Immunoisolation Approaches for Islet Transplantation

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Abstract

The widespread use of islet transplantation as a treatment for type 1 diabetes is limited in part by the necessity for long term immunosuppression. The principle of immunoisolation is to create a physical barrier surrounding the islets to prevent immune destruction after implantation, therefore removing the requirement for immunosuppression. Alginate microcapsules are the most commonly used immunoisolation device which are typically implanted intraperitoneally. Intraperitoneal microencapsulated islet grafts are suboptimal, requiring at least double the number of islets to reverse hyperglycaemia compared to non-encapsulated islet grafts. The large size of microencapsulated islet grafts also restricts the selection of implantation site. We aimed to determine if the subcutaneous site is a suitable alternative site to the intraperitoneal site for microencapsulated islet grafts. It was discovered that equivalent microencapsulated islet grafts that were efficacious at the intraperitoneal site were ineffective at the subcutaneous site. Helper cells can be used to improve islet transplantation by secreting beneficial factors. L cells produce glucagon-like peptide-1 which is known to have several positive effects on islets. Co-encapsulation of islets with L cells increased islet insulin secretion but did not improve graft outcome. Mesenchymal stem cells (MSCs) can be co-transplanted to improve the graft outcome of non-encapsulated grafts. Islets co-encapsulated with MSCs had improved insulin secretion and also improved graft outcome. Immunoisolation of islets by conformal coating has the potential to maximise the diffusion of vital molecules and minimise the graft volume, enabling transplantation to preferred sites. Using allogeneic islets implanted at the kidney subcapsular site it was found that non-encapsulated grafts rejected whereas a novel conformal coating protected grafts from rejection in 5/7 of recipients. In summary, immunoisolated graft efficacy can be optimised by the selection of site, the use of helper cells, and with a novel conformal coating approach.

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List of Abbreviations

APA	Alginate-poly-L-lysine-alginate microcapsules
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BALB/c	Bagg Albino C inbred mouse strain
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
C57BL/6J	C57 black 6J inbred mouse strain
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CD	Cluster of differentiation
DAB	3,3'-diaminobenzidine
DCCT	Diabetes control and complications trial
DNA	Deoxyribonucleic acid
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPA	Diprotin A
DPP-IV	Dipeptidyl peptidase-4
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
G	Guluronic acid
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen

IBMIR	Instant blood-mediated inflammatory response
ICR	Institute for Cancer Research outbred mouse strain
IEQ	Islet equivalent
IFN-γ	Interferon gamma
IL	Interleukin
IMS	Industrial methylated spirit
K_{ATP}	ATP-sensitive potassium channel
M	Mannuronic acid
MCP-1	Monocyte chemoattractant protein-1
MEM	Modified Eagle's medium
MIN6	Mouse insulinoma β cell line
MHC	Major histocompatibility complex
mmHg	Millimetre of mercury
MMP	Matrix metalloprotease
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
NaOH	Sodium Hydroxide
NK	Natural killer cell
NO	Nitric Oxide
NOD	Non-obese diabetic mouse
PBS	Phosphate buffered saline
PC	Phosphorylcholine
PC(1/2/3)	Prohormone convertase 1/2/3
PDX-1	Pancreatic duodenal homeobox-1
PES	Polyethersulfone
PI-9	Serine protease inhibitor 9
PLL	Poly-L-lysine
PLO	Poly-L-ornithine
PSS	Polystyrene sulfonate
STZ	Streptozotocin
SV40Tag	Simian vacuolating virus 40 large T antigen
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta

TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal polypeptide
XIAP	X-linked inhibitor of apoptosis protein

