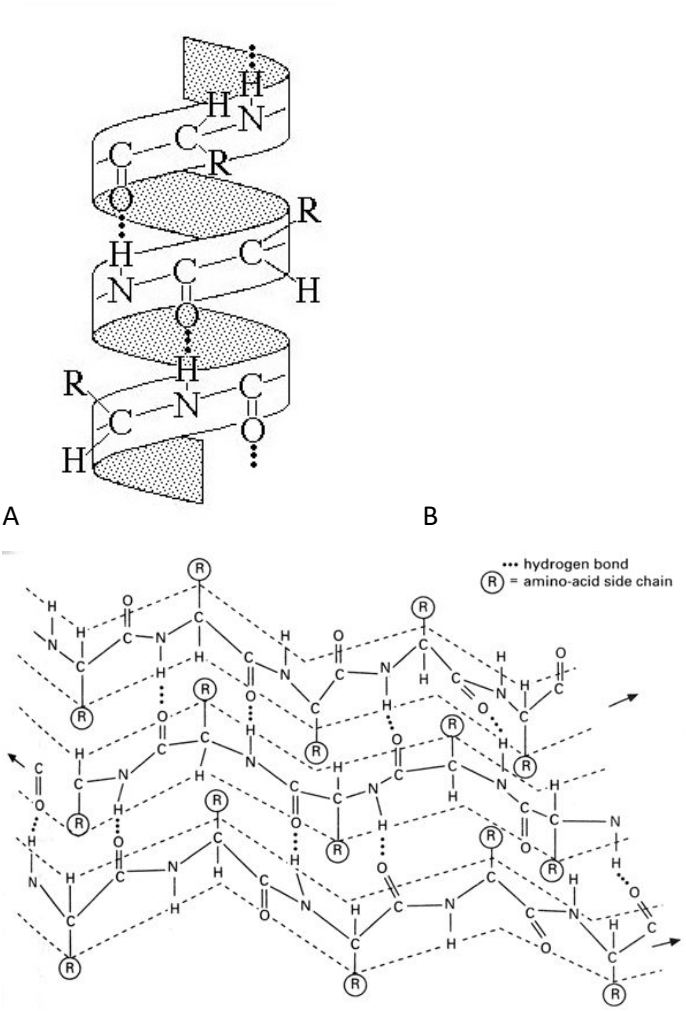


Figure 5: (A) structure of the alpha helix and (B) structure of the β sheet. Both show the H-bonding that leads to the above structures. R= amino acid side chain. Source: Oxford Dictionary of Chemistry, 2008.

The first study to take advantage of peptides in the generation of self-assembling systems for cell culture was work by Zhang *et al*, with the design of an oligopeptide capable of self-assembling into a macroscopic membrane with the β-sheet conformation [Zhang *et al.* 1993]. The peptide blocks of Zhang's system contained a sixteen residue sequence of alternating hydrophobic and hydrophilic amino acids, EAK16 that assembled into an insoluble membrane when in the presence of a salt. The structure of the resulting membrane was due to the β-sheet formation, allowing the charged amino acids (positively charged lysine and negatively charged glutamic acid) to align, and so interact with complementary positive and negative ionic groups [Zhang *et al.* 1993]. Further work by Zhang in the design of another amphiphilic oligopeptide RAD16, containing positively

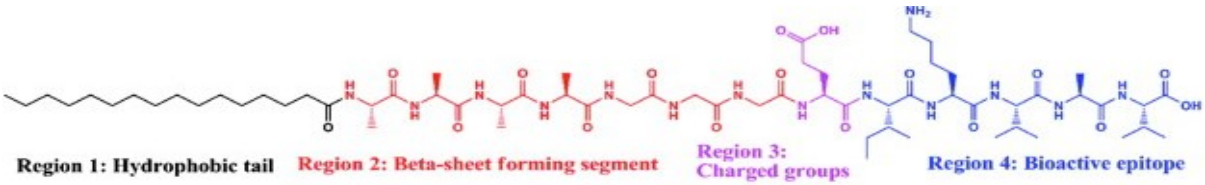
charged arginine and negatively charged aspartic acid, separated by hydrophobic alanine residues was shown capable of supporting mammalian cell types. The peptides of the RAD16 gel interact to form β-sheets (see figure 5B), which then further interact forming filaments. It is these filaments that lead to formation of the membrane like structure to which cells can attach. The cells tested, e.g. mouse and human fibroblasts, were able to adhere to the RAD motif. This work demonstrated the possibility of using peptide based biomaterials as substrates in cell culture, allowing a variety of cell types to attach to the self-assembled matrix and survive for a prolonged period of time (2 week incubation) [Zhang *et al*. 1995]. RADA peptides have been shown to support a wide range of cell types such as hepatocytes, chondrocytes, osteoblasts and murine embryonic cells amongst many others [Mershin *et al*. 2005].

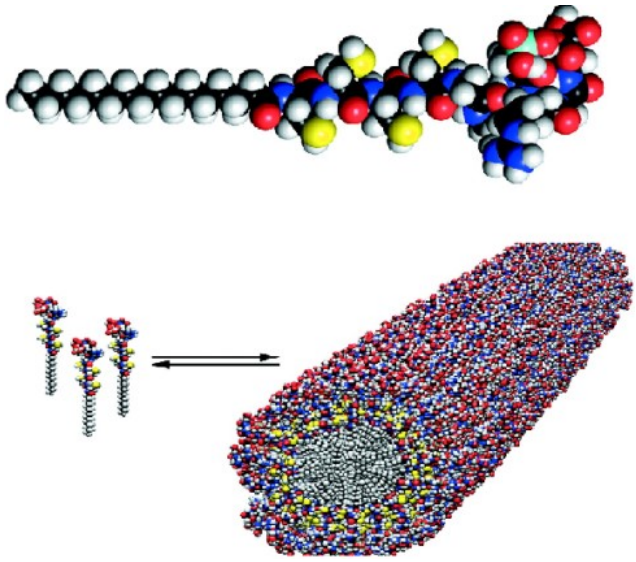
Following the work by Zhang, a study by Pochan *et al* demonstrated the formation of a peptide hydrogel containing the β-hairpin conformation. These β-hairpins, present within the β-strands, occur between two strands that are adjacent in the anti-parallel configuration, and link the strands together in a short loop of 2-5 amino acids. An example of this is the peptide MAX3, which contains a repeating hydrophobic/hydrophilic VKVK motif, KVKV after the β-hairpin turn, which is essential to the self-assembly of the peptide into a hydrogel. Hydrogelation was thermally triggered, allowing self-assembly to occur when the temperature increased, a process which was reversible upon cooling of the peptide, leading to the unfolding of the β-hairpin structure [Pochan*et al.* 2003]. A similarly designed peptide, MAX1, was shown to be able to self-assemble into a hydrogel when in the presence of DMEM cell media. MAX1 contained a 20 amphiphilic amino acid residue sequence of repeating lysine and valine residues with the β-hairpin turn within a β-sheet conformation. The self-assembly was thought to occur due to charge screening, the reduction of the strength of the charged residues induced by the cell media. The hydrogel formed from this peptide sequence was also shown to be cyto-compatible with mouse fibroblast cells, allowing for their attachment and proliferation [Kretsinger*et al*. 2005]. This study clearly demonstrates the advantage of peptide systems, particularly in their application for cell culture.

Another example of peptide-based systems for use in cell culture are peptide amphiphiles (PA). Peptide amphiphiles are synthetically designed peptide systems that have both a hydrophobic and hydrophilic nature. They contain a hydrophobic alkyl tail that promotes

self-assembly through collapse when in the presence of water. The tail is covalently bonded to a hydrophilic peptide sequence, conferring the amphiphilic nature of the molecule. The peptide sequence also allows for self-assembly into the β-sheet structures, as well as conferring characteristics such as flexibility e.g. through glycine residues. It also contains a short bioactive peptide group (such as IKVAV in figure 6) that can be manipulated and changed depending on specific needs [Ghanaati et al 2009].

[A]





[B]

Figure 6: [A] Structure of a peptide amphiphile:(left to right) the hydrophobic, alkyl tail, section of the peptide sequence that allows for β-sheet formation, the charged groups that along with the hydrophobic tail confers the amphiphilic nature of the peptide, and the bioactive epitope (active group) that provides the functionality of the peptide. [B] Molecular model of the amphiphile from the alkyl tail (left) to the peptide region (right) as well as the self-assembly process into a cylindrical micelle (bottom). Adapted from Tan *et al.* 2012 and Hartgerink *et al.* 2001.

PAs can be designed for a multitude of specific purposes by variation of the incorporated active groups and can mimic a wide range of biological systems. Biological mimicry can be achieved by addition of key peptide sequences found in proteins that are vital to the natural role of the protein. Specific peptide amphiphiles have been designed [Lao and Tong, 2011] to imitate the function of collagen, one of the ECM proteins, which has already been shown to improve the adhesive qualities of pancreatic cells [Daoud *et al.* 2010], with implications for their survival *in vitro*. An example is a study by Lao and Tong (2011), where a self-assembling peptide amphiphile was developed that included the key sequence found within the collagen protein, GFOGER, which was subsequently displayed by the nanofibres of the PA. The resulting peptide nanofibre was able to mimic the biology of collagen fibres by displaying this GFOGER sequence, allowing cell adhesion when recognised by cell membrane receptors [Lao and Tong, 2011]. The collagen-mimetic PA is depicted in figure 7.

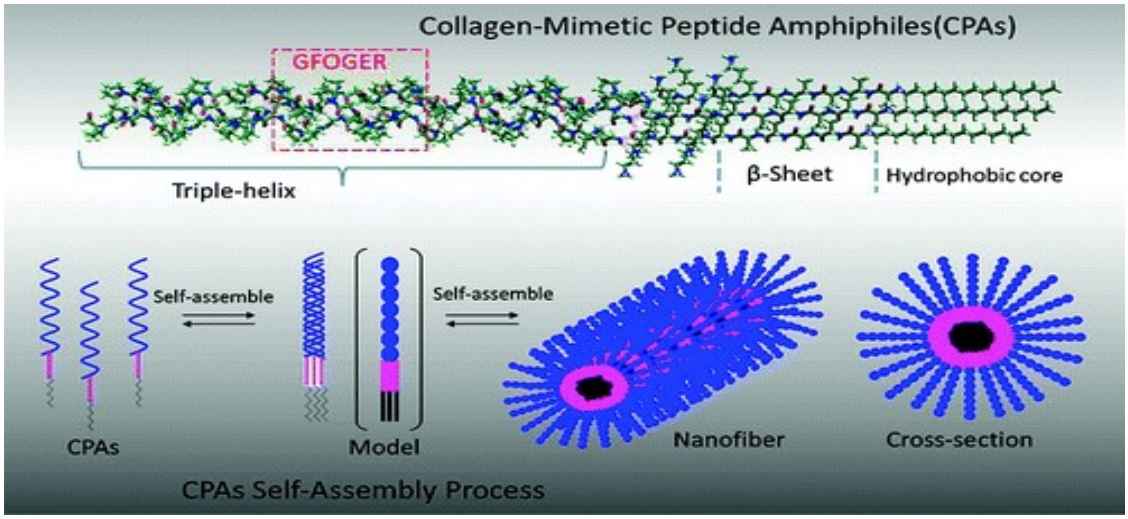


Figure 7: The structure of the collagen-mimetic peptide amphiphile, with the GFOGER sequence that is subsequently displayed by the nanofibres highlighted. Adapted from Lao J and Tong Y.W (2011)

1.9.1 Fmoc-Peptide Systems

Fluorenylmethoxycarbonyl (Fmoc)-peptide based hydrogels are able to self-assemble largely through the overlap of side-to-side p orbitals, which form π-bonds in the aromatic rings of fluorenyl. The π-bonds of the fluorenyl molecules further interact with π-bonds in other fluorenyl molecules in the form of π-π stacking. H-bonding of the carbonyl and NH functionality in the peptide backbones also contributes to the self-assembly process [Tang *et al.* 2009]. Fmoc-peptide gels can be designed with a range of properties by variation of the amino acids sequences. Examples of Fmoc-peptide gels include the di-peptide sequence diphenylalanine (FF), with the structure shown in figure 8(A), which is capable of self-assembling into a hydrogel at neutral pH, forming anti-parallel β-sheets. Four of these β-sheets twist and form a cylindrical structure via π-π interactions [Smith *et al.* 2008]. It also forms fibres 43-69nm in diameter, comparable to those of the ECM, making it a suitable hydrogel for cell culture [Jayawarna *et al.* 2006]. Fmoc-FF can be further functionalised via co-assembly with Fmoc-amino acids such as Fmoc-Serine (S), the structure of which is shown in figure 8(B). Fmoc-S is thought to assemble on the surface as a surfactant, coating an Fmoc-FF core similar to that reported for the Fmoc-Tyrosine Leucine (YL)/S system (Fleming *et al*, 2014) Fmoc-FF/S has been shown to be compatible with many cell types e.g. bovine-derived chondrocytes and human dermal fibroblasts and has a tuneable stiffness important for cell culturing purposes [Jayawarna *et al.* 2009].

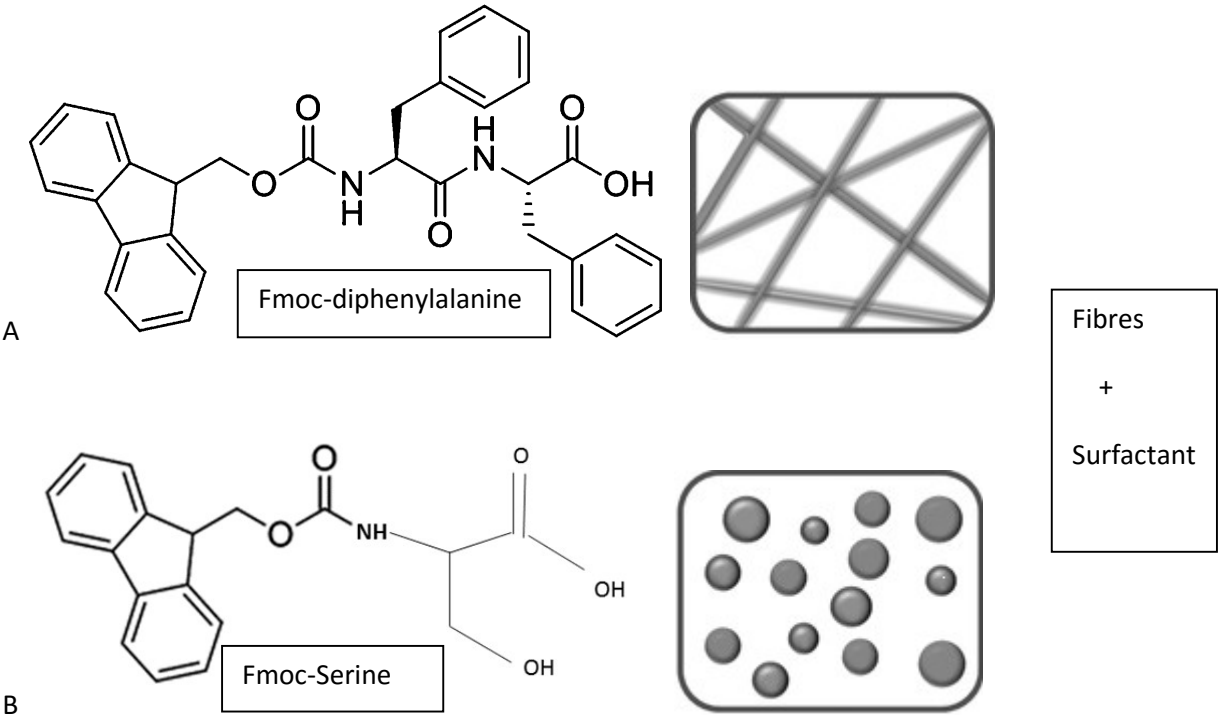


Figure 8: (A) Structure of Fmoc-FF, which forms nanofibres and co-assembles with (B) Fmoc-S, which forms a surfactant on the Fmoc-FF/S core. Adapted from Abul-Haija *et al* 2014.

1.11 Aims and Objectives

The aim of this study was to explore different protocols to develop reproducible Fmoc-FF/S hydrogels with a range of stiffness that are suitable for optimal pancreatic β-cell culture by enabling maintenance of MIN6 cell viability and insulin secretion capabilities.

Various protocols for hydrogel preparation were explored to create hydrogels of varying stiffness in order to generate a wide range in which to test compatability with the MIN6 cell type due to the unknown matrix stiffness preference of this cell type. Ranges from 0.5 kPa, the known upper stiffness value for brain tissue, to 23 kPa, in the range of skeletal muscle were analysed as it was hypothesised that pancreatic tissue should fall between these measurements The Fmoc-FF/S hydrogels were assessed by fluorescence spectroscopy and rheology to ascertain gel formation and matrix stiffness values. Suitability of the peptide hydrogels to preserve viability and the glucose-induced insulin secretion of the MIN6 cells

was examined by use of the Alamar Blue viability assay and cell function assessed using an insulin immunoassay.

2. Materials and Methods

2.1 Materials

The cell line used in these experiments was MIN6 donated by Prof Peter Jones, Kings College, London with permission from Prof J Miyazaki, University of Osaka. Cell culture materials were all purchased from Invitrogen, UK. All chemicals were purchased from Sigma Aldrich, UK except for Fmoc-FF which was purchased from Biogelx and Fmoc-S which was obtained from Bachem. The insulin immunoassay kit was ordered from Millipore and the inserts used in hydrogel formation from Greiner Bio-One.

2.2 Methods

2.2.1 Peptide Solution Preparation

Peptide solutions were prepared by mixing various volumes Fmoc-FF and Fmoc-S with 4 ml of distilled H_2O and 0.5 mM NaOH. The mixture was then exposed to vortex and sonication until a homologous solution was achieved. O.1 mM HCl was then added and the solution before brief vortexing in order to control pH. The peptide solutions were then refrigerated overnight.

2.2.2 Vortex and Sonication Controlled Hydrogel Preparation

Fmoc-FF/S peptide solutions were prepared at a concentration of 20 mM by the addition of 0.0214 g of Fmoc-FF [Bachem] and 0.0138 g of Fmoc-S [Bachem]. 150 µl of 0.5mM NaOH was then added to the solution, which was subsequently dissolved via the process of vortexing and sonication. The exposure time to each of these processes was varied, with the samples alternated between the vortex and sonicator after one minute.

The samples were mixed using a Scientific Industries Vortex Genie 2 at the highest rpm setting for one minute, followed by sonication using a VWR USC100TH, Ultrasonic Cleaner sonicator for one minute, alternating for a total of 5,6,8,10 and 12 minutes.

2.2.3 pH Controlled Hydrogel Preparation

To produce peptide samples with different pH values a 20 mM Fmoc-FF/S was prepared using 0.0214 g of Fmoc-FF [donated by Dr Sangita Roy], 0.0138 g of Fmoc-S, 4 ml distilled H_2O and 150 µl 0.5 mM NaOH. To dissolve the peptides, the samples were exposed to 30 seconds of sonication, followed by 1.5 minutes of vortexing, repeated for a minimum of five minutes, until a homogenous, clear solution was observed. The solutions were then left for 30 minutes to ensure they were fully dissolved.

To adjust the pH of each of the samples, 10, 15 and 20 µl of 0.1 mM HCL was added to bring the pH to 8.7, 7.2 and 6.9 respectively. The pH of the samples were determined using the Jenway 3510 pH probe reader.

2.2.4 Peptide Concentration Controlled Hydrogel Preparation

The concentration of Fmoc-FF/S samples were prepared initially at 6 mM (0.0128g Fmoc-FF, 0.0082g Fmoc-S), 20mM (0.0214g Fmoc-FF, 0.0138g Fmoc-S) and 30mM (0.0321g Fmoc-FF, 0.0207g Fmoc-S) in 4ml of distilled water. The volume of 0.5mM NaOH was added as needed to fully dissolve the samples (ranged from 85 to 250µl).

The samples were alternated between the vortex for 1.5 minutes and the sonicator for 30s until the samples appeared dissolved. The volume of 0.1mM HCl added was dependent on the pH of the samples after they had dissolved, being added until the pH of all the samples were 7.2.

Subsequent concentrations of 10, 15, 18, 20, 22, 25 and 30 mM were prepared to generate a range of hydrogels for cell compatibility testing. The samples were exposed to vortex and sonication for 5-9 mins with the pH kept below 11. 0.1 mM HCl was then added until the pH of all samples was 7.5, with the suspension syringe filtered and left overnight.

2.2.5 Hydrogel Formation

For use in cell culture peptide solutions were sterilised under UV light for 1 hr prior to hydrogelation and the hydrogel prepared within a cell culture hood cabinet to prevent contamination. For the 2D cell culture studies, MIN6 cells were diluted in media to concentrations of 0.5, 1 and 1.5 x10^5/µl, with 300 µl of the cell-containing media placed on the surface of the peptide hydrogels. For 3D cell culture studies MIN6 cells were incorporated throughout the layers of the hydrogels by the addition of the MIN6 cells to the peptide solutions used to form the hydrogels. This method is illustrated in figure 9.

To form the hydrogels from the peptide solutions 1.4 ml of Dulbecco's Modified Eagle Medium (DMEM) cell media containing 4.5g/l glucose, L-glutamine, penicillin/streptomycin and 10% FBS was added to a 12 well plate, with 300µl of the peptide solution added to inserts that were positioned in the media containing well. The plate was placed in an incubator set at 37˚C and 5% CO_2 and left for 1.5 hrs, after which time the media in the wells were replaced and 300µl of fresh media added to the inserts. The plate was placed back inside the incubator and left overnight. The plate was only removed immediately prior to analysis.

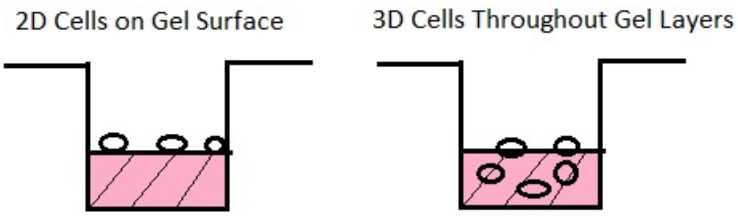


Figure 9: Gel formation within inserts with cells added to the surface after gel formation in 2D cell culture and embedded throughout the gel layers due the cells being added to the peptide solution prior to gel formation in 3D culture.

2.2.6 Fluorescence Spectroscopy

Fluorescence spectroscopy was used to assess the formation of π-π stacking to determine if the peptide solutions had formed a hydrogel. Fluorescence emissions of all samples were measured on a Jasco-FP-6500 spectrofluorimeter. 1 ml of the pre-gel solution and 0.5 ml of cell media were added to a 1 cm^2 quartz cuvette. Excitation of the samples was 280nm, with the emission detection range between 300 and 600 nm. The band width was 3nm with a medium response. Experimental data were gathered in triplicate with average data shown. Decreasing intensity of the peaks correlates with an increase in fluorenyl activity and therefor matrix stiffness of the peptide hydrogels.

2.2.7 Rheology

Rheological analysis was used to determine the mechanical stiffness of the peptide hydrogels. Hydrogels were transferred for analysis by removing the membrane from the bottom of the insert directly onto the rheometer platform immediately prior to analysis. Rheological properties of all samples were analysed using a Malvern Kinexus rheometer, with the temperature controlled at 25°C using a 20mm diameter parallel plate geometry with the gap set to 0.9mm. A strain sweep was first performed at a constant frequency of 1 Hz from strains of 0.01-100%, with no variation in G' or G" observed up to a strain of 0.7% for the Fmoc-FF/S samples. Dynamic moduli of the samples were then measured as a function of frequency in the range of 0.1 - 100 rad s^{-1} with a constant strain value. Experimental data were acquired in triplicate and the average data shown.