1 General Introduction

1.1 Diabetes and the Islets of Langerhans

1.1.1 Diabetes Mellitus

Insulin is the central regulator of blood glucose in the body, causing cells in the liver, muscle, and fat tissues to take up glucose from the blood and thereby reduce the blood glucose level. Diabetes mellitus (DM) is characterised by elevated blood glucose levels (hyperglycaemia) caused by a lack of insulin production by the β cells in the pancreas or a deficiency in the utilisation of insulin in the body. The condition is estimated to affect 5% of the population worldwide (346 million) and the related mortality is predicted to double between 2004 and 2030 (WHO, 2012). DM is classified into three main types; type 1 diabetes mellitus (T1DM), formally known as insulin-dependent diabetes mellitus or juvenile-onset diabetes, type 2 diabetes mellitus (T2DM), formally known as insulin-independent diabetes mellitus or adult-onset diabetes, and finally gestational diabetes. T1DM and T2DM can both occur at any age, however onset for T1DM is typically in children and young people and onset for T2DM is typically in older people. T1DM is caused by the autoimmune destruction of the insulin producing β cells in the islets of Langerhans in the pancreas resulting in an absolute lack of insulin. It is not fully known what causes the autoimmune response, and preventing T1DM is not currently achievable. Insulin replacement therapy is essential to keep T1DM patients alive. T2DM is caused by a combination of insufficient insulin production and peripheral insulin resistance in the tissues of the body which prevents the utilisation of insulin, thereby inhibiting the uptake of glucose from the blood. The major risk factors of T2DM are excessive body weight and physical inactivity, and disease progression can, in some cases, be prevented or reversed by lifestyle changes. Gestational diabetes is typically a transient form of diabetes which onsets during pregnancy and reverts after child birth. The causes are related both to insufficient insulin production and insulin resistance.

The symptoms of hyperglycaemia include excessive excretion of urine (polyuria), thirst (polydipsia), weight loss, hunger, distortion of vision, and fatigue. These symptoms are severe and may occur suddenly in T1DM patients whereas they are subtle and gradual in T2DM and gestational diabetes patients. Following diagnosis, the management of DM involves medical treatments and changes in lifestyle to keep the patient's blood glucose in the normal range (normoglycaemic, 4-7 mM). Achieving consistent normoglycaemia with disease management is difficult and consequently an accumulation of time spent in an aberrant glycaemic state leads

to secondary complications in the long term. As gestational diabetes is typically transient and usually well managed due to prenatal screening, minimal impact from hyperglycaemia is predicted in the long term, although the condition does complicate pregnancy. Due to the gradual presentation of symptoms with T2DM, diagnosis may be several years after initiation of hyperglycaemia when secondary complications may have already occurred. Secondary complications of DM include cardiovascular disease, nephropathy, retinopathy, neuropathy, poor wound healing, and pregnancy complications. Recently T2DM has been described as pandemic and aside from the human suffering caused by T2DM and its related secondary complications they are a major economic burden to individuals and governments.

1.1.2 Type 1 Diabetes

T1DM accounts for 5-10% of DM cases worldwide (ADA, 2004) and is increasing in incidence at a rate of 3-5% per year (Forlenza and Rewers, 2011). More than 85% of all DM cases in people <20 years of age are T1DM, with a peak incidence occurring at 10-14 years of age (Maahs et al., 2010). The increase in incidence of T1DM is particularly evident in young children and it is predicted that incidence of T1DM will double in children under 5 years old by 2020 (Patterson et al., 2009). The rate of change cannot be attributable to genetics alone and is likely to be related to the interaction between genes and the environment. The proportion of children with the highest risk human leukocyte antigen (HLA) haplotype has not increased concordantly (Gillespie et al., 2004) suggesting that changes in lifestyle and environmental factors such as diet, physical activity levels, infections, vaccinations, and pharmaceuticals may be contributing to the increased incidence.

T1DM is classified into two main forms according to aetiology. Type 1a constitutes 90% of cases and is caused by T-cell mediated autoimmune destruction of the β-cells. Type 1b makes up the remaining 10% of cases and is characterised by β-cell destruction in the absence of evidence of autoimmunity. T1DM is thought to be initiated by environmental triggers in genetically predisposed individuals, resulting in the destruction of the insulin producing β cells of the islet of Langerhans of the pancreas (Eisenbarth, 1986). In the absence of insulin, glucose cannot be transported into the liver, muscle and fat tissue which results in ketosis and abnormal elevation of blood glucose levels resulting in osmotic dieresis. Together these cause ketoacidosis, dehydration, and unbalanced plasma solutes, which results in a progressive homeostatic imbalance which can ultimately lead to cerebral oedema, coma, and death.