2.2.8 Cell Culture

The MIN6 cells were maintained in DMEM cell medium containing 1 g/l of glucose and L-glutamine, 3.4g sodium bicarbonate, 75 mg penicillin, 50 mg streptomycin and 110 ml of heat inactivated FBS per 1000 ml of media (10%). The cells were then incubated at 5% CO_2, 95% air and 37°C in an incubator, with the media changed as needed. Cells were split when 70% confluency was reached. For incorporation into the hydrogels the media was

removed from the flask, with the flask then washed with Hanks Salt Solution to prevent remaining medium interacting with the 7 ml of trypsin added to free the adherent cells. 8 ml of fresh DMEM media was added to the flask to stop the trypsin reaction, with the cells then transferred to a centrifuge tube. After 2 min of centrifugation at 1200 rpm the supernatant was decanted, the pellet dislodged and re-suspended in 10 ml of DMEM. From this stock a dilution was prepared and mixed with the Fmoc-FF/S suspension to be used for hydrogel formation.

2.2.9 Alamar Blue Viability Assay

To assess the viability of the cells, the Alamar Blue Reagent was used. The MIN6 cells were cultured on a 12 well plate with the Alamar blue reagent, Resazurin added at a 10% to volume ratio (30µl) and the plate then incubated at 37˚C, 5% CO_2 for 1 to 6 hours. The reduction of the compound by living, respiring cells to resorufin was observed by a colorimetric change (blue to red), detected on a Wallac Victor 2 1420 multi-label counter. The plates analysed contained samples with varying cell concentrations (1.5, 1 and $0.5x10^5$), negative controls (gels not containing any cells) and positive controls (cells only, at the same concentrations as samples). The cell numbers were automatically generated by the multi-label counter as the intensity is proportional to the cell number.

2.2.10 Insulin ELISA

10 µl of media from the top of each of the gels was removed and used for the insulin ELISA test. The plate was read at 450 and 590 nm to detect colorimetric changes observed when the anti-insulin antibodies and enzyme conjugate bound the insulin molecules. The concentration of insulin released from the cells embedded within the gels was calculated from the dose response curve generated using the insulin standards provided of 0.2, 0.5, 1, 2, 5 and 10 ng/ml.

3. Results and Discussion

3.1 Peptide Solution Preparation Methods

Pre-gel solutions (Fmoc-FF, Fmoc-S, H_2O and NaOH, HCl) were prepared using various methods (variation of time exposed to vortex and sonication, variation of the pH of the solutions and varying the concentration of Fmoc-FF and Fmoc-S) before being used to form hydrogels. These gels were then analysed using fluorescence spectroscopy to establish if π-π stacking was present and rheology to ascertain the formation and mechanical properties of the resulting hydrogels.

3.1.1 Control of Sonication and Vortex

Pre-gel solutions were prepared with time exposed to vortexing and sonication varied in order to study the effects of such process on the formation and mechanical properties of resulting hydrogels. Fluorescence data was gathered on each of the solutions that were exposed for a total of 5, 6,8,10 and 12 min of alternation between vortex and sonication. The data was analysed for evidence of π-π interactions that are present if a self-assembled hydrogel is formed [Kang *et al.* 2005] using fluorescence spectroscopy as shown in figure 8. π-π stacking is indicated by a peak at 330nm, shifted from the 320 nm peak associated with fluorenyl excitation that has been observed in previous studies in non-gelating samples [Jayawarna *et al.* 2006].

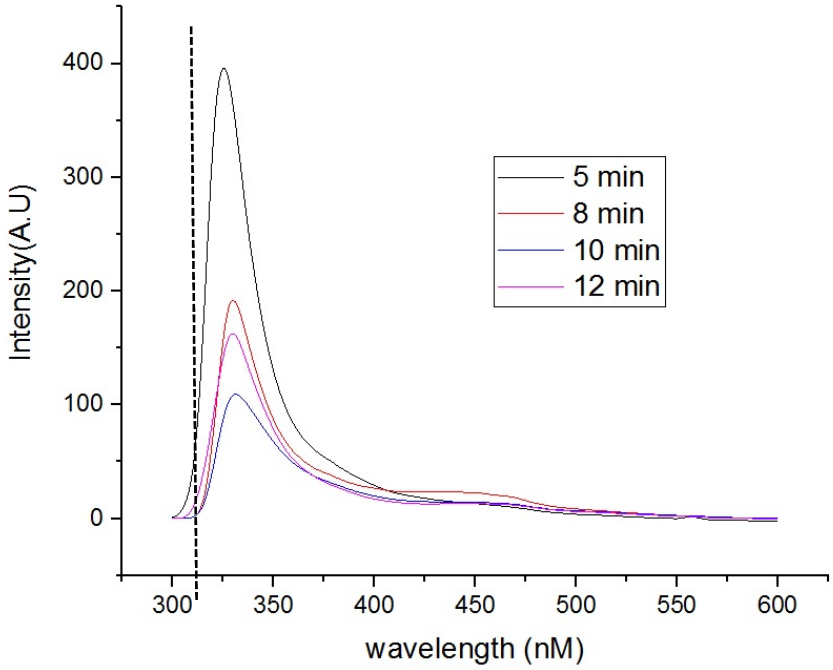
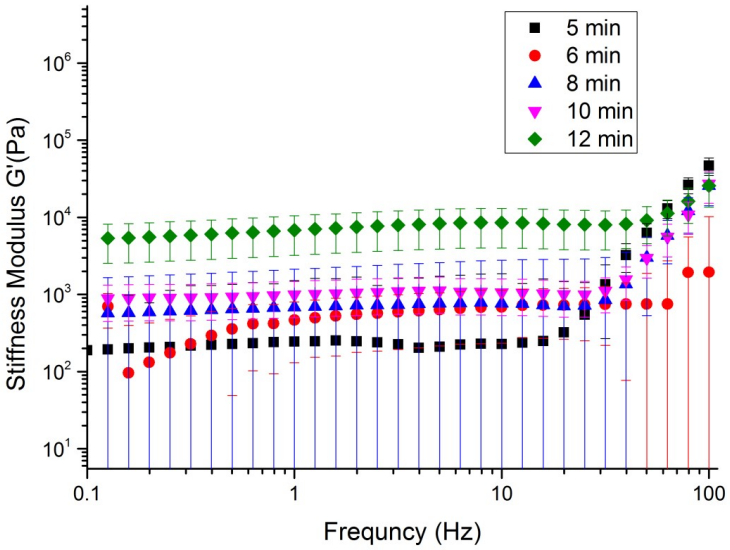


Figure 10: Fluorescence emission spectra graph representative of each sample, exposed to vortex and sonication alternatively, for a total of 5,8,10 and 12 minutes. Excitation was at 280nm with emission detected at a range from 300 to 600 nm. Dotted line at 320 nm to highlight 330 nm shifted peak that indicated gel formation.

An initial peak at 330nm and a secondary peak at 450nm were observed in each of the samples. The sample exposed for a total of 5 minutes of the alternating process showed the greatest initial peak at an intensity of 400 a.u. The intensity of both peaks (330nm and 450nm) of the samples exposed to 8 and 10 min decreased with increased time exposed to vortex and sonication (refer to figure 10). This decrease is likely due to quenching, a reduction of the fluorescence intensity as a result of enhanced interaction, which has been observed in other Fmoc-amino acid peptide systems [Zhou *et al*. 2009].

The gels made from each of the pre-gel solutions exposed to alternating vortex and sonication were then analysed by rheology to examine the mechanical properties of each of the gels, shown in figure 11.



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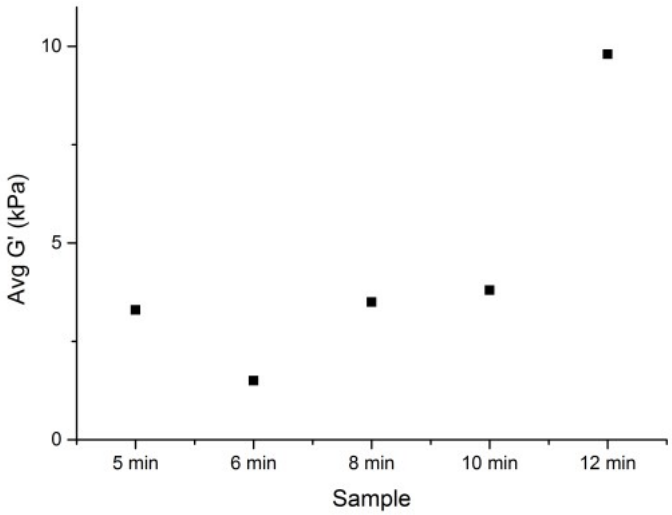

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Figure 11: (A) Stiffness modulus (G') values (Pa) as determined through rheology for the samples exposed to alternating vortex and sonication for 5, 6, 8, 10 and 12 min. All data gathered in triplicate, with average data and standard deviation error bars shown. Standard deviation error bars are (B) Avg stiffness value for each of the samples (kPa) derived from the data shown in figure 11(A).

During preparation only the sample exposed to sonication and the vortex mixer for a total of 12 minutes resulted in a fully homogenous solution by visual inspection.

From the rheological data in figure 11A all of the samples on average had a G' modulus value greater than the G" modulus value, expected if a hydrogel has formed [Kavanagh and Ross-Murphy, 1998]. In general, this data indicated that as the exposure time to alternation between vortex and sonication increased, there was a slight increase in the G' modulus values, with the exception of the 6 min sample. This may be due to increased interaction of the peptide components, for example, the sample at 5 minutes exposure to vortex/sonication gave a modulus value of $3.3x10^3$Pa and the 6 min sample giving a lesser value at $1.5x10^3$Pa. In the sample exposed for a total of 8 minutes the G' value was increased from the modulus value of the 6 min sample, at $3.5x10^3$Pa. The G' values for the rest of the samples continued to increase with the exposure time to vortexing and sonication, with the 10 min sample generating a G' value of $3.8x10^3$Pa and the 12min sample giving the greatest modulus value of $9.8x10^3$Pa. However the G' values were all within the same order of magnitude, therefore any difference in mechanical properties is likely to be minimal. Also the sample at 6 minutes gave a rheological value lower than the sample exposed for a total of 5 mins, most likely due to a failure of the particles to dissolve, observed upon visual inspection. The increase of the modulus values at higher frequencies observed in all of the samples is likely due to thickening instability, where the viscosity appears to increase as the shear rate increases that has been noted previously in Fmoc-tripeptide systems [Cheng *et al* 2010].

The effects of vortexing Fmoc-peptide solutions have previously been shown to affect the mechanical properties of the resulting gels, with the longer exposure times resulting in reduced modulus values attained through rheology [Helen *et al*. 2010]. However the current study using Fmoc-FF/S peptide solutions shows that there is a slight increase in the G' modulus values on average with longer exposure to both vortexing and sonication. This is most likely due to the increased mixing time the samples were exposed to, allowing the Fmoc-FF/S particles to fully dissolve and so form a more homogenous gel. This possibly

leads to more interaction of the components within the sample, which can affect the mechanical properties of the resulting gel.

Based on the fluorescence and rheological data this method of preparation did not allow for precise control of the resulting mechanical properties of the gels formed from the peptide solutions exposed to various time of vortexing and sonication, and so was not suited for use in cell culture. As the stiffness value of the matrix is crucial for cell viability and functionality [Engler 2006] it is key that the hydrogel preparation method is predictable and reproducible, therefore other methods of controlling peptide hydrogel formation were subsequently explored as means of producing hydrogels with predictable mechanical properties, for use in the culture of MIN6 cells.

3.1.2 Control of pH

Pre-gel solutions were also prepared with various pH values in order to assess the method's ability to control the resulting mechanical properties of hydrogels formed from these samples. It has previously been shown that alteration of the pH levels can affect the morphology of Fmoc-FF hydrogels [Tang *et al*. 2009] with for example fibril morphology being observed at high pH levels. The effect of pH to control the mechanical properties of the Fmoc-FF/S hydrogels is explored.

Fluorescence emission data was collected in triplicate and analysed for evidence of π-π interactions in each of the samples (figure 12).

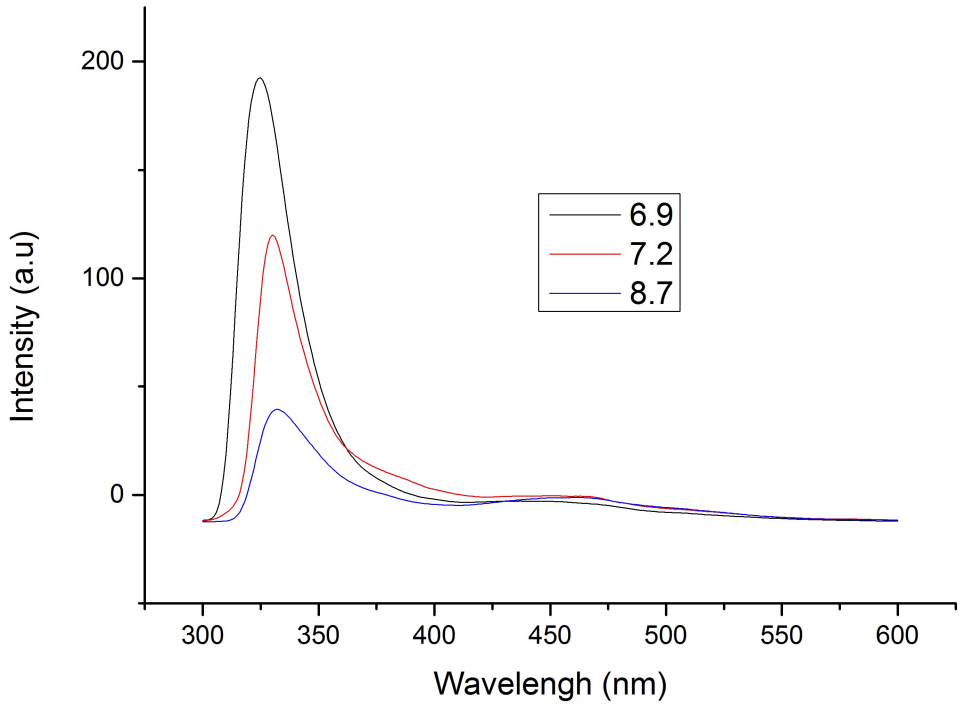


Figure 12: Fluorescence emission spectra for the pH controlled samples at excitation 280 nm, with an emission range from 300 to 600 nm. Image is representative of n=3 samples at each pH value.

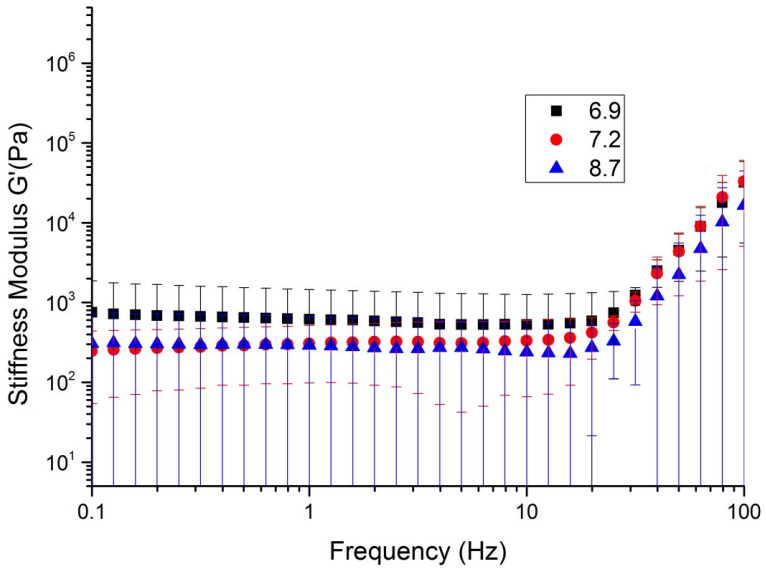
Red-shifted fluorenyl emission peaks were observed for all samples at 330 nm, evidence of π-π stacking (figure 12). The 330 nm peak for sample at pH 6.9 gave the highest intensity of 190 a.u, followed by the sample at pH 7.2 at 120 a.u and sample at pH. 8.7 the lowest at 40 a.u. A broad peak was observed in each of the samples at 450 nm, indicative of strong π-π stacking and previously proposed to indicate formation of extended pi stacked aggregates [Jayawarna 2008]. The data shows that intensity of the peaks both at 330nm and 450nm decreased as the pH of the peptide samples increased. It has been shown previously that high pH values (> pH 9) is unfavourable to the self-assembly of Fmoc-FF gels [Tang *et al*. 2009], therefore lower pH values may allow for more π-π interactions to take place in the Fmoc-FF/S gels. It should also be noted however that storage of the Fmoc-tripeptide solution can lead to an altered pH value from that of the resulting hydrogel [Raeburn *et al* 2011].

The hydrogels formed from the peptide solutions of varying pH values were also assessed through rheology to examine the mechanical properties of the samples (figure 13). G' values for each of the samples was greater than the G" on average, suggestive that the samples had formed a hydrogel [Kavanagh and Ross-Murphy, 1998]. For samples at pH 8.7 and 7.2 instability of the gel was observed at frequencies greater than 10 Hz. For the sample at pH 6.9, instability was observed at frequencies greater than 5 Hz.

The rheological data (figure 13) from the pH controlled method of peptide solution preparation showed variation between individual gels at the same pH. For example, samples prepared at pH 6.9 resulted in gels with a rheological G' value of $6.9x10^2$ Pa, up to $3.7x10^3$ Pa and an average of $2.0x10^3$, the sample of pH 7.2 giving modulus values from $4.0x10^2$ to $1.6x10^3$ Pa, with an average of $1.0x10^3$

Pa and the sample at pH 8.7 ranging from $1.1x10^2$ to $5.5x10^2$ Pa with an average modulus value of $3.3x10^2$ Pa. Given the intended use of the gels for cell culture this can make a considerable difference as cells require a matrix that is consistent in terms of its mechanical properties with that of their natural environment [Engler *et al.* 2006].

Also, samples prepared above a pH of 8 started to give much lower G' values than those around pH 7 possibly due to the unfavourable condition for self-assembly at higher pH values that has been observed for Fmoc-FF gels [Tang *et al*. 2009]. The upturn in modulus values at the higher frequencies seen in all samples is again likely to be due to thickening instability observed previously in Fmoc-tripeptides [Cheng *et al* 2010].



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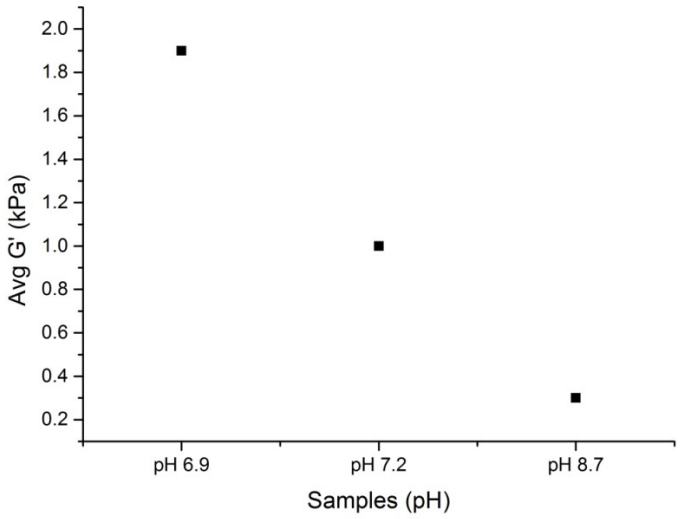

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Figure 13: (A) Stiffness modulus (Pa) of the samples of increasing pH determined via rheology. Data was gathered in triplicate, with the average data and standard deviation error bars shown. (B) Avg stiffness for each of the pH controlled samples (kPa), derived from data shown in figure 13(A).

From the data in figure 13 it was concluded that this method was not reproducible, generating gels with too wide a range (0.3 to 1.9 kPa) of rheological values within each pH

level and so were not suitable for cell work as each tissue type requires a specific matrix stiffness range for their survival and functionality [Collier *et al* 2010]. As a result of this data controlling concentration of the peptides was explored as means of generating peptide hydrogels of predictable stiffness for use in the MIN6 cell culture studies.

3.1.3 Control of Peptide Concentration

Three different concentrations of Fmoc-FF and Fmoc-S were chosen to provide a range of hydrogels to assess their suitability in supporting the functionality of the MIN6 cells. 6, 20 and 30 mM peptide solutions were prepared as described in section 2.2.3, with the samples first analysed by fluorescence spectroscopy to investigate the presence of π-π stacking, as shown in figure 14.

All samples showed peaks at 330nm and a broad peak at 450nm. The greatest initial peak was the 30mM concentration sample with an intensity of 360 a.u, followed by the 20mM sample at 340 a.u and the lowest for the 6mM sample at 260 a.u. Fluorescence data described in figure 10 for the samples at different concentrations show that all samples have evidence of strong π-π stacking, shown by both the 330nm peak (shifted from 320nm in non-gelating samples) [Jayawarna *et al.* 2006] and the broad peak at 450 nm. The spectra shows that as the concentration of the peptide solutions increased the intensity of the 330 nm peaks also increased. This may be due to the higher concentrations of Fmoc-peptides allowing for more π-π stacking, although the quenching effect is not observed.

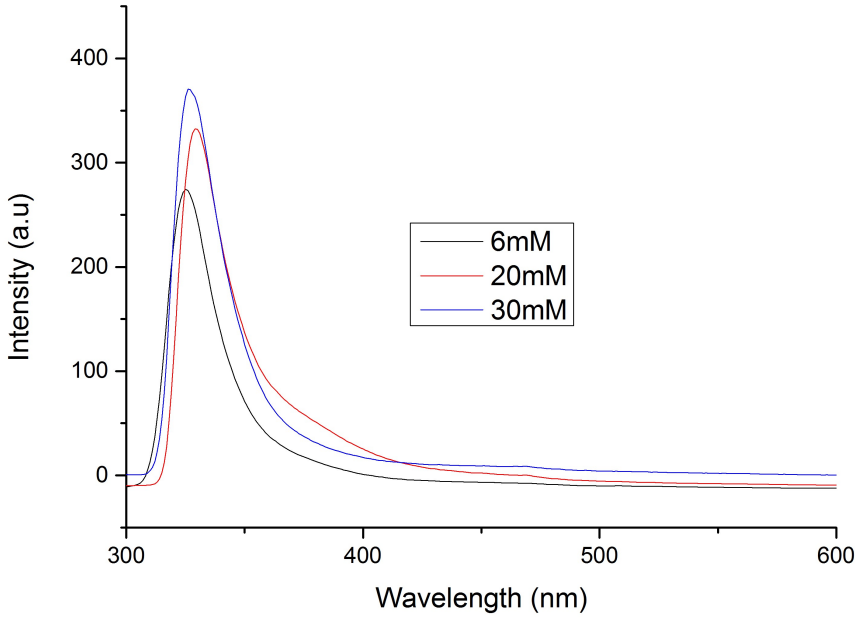


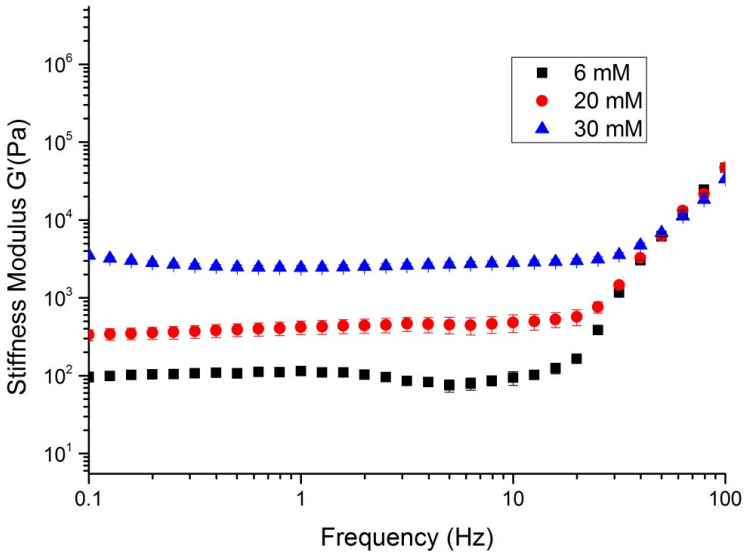
Figure 14: Fluorescence spectra for the concentration dependent sample. Excitation was 280nm with emission detection over a range of 300-600nm. Image is representative of all hydrogels at each peptide concentration.

The peptide hydrogels of each concentration were analysed by rheology to ascertain the mechanical properties, shown in figure 15.

The G' values for each of the samples were greater than the G" values, suggesting that each peptide solution had formed into a visco-elastic hydrogel. However, instability of the samples at 6mM and 20mM concentrations were observed at frequencies greater than 2Hz. Differing the concentration of samples opposed to the composition has already shown to affect mechanical properties of many different types of hydrogel in a predictable manner [Killion *et al.* 2007], although this is yet to be established for the Fmoc-based gels. However, rheological data from the concentration controlled samples of 6mM, 20mM and 30mM showed that as the concentration of the peptide increased the G' modulus values also increased e.g. the 6mM sample gave an average modulus value of $3x10^3$Pa, the 20mM $3.4x10^3$Pa and the 30mM $7.5x10^3$ Pa. As the G' modulus values for all samples were of the same order of magnitude, the increase in mechanical strength between the samples of increasing concentration is only slight and likely to be due to the presence of more Fmoc-FF/S allowing for greater interaction of the components. Again, an increase in modulus

values at higher frequencies is observed in all samples, attributed to thickening instability seen previously in other Fmoc-tripeptide systems [Cheng *et al* 2010].

From this work it was determined that the peptide concentration-dependent method provided the most reliable and reproducible means of forming a range of hydrogels of varying kPa to be used for MIN6 cell culture. Hydrogels generated from a particular peptide concentration were shown to result in a predictable stiffness value range, essential for use as a matrix in cell culture. As a result of the data described in figure 15 MIN6 cells were cultured in Fmoc-FF/S peptide hydrogels prepared using the concentration controlled method described in section 2.2.4.



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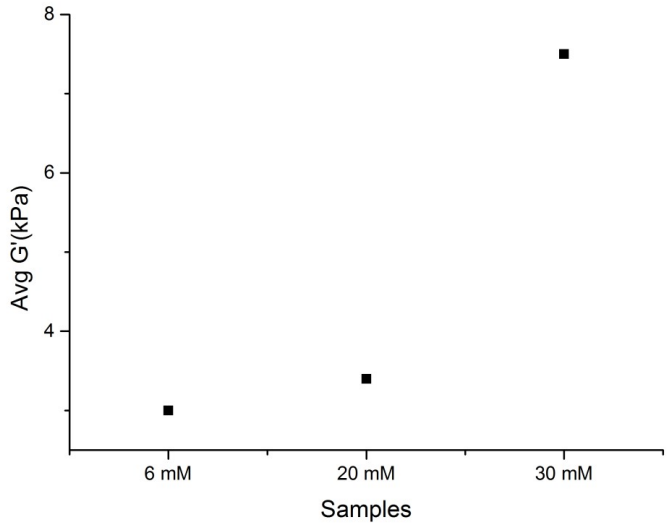

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Figure 15: (A) Stiffness modulus (G') for each of the hydrogels prepared through variation of peptide concentration (Pa). Data was gathered in triplicate and the average data with standard deviation error bars shown. (B) Average stiffness value for the concentration dependent samples (kPa) derived from the data displayed in figure 15(A).

3.2 Pancreatic Cell Culture in 2D

MIN6 cells were incorporated onto the surface of the hydrogels as described in section 2.2.5, formed through controlling the concentration of the peptides as described in section 2.2.4. The pH of all samples was 7.2 and cells were added to the gels at varying cell concentrations (0.5, 1 and 1.5, $1.7x10^5$/µl) to determine optimal cell concentration for MIN6 cell viability as these cell types are particularly dependent on cell-cell communication due to the clustering nature observed in the pancreas [Park *et al* 2005]. To further explore the issue of overcrowding discussed previously the experiment was repeated with different cell concentrations (1.5, 1 and $0.5x10^5$/µl) in gels of varying peptide concentration at pH 7.2, with results shown in figure 16.

Gel concentrations of 6, 20 and 30 mM were used to ascertain Fmoc-FF/S hydrogel compatibility with the MIN6 cell line. The number of viable cells within each of the gels were ascertained through the Alamar Blue assay. Hydrogels with no cells were used as a negative control and cells grown without the hydrogels, in standard cell culture welled plates at the same densities as the samples under investigation were used as a positive control. Viable cell numbers in each hydrogel of various peptide concentrations are shown in figure 17.

From the Alamar blue viability assay all of the hydrogels contained an order of magnitude lower number of respiring cells than in the positive control, where cells were cultured without the gels. The gels at a concentration of 30mM contained a slightly larger number of respiring cells than did the gels at a concentration of 20mM and 6mM, with a cell count of $5.1x10^3$ compared to a count of $4.2x10^3$ for the 6mM sample and $4.5x10^3$ for the 20mM sample (figure 17). The values attained for the negative controls are likely to be due to the background fluorescence generated by the Fmoc-FF/S gels themselves [Jayawarna *et al*. 2009]. The reason for such a low number of live, respiring cells in the concentration-dependent gel may be as a result of the cells not being able to grow and survive well on the specific gels examined due to issues such as overcrowding (too high a cell concentration), not having a high enough cell seeding density or the particular assay used may not be suited for studies involving the Fmoc-FF/S gels (may not be able to penetrate the gel and

reach the cells within). Due to the possible limitation of the reagent being unable reach the MIN6 cells within the peptide hydrogel during the Alamar blue assay other methods could be used to examine this issue such as the incorporation of intracellular dyes to label the cells (Parish *et al* 2009) and confocal microscopy to visualise the cells in-situ (Matsumo 2002).

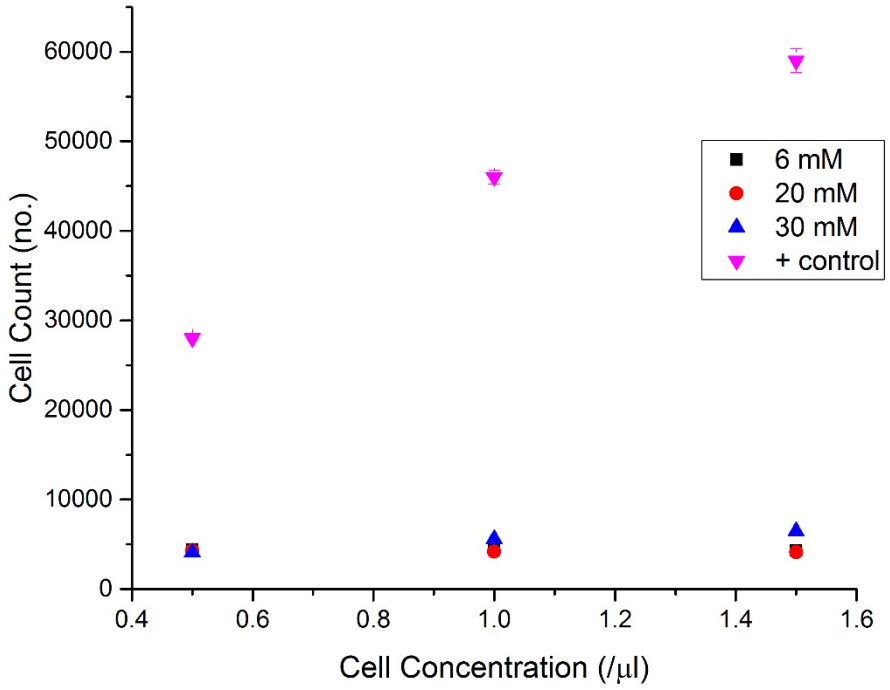


Figure 16: Number of viable cells in each of the hydrogels as the concentration of MIN6 cells was varied (0.5, 1 and $1.5x10^5$/μl). Data was gathered in triplicate with the average and standard deviation error bars shown.

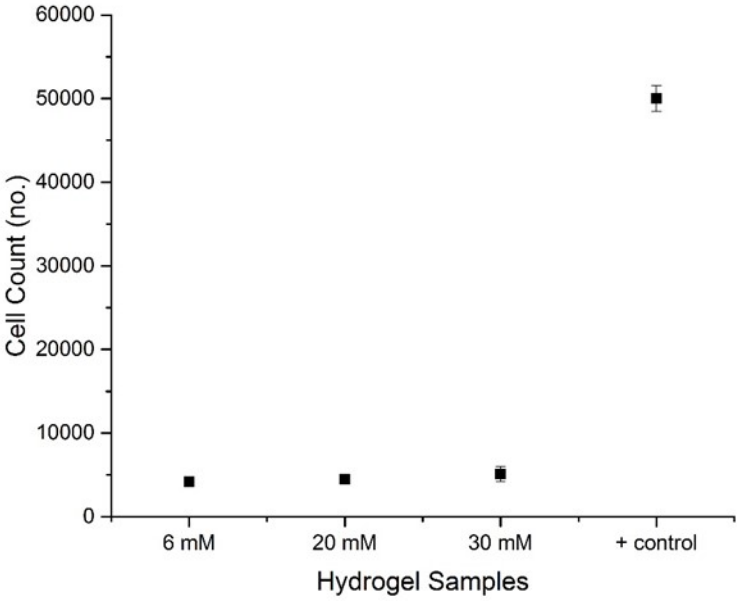


Figure 17: Number of viable cells in each hydrogel of varying peptide concentration as determined by the Alamar blue cell viability assay. Data was gathered in triplicate with the average shown along with standard deviation error bars.

Figure 17 shows that all of the samples contained respiring cells at a number lower than in the positive controls, where the cells were cultured without the gels. The hydrogel at a peptide concentration of 20 mM, with a cell concentration of $1.5x10^5$/µl contained the lowest number of respiring cells. The hydrogel of a peptide concentration of 30 mM and a cell concentration of $1.5x10^5$/µl contained the highest number of respiring cells as determined by the Alamar blue viability assay. Again, values obtained for the negative control can be attributed to the background fluorescence of the hydrogel [Jayawarna *et al*. 2009].

The data gathered from the Alamar blue assay of samples at different cell concentrations showed a slight improvement between samples at different cell concentrations. For example, in the 6 mM gel there was an increase in the cell count of 175 from the sample at a cell concentration of $1.5x10^5$/µl to the sample at $0.5x10^5$/µl. However, this trend was only observed for the samples at peptide concentration of 6mM and 20mM, with the greatest cell count being observed for the sample at a peptide concentration of 30mM and a cell concentration of $1.5x10^5$. Another feature to note from these experiments is the possible

preference of the cells for the 30mM concentration gel, which provided an average G' value of 7.5 kPa.

3.3 Fmoc-FF/S Hydrogel Characterisation for 3D Cell Culture

As is was determined in section 3.1.3 that the varying of peptide concentrations to control the mechanical properties of the resulting hydrogels was the most reliable and consistent methodology, hydrogels formed in this manner as outlined in section 2.2.4 were generated to expand upon data gathered in section 3.3.

To determine the peptide concentration, and therefore stiffness modulus that promotes the optimal survival and functionality of the β-cells a range of different peptide concentrations were prepared to generate a variety of hydrogels with which to test with the MIN6 cells. Initially peptide concentrations of 10, 20 and 30 mM were selected to provide a wide range of stiffness moduli to assess the preference of the MIN6 cells for hydrogels with particular mechanical properties.

3.3.1 Fluorescence Spectroscopy

Fluorescence spectrometry was performed on the hydrogels of peptide concentrations 10, 20 and 30 mM to assess the π-π stacking between fluorenyl moieties that are present if a hydrogel is formed (Kang *et al*, 2005) as shown in figure 18. Data from figure 18 was compared to data previously gathered in figure 14, which did not show the quenching result seen in figure 18 though to be due to the difference in source of the Fmoc-FF and therefor formulation of the dipeptide that can effect many charactersitics [Raeburn *et al* 2012].

Fluorescence data for the hydrogels at different peptide concentrations reveals that all have evidence of strong π-π stacking, shown by both the 330 nm peak (shifted from 320 nm in non-gelating samples) and the broad peak at 450 nm (Jayawarna *et al*, 2006). The spectra in figure 18 shows that as the concentration of the peptide solution increased, intensity of the peaks decreased. This decrease is likely due to quenching as a result of

enhanced interaction, which has been observed in other Fmoc-amino acid peptide systems (Zhou *et al*, 2009).

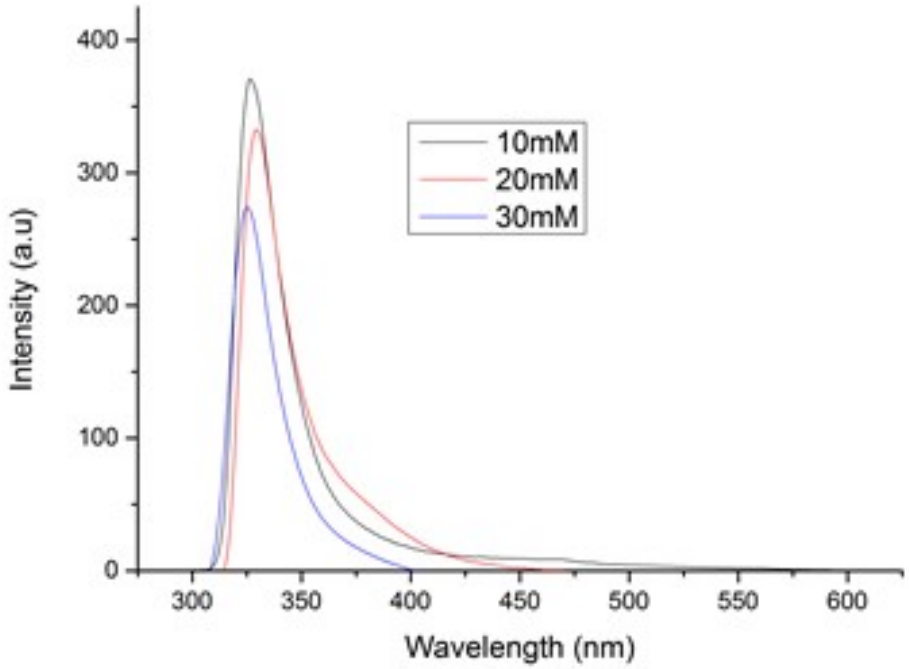
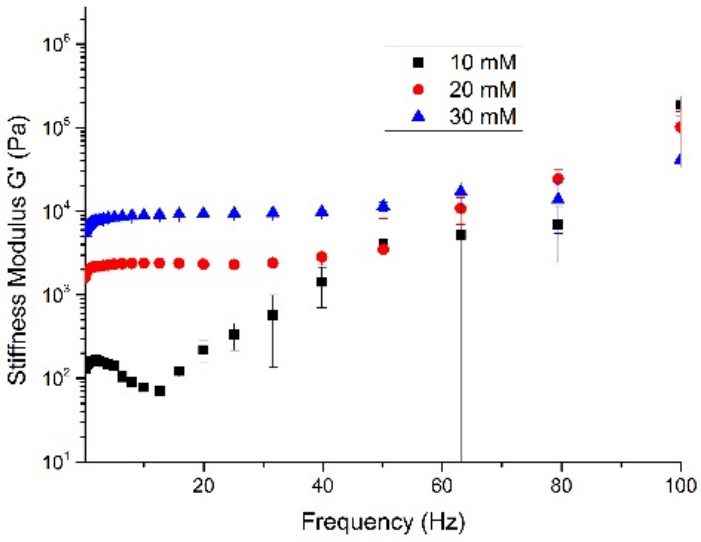


Figure 18: Fluorescence spectra for the peptide concentration-dependent hydrogels at 10, 20 and 30 mM. Image is representative of all hydrogels of various peptide concentrations.

3.3.2 Rheological Analysis

Rheological analysis was performed on the cell free hydrogels at concentrations 10, 20 and 30 mM in order to determine the mechanical properties of each and is shown in figure 19. It is important to test a range of hydrogel stiffness's with the MIN6 cells as the pancreas stiffness is not known and the effect of matrix stiffness on survival has yet to be determined for the islet cells (Collier *et al*, 2010). Variation of the concentration of the peptide derivatives was expected to effect the rheological properties of the hydrogels as previously reported in other hydrogel systems (Killion *et al*, 2011) (Tan *et al*, 2012).

It was observed that the elastic modulus (G') increased with concentration (0.5 to 23 kPa) to generate a range of hydrogels to be tested for compatibility with the MIN6 cells. These results can be explained by the increased density of the fibre network, resulting in greater interaction of the components and a more stable hydrogel structure as described for other hydrogel systems (Abul-Haija *et al*, 2014). The wide range of stiffness values generated provides a basis in which to test to the compatibility of the pancreatic cells. Stiffness values for other biological tissues have previously been defined, representing a scale of tissue types of varying kPa (Levental *et al*, 2007).



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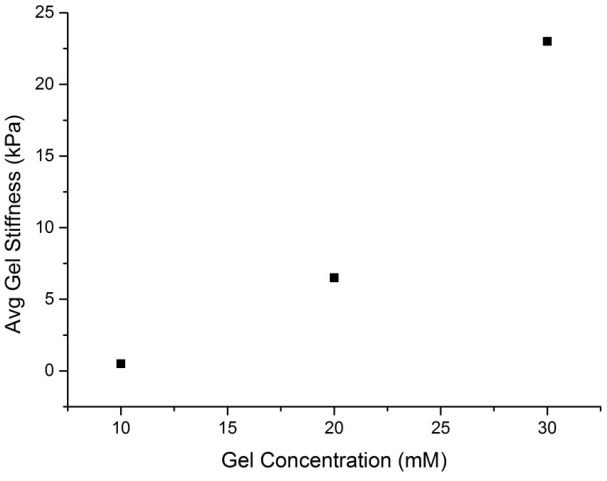


B

Figure 19: (A) Rheological data from Fmoc-FF/S hydrogels of 10, 20 and 30 mM peptide concentrations, showing the G' or storage modulus values (Pa) v increasing frequency (Hz). Data was gathered in triplicate with average shown as well as standard deviation error bars. (B) Average modulus values of the different hydrogels (kPa) derived from (A).

3.3.3 MIN6 Cell Viability within 3D-Like Fmoc-FF/S Hydrogels

The Fmoc-FF/S hydrogels of three different, wide ranging stiffness values were tested for their compatibility with MIN6 cells grown in 3D like culture, with the cells added to the peptide solutions used for gel formation, as described in section 2.2.5. The Alamar Blue viability assay was used to establish the number of live cells within the gels with varying kPa values (0.5, 6.5 and 23 kPa) after 48 hrs incubation. As high levels of apoptosis (Rosenberg *et al*, 1999) and slow proliferation have been reported in isolated islet cells (Beattie *et al*, 2002) it is important that the Fmoc-FF/S hydrogel is capable of supporting survival of the MIN6 cell line.

The results shown in figure 20 reveal that the 6.5 kPa hydrogel supported by far the greatest number of viable cells. These results suggest that matrix stiffness matching is critical for β-cell culture.

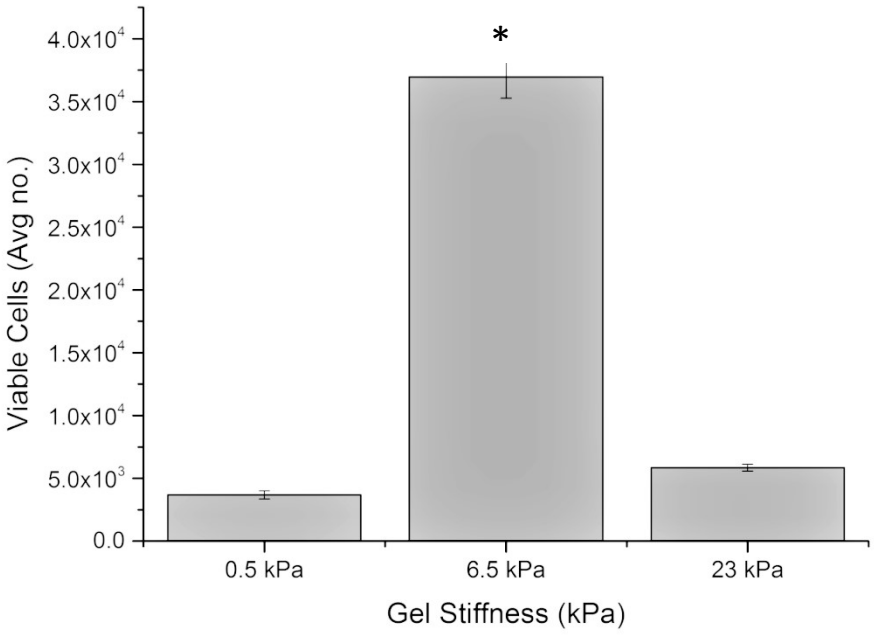


Figure 20: Number of live, viable cells in each of the Fmoc-FF/S hydrogels of varying stiffness values; 05, 6.5 and 23 kPa (peptide concentrations 10, 20, 30 mM respectively). The data was gathered in triplicate, with the average data and error bars representing standard deviation shown. *p<0.05 for 6.5kPA versus 0.5kPa and 23kPa, ANOVA followed by Dunnett's t-test showing that the cell number was significantly different in the 6.5kPa gel against cell numbers in the 0.5 kPa and 23 kPa hydrogels.

ANOVA testing was carried out on the data depicted in figure 20 to evaluate if the concentration of the peptide and therefore stiffness of the matrix had a significant effect on the number of cells within the hydrogels. There was a significant effect at the p<0.05 level for the three matrices, F (2,6) = 11.495, p = .009]. Post hoc Dunnett comparisons were then carried out and found that there was significant difference between 6.5 kPa (20mM Gel) and the 0.5 kPa (10 mM) and the 23 kPa (30 mM), however, no significant difference was found between 0.5 kPa (10mM gel) and 23 kPa (30 mM gel)

The hydrogels of 0.5 and 23 kPa were no longer investigated as suitable matrices for β-cell culture due to the low levels of cell survival observed, while a range of hydrogels in the 6.5 kPa range were further examined. This data further illustrates how crucial the role of mechanical signalling is within biological tissues, able to effect cell numbers and function as previously discussed in section 1.7. It also demonstrates that variation in the stiffness modulus of the Fmoc-FF/S peptide hydrogels is as important for MIN6 cells as it for many other cell lines [Jayawarna *et al* 2009], with the hydrogel of 6.5 kPa (20 mM peptide concentration), a stiffness value which falls within the reported range for lung tissue (3 to 10 kPa) [Collier *et a*l 2010], able to support greater cell numbers than hydrogels of other stiffness values, 0.5 and 23 kPa gels of peptide concentrations 10 and 30 mM, which are stiffness values within the range reported for brain tissue and skeletal muscle respectively [Collier *et al* 2010].

3.4 Hydrogel Optimisation

Based on the results detailed in figure 20 which showed the hydrogel of 20 mM concentration (6.5 kPa stiffness modulus) supported the greatest number of cells, subsequent peptide concentrations in this range of 15, 18, 22 and 25 mM were also

prepared to further optimise the Fmoc-FF/S hydrogels for pancreatic cell culture through the generation of further stiffness values with which to test the MIN6 cells.

3.4.1 Fluorescence Spectroscopy

Fluorescence spectroscopy was performed on samples of Fmoc-peptide concentrations 15, 18, 22 and 25 mM to assess the formation of the expected π-π stacking of the fluorenyl moieties if a hydrogel has been formed (figure 21).

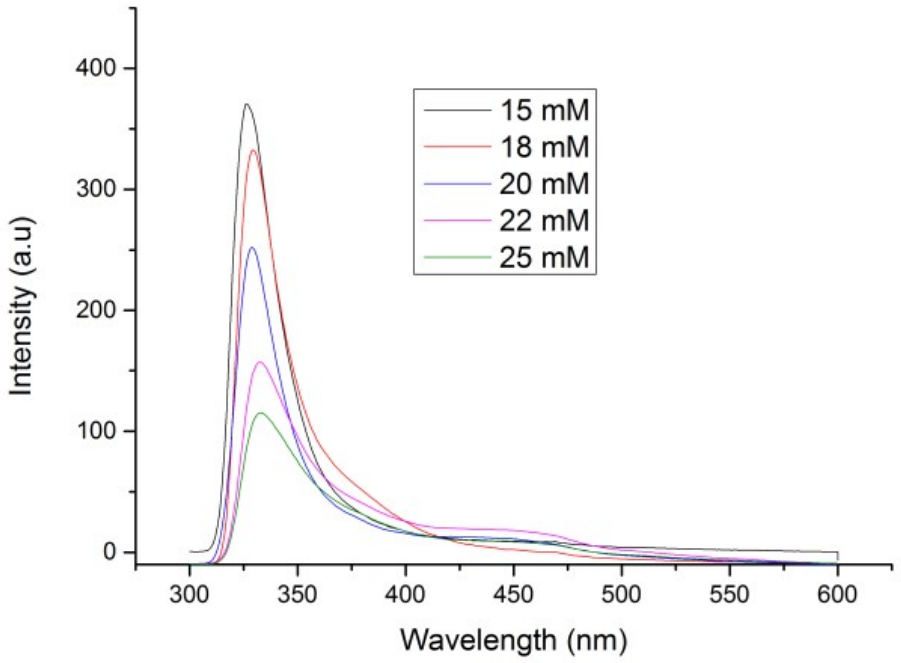
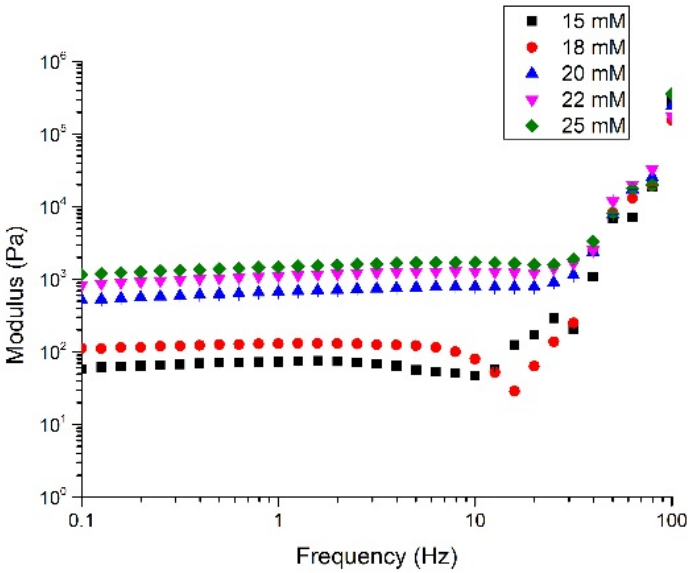


Figure 21: Fluorescence spectroscopy data for the FF/S peptide concentration dependent samples 15, 18, 22 and 25 mM. Excitation was 280 nm with an emission detection range of 300-600 nm. Image is representative of all samples of varying concentration.

3.4.2 Rheological Analysis

To add to previous results depicted in figure 19, rheology was performed on Fmoc-FF/S hydrogels of peptide concentrations 15, 18, 22 and 25 mM, as shown in figure 22, to obtain kPa values of each hydrogel in order to establish the ideal value for the β-cell viability.

The results shown in figure 20 suggest that within this stiffness range there is a near linear correlation between concentration of the Fmoc-peptides and the resulting kPa values of each due to the increased fibre density leading to a more stable structure (Abul-Haija *et al*, 2014). Rheological analysis shows that variation of the peptide concentrations provides an easy method of generating a range of hydrogels with differing mechanical properties. These gels were then tested with MIN6 cells to establish the ideal stiffness value to promote cell survival and functionality.



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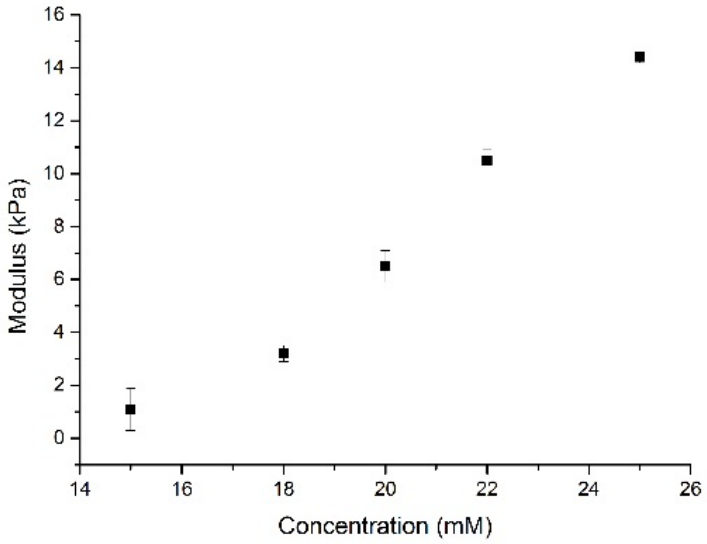


B

Figure 22: (A) Rheological data for the hydrogels of concentration 15, 18, 22 and 25 mM, giving the G' or storage modulus values (Pa) as the frequency of oscillations (Hz) increased. Data was gathered in triplicate with the average data and error bars derived from standard deviation shown. (B) Average stiffness modulus value (kPa) for each of the hydrogels, derived from data in (A), with standard deviation error bars shown.

3.4.3 Cell Viability in 3D like Culture

The Alamar Blue viability assay was used to determine the number of live cells within each of the hydrogels in 3D like manner (cells added to the peptide solution prior to gelation) of concentration 15-25 mM and 1.1 to 14.4 kPa in order to establish the ideal hydrogel matrix for cell viability, shown in figure 23.

The results described in figure 23 show the greatest cell survival within the 25 mM, 14.4 kPa hydrogel. This work further demonstrates the importance of matrix stiffness on cell survival. ANOVA testing showed there was a significant effect of peptide concentration and so matrix stiffness on the number of cells at the $p<.05$ level for the three conditions $F(4,10) = 19.76$, $p = .000$]. Post hoc Dunnett t-tests were carried out and found that there was

significant difference in cell number in the hydrogels of the higher stiffness values, 6.5, 10.5 and 14.4 kPa to the hydrogel of the lower stiffness value of 1.1 kPa but no significant difference between the hydrogels of 1.1 kPa and 3.2 kPa.

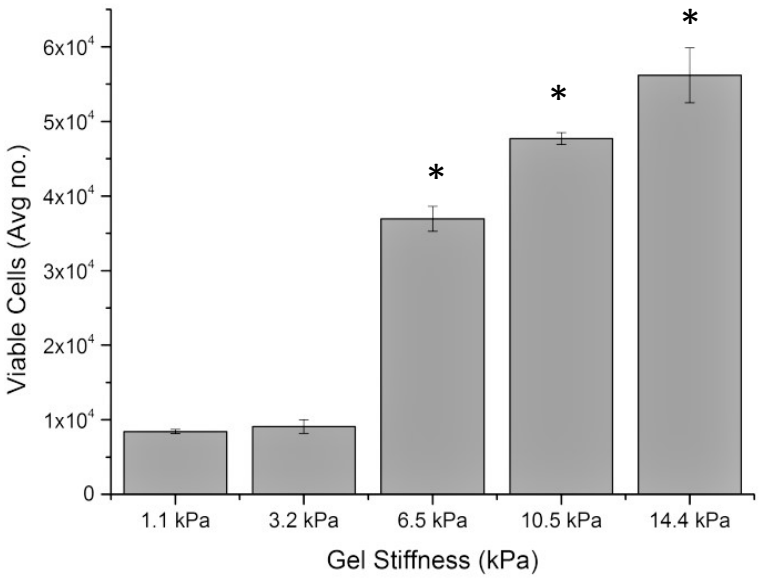


Figure 23: Number of live, respiring cells within the hydrogels of stiffness values 1.1 to 14.4 kPa and peptide concentrations of 15 – 25 mM. Data was gathered in triplicate with average data shown with error bars representing the standard deviation. *p<0.05 versus 1.1kPa, ANOVA, followed by Dunnett's t-test.

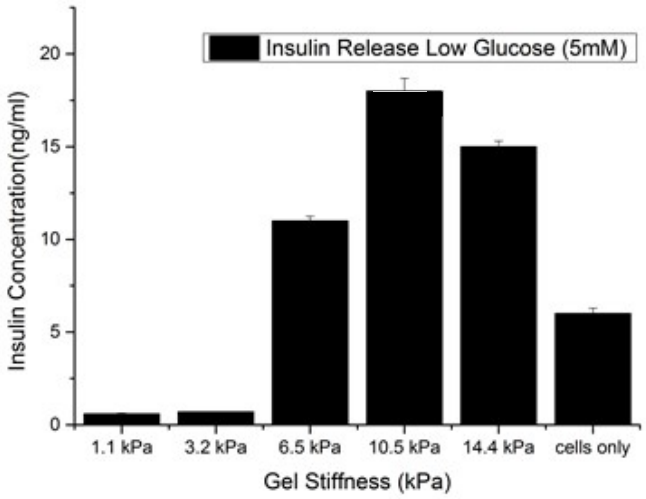
3.4.4 Insulin ELISA

Having established the effect of stiffness on cell survival in 3D-like cell culture matrix stiffness was further explored for its effect on the dose responsiveness of insulin to glucose. The glucose-induced insulin secretion of the MIN6 cells within each of the hydrogels was examined through an ELISA in order to demonstrate the preservation of this vital function as shown in figure 24. The insulin response in both high glucose (25 mmol) and low glucose (5 mmol) were both examined to assess the ability of the MIN6 cells embedded within the hydrogels to behave in a physiological manner as healthy β-cells increase insulin production when glucose levels rise to combat the dangerous effects of high glucose levels in the

bloodstream and lower their insulin production when glucose levels return to homeostatic levels to ensure enough glucose is available for metabolic functions (refer to section 1.1).

The ELISA test results (figure 24) showed that cells within the Fmoc-FF/S hydrogels retained their ability to secrete insulin in response to an increase in glucose concentration (5 to 25 mM). As cells lose their ability to secrete insulin in response to increasing glucose levels both *in vitro* (GSEDNu, 2006) and *in vivo* following islet transplantation (Ali Kondi *et al*, 2010) these results clearly demonstrate the advantage of using a hydrogel matrix in order to support viability of the β-cells.

The greatest increase in insulin secretion when the glucose concentration was raised from 5 mM to 25 mM was observed in the hydrogel of 10.5 kPa despite the 14.4 kPa hydrogel containing the largest number of live cells, however the dose response in the 14.4 kPa hydrogel was appropriate with a corresponding insulin increase with an increase in glucose concentration. In the ANOVA testing there was a significant effect of stiffness of the gel on the insulin concentration in low glucose conditions at the $p<.05$ level for the three conditions $F(5,12) = 14.463$, $p = .000$ Dunnett t tests show that the only significant difference from the positive control was in the 22 mM, 10.5 kPa gel stiffness. This work again highlights the importance of finding the ideal stiffness (kPa) in order to best support the survival and function of the β-cells.



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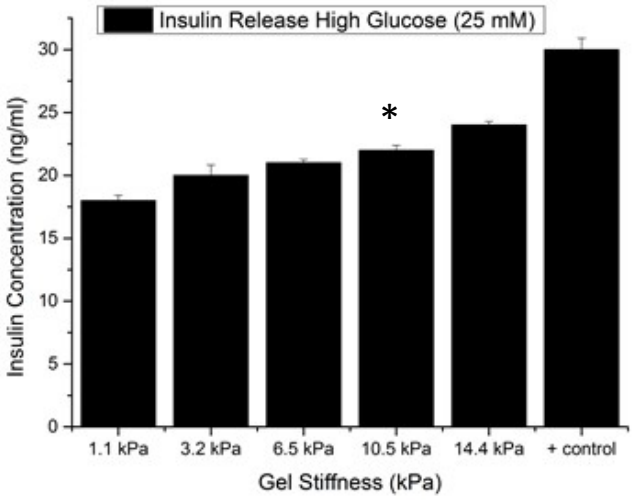

B

Figure 24: Concentration of insulin (ng/ml) released from MIN6 cells grown within each of the hydrogels of varying stiffness values (kPa) in a 3D manner as the concentration of glucose was increased from 5 mM (A) to 25 mM (B). These results were compared against the positive control, where MIN6 cells were grown outside the hydrogels in 2D. *$p<0.05$ versus control, ANOVA, followed by Dunnett's t-test.

3.5 Towards 3D Pancreatic Cell Culture

MIN6 cells were previously shown in figure 24 to secrete insulin in response to increased levels of glucose in the Fmoc-FF/S gels. Not only is it important that the cells are able to adjust insulin secretion to differing levels of glucose, but that they do so in a time appropriate manner. Under normal physiological conditions onset of insulin release in response to the metabolism of glucose occurs very quickly, reaching a peak as rapidly as one to two minutes [Nesher and Cerasi, 2002]. However, in the diabetic state rapid-acting insulin is injected during meal times to ensure that physiological glucose level remain within homeostatic parameters. Post injection the onset of insulin action is expected after 15 min, with peak insulin action occurring approximately at 90 min. The total duration of insulin action varies between individuals and brands of synthetic insulin but can last between 2 and 5 hours. After this time insulin levels should return to basal levels. MIN6 cells were cultured in low glucose (5 mM) media and the base insulin concentration measured. The

cells were then incubated in high glucose (25 mM) media and insulin concentration measured after 15 and 90 min. The high glucose media was then replaced with the low glucose media and insulin concentration measured after 2 and 5 hours.

The results in figure 25 show that the MIN6 cells within the Fmoc-FF/S hydrogels of 10.5 and 14.4 kPa were dose-responsive to varying concentrations of glucose in a time appropriate manner, with insulin secretion increasing after 15 min and peaking after 90 min in response to an increase in glucose levels (5 to 25 mM) and decreasing again after 120 and 300 min to within basal levels after glucose concentrations were reduced from 25 mM to 5 mM. The MIN6 cells grown outside of the hydrogels were dose-responsive to the varying concentrations of glucose, however insulin secretion did not return to base levels within the 300 min time-frame. A two way analysis of variance was carried out to analyse the influence of hydrogel stiffness on insulin concentrations at different time points, under either low or high glucose concentrations. Time was shown as a significant factor $F(4, 15) = 32.779$, $p < .001$. Effect of the hydrogel stiffness $F(2,15)= 71.054$ $p < .001$, was also significant. Interaction of both was $F(8,15)= 5.730$, $p< .003$. Dunnet's test showed the difference between 15 mins and 5 hours to be insignificant $p=.979$.

This shows that the Fmoc-FF/S peptide hydrogel are successful at maintaining the MIN6 cells' ability to respond appropriately with insulin to varying glucose concentrations compared with the cells grown freely within standard culture plates.

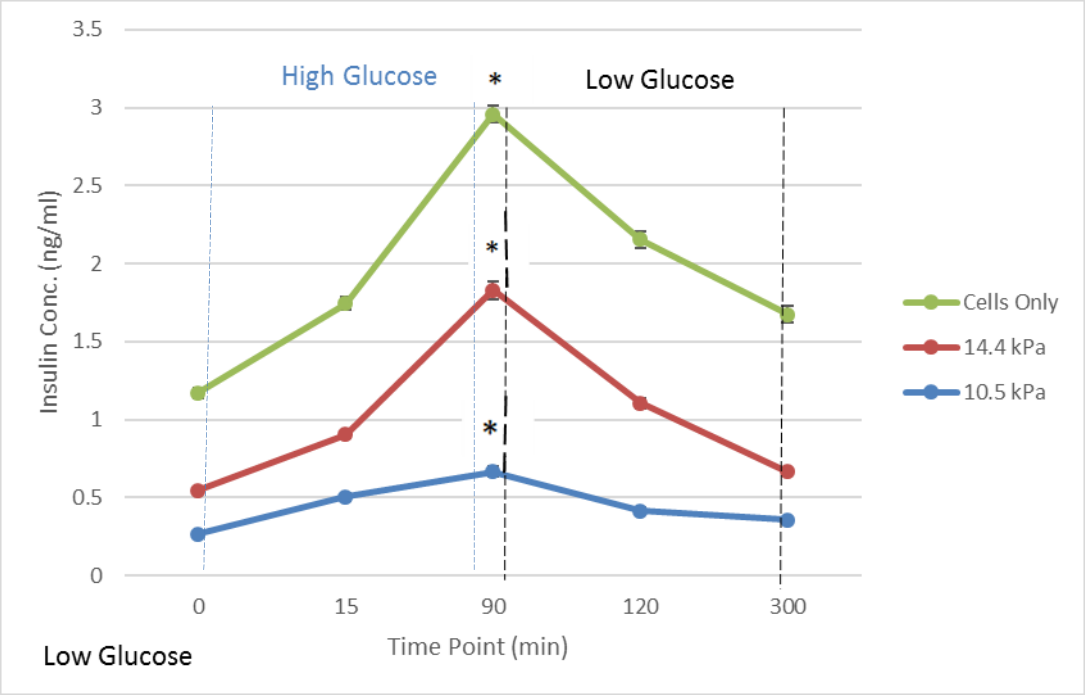

Figure 25: Insulin concentration (ng/ml) determined via the insulin ELISA at 15, 90, 120 and 300 min as glucose was increased from 5 to 25 mM and decreased again to 5 mM in the hydrogel of 10.5 kPa (blue), 14.4 kPa (red) and cells only (green) within a well of a standard 12 well culture plate. Data was gathered in triplicate with the average and error bars derived from standard deviation shown. *p<0.03 in two way analysis of variance, insulin concentration versus time and glucose concentration, followed by a Dunnett's t test.

4. Conclusion

Peptide hydrogels have previously been shown to be effective in the area of cell culture [Zhang *et al* 1993], with the Fmoc-FF/S in particular showing promising results for use in 3D cell culture [Jayawarna et al 2009]. This system was demonstrated to have tuneable mechanical properties, an important feature for cell survival and function [Disher *et al* 2005], although had yet to be tested with the β-cell type. Many other hydrogel systems have been studied for use with β-cells, with varying successes and limitations. For example, in the work by Weber *et al* 2008 outlined in section 1.2 a 3D-gel matrix was developed that incorporated different components of the β-cell ECM and was successful at maintaining healthy, functional cells. However this system required a complex formation in comparison

to the Fmoc-tripeptide system due to the many different factors incorporated [Weber *et al* 2008], [Jayawarna *et al* 2006]. Another study by Labriola *et al* 2007 developed a laminin-based matrix, where the β-cells were co-cultured with the growth factor hormone, pro-lactin. Again, although this matrix was able sustain healthy cells, the system was limited to 2D cell culture [Labriola et al 2007], while the Fmoc-FF/S system can be adapted for both 2D and 3D culture [Jayawarna *et al* 2006]. Due to the ease of formation, tunable mechanical properties and potential for 3D cell culture, the Fmoc-FF/S system was selected as a matrix to study the β-cell type.

This study analysed the effectiveness of different methods to control the matrix stiffness of the Fmoc-FF/S hydrogels (sonication/vortexing, pH and concentration) with the peptide concentration dependent method proving to be the most reliable and reproducible (section 3.1.3). The research also demonstrated that variation of the peptide concentration and therefore matrix stiffness influenced cell numbers within the hydrogels and the cell's ability to retain their glucose-induced insulin secretion functionality (sections 3.4 and 3.5). From this it was concluded that the Fmoc-FF/S hydrogels provided a suitable matrix for the study of the MIN6 cell line.

Due to the importance of matrix stiffness and as the stiffness value of the pancreas tissue is undefined in the literature a range of hydrogels of varying mechanical strength were generated to test with the MIN6 cells. However, data on optimal stiffness values of many other tissue and cell types exist and were used to guide the selection of stiffness ranges generated. For example the known values for soft tissue such as neural being 0.1 – 0.9 kPa, and the range for harder tissues such as cardiac muscle being 20kPa to 120 kPa, with pancreatic tissue hypothesised to fall between these values [Collier *et al* 2010]. As such one major aim of this research was to establish the preferred stiffness range for the MIN6 cell type, used to represent pancreatic cells.

MIN6 cells were initially cultured on the surface of the Fmoc-FF/S hydrogels in a 2D manner to first assess the compatibility of this cell line with the peptide hydrogel which had yet to be established in the literature. Results from figures show that varying the peptide concentration and therefore matrix stiffness is able to influence both MIN6 cell survival and functionality. Further to this, MIN6 cells were then cultured within the peptide hydrogels in a 3D like manner and the gels optimised to best support cell number and insulin releasing capabilities. The cell viability assay revealed the greatest cell survival in the 25 mM

hydrogel and may indicate a possible preference for a matrix of 14.4 kPa (fig 23), a value in the upper range of lung tissue (5 to 15 kPa) [Collier *et al* 2010]. The Fmoc-FF/S hydrogel of 14.4 kPa was also demonstrated to protect the insulin secreting functionality of the cells (fig 24), with insulin exhibiting dose-responsiveness to glucose concentrations in a time appropriate manner (fig 25). This was the first time the Fmoc-FF/S system has been shown to support these features of a β-cell type.

Future studies should include further exploration of the response of the cells within the hydrogels to varying levels of glucose through repetition of the experiments outlined in section 3.5. Another possible avenue of investigation is the production of C-peptide within the hydrogels, which alongside insulin fails to be or low levels are produced by the β-cells in the diabetic state. The ability of Fmoc-FF/S hydrogels to sustain other characteristics typical of healthy β-cells such as those described in section 1.9 could also be explored in order to fully establish the hydrogel's capacity to represent the microenvironment of the β-cell. Furthermore, the capability of the hydrogel to protect the embedded MIN6 cells from destructive cytokines and inflammatory molecules that can damage cells may also be examined as well as the possible biocompatibility of the system in order to establish a matrix that can be developed further for future therapeutic uses.

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