Genetic predisposition to T1DM is largely inherited and resides predominantly in the HLA class II region of the major histocompatibility complex (MHC). The DR and DQ HLA genotypes are

thought to account for 50% of the genetic predisposition to T1DM (Redondo et al., 2001), and two specific haplotypes are present in 90% of children with T1DM (Devendra and Eisenbarth, 2003). These regions affect the ability of antigen-presenting cells to present β cell autoantigens to T lymphocytes to confer self-tolerance to β cells. The second most important genetic susceptibility factor is the insulin gene which contributes to 10% of the genetic predisposition (Bell et al., 1984). Two other genes associated with inhibition of T cell activation, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and lymphoid tyrosine phosphatase (LTP) are considered the third and fourth most important susceptibility genes. All of these genes can have susceptibility and protective alleles. For example, a variable number tandem repeat in the insulin promoter is associated with susceptibility with fewer repeats, and protection with a greater number of repeats (Bennett et al., 1995).

The genetic predisposition to T1DM couples with exposure to one or more environmental triggers that alter immune function to initiate β cell destruction. Putative triggers include viruses (enteroviruses, coxsackie, and congenital rubella), bacteria, cow's milk, wheat proteins, and vitamin D deficiency (van Belle et al., 2011). Viruses are hypothesised to act in a number of ways to initiate an autoimmune response. For example, β cell death from viral infection may cause β cell antigen shedding, or viral antigens with sequence homology with β cell antigens may cross-react with β cell auto-antigens, re-stimulating resting β cell autoreactive T cells. Antigen cross-reactivity is also proposed to be the mechanism by which bacteria, cow's milk, and wheat proteins may initiate the autoimmune response. Vitamin D inhibits immune activation and is thought to be protective. An alternative theory to environmental triggering is the hygiene hypothesis which suggests that modern living conditions create a lack of exposure to antigens and infectious organisms which causes a hyperactive and deregulated immune system which leads to autoimmunity (Gale, 2002). Tolerance to self antigens is normally achieved by a combination of deletion of autoreactive T cells in the thymus during T cell maturation and peripheral control by regulatory T cell populations such as cluster of differentiation 25 positive (CD25+) CD62L+ CD4+ T cells and natural killer (NK) T cells. If self antigens are not presented correctly in the thymic cortex tolerance may not be established during T cell maturation. The failure of peripheral tolerance is likely to be significant as islet antigen reactive T cells can be detected without the development of diabetes (Roep et al., 1999).

Activation of the autoimmune response directed at β cells leads to inflammation in the islets (insulitis) and humoral production of β cell antibodies. Presentation of β cell antigens activates T helper lymphocytes causing the release of various cytokines that activate other immune

cells. Antibodies produced by B lymphocytes flag β cells for destruction by complement. The autoimmune destruction of the β cells is carried out by CD4+ and CD8+ T cells and macrophages. Activation of NK cells and cytotoxic (CD8+) T lymphocytes stimulate cell-mediated destruction of β cells. β cell destruction occurs predominantly through apoptotic mechanisms (Kurrer et al., 1997) such as interaction of cell surface Fas with Fas ligand on infiltrating cells, perforin and granzyme B mediated membrane destruction by cytotoxic T cells, and macrophage-derived nitric oxide (NO) and toxic free radicals. T cell cytokines such as interleukin-1 beta (IL1β), tumour necrosis factor alpha (TNF-α), and Interferon gamma (IFN-γ) upregulate both Fas and Fas ligand, and also induce NO and toxic free radical production. Rodent models have demonstrated that it is possible to transfer the autoimmune response by grafting islet reactive T cells from a diabetic animal to a healthy animal (Matsumoto et al., 1993). It is not possible to transfer the autoimmune response using only antibodies, which suggests that T1DM is a predominantly T cell mediated disease. The β cell specific destruction in T1DM is demonstrated by the presence of T lymphocytes and macrophages associated with residual β cells and the persistence of other islet cell types in the islets of patients. Major markers of insulitis are islet cell autoantibodies (ICAs), insulin autoantibodies (IAA), protein tyrosine phosphatase (IA2) antibodies and glutamic acid decarboxylase (GAD) antibodies. The presence of the antibodies does not necessarily indicate insulitis and an antibody positive prodromal period of months or years can occur.

1.1.3 Architecture of the Islets of Langerhans

The pancreas is composed mostly of exocrine tissue which produces the digestive enzymes that facilitate the breakdown of food in the gastrointestinal tract. The endocrine component of the pancreas is composed of approximately 1 million islets of Langerhans which constitute approximately 2-3% of the total gland mass. Islets vary in size from 50-400 μm with approximately 1,000-3,000 cells per islet. Islets are distributed throughout the exocrine tissue of the pancreas but at a higher density in the tail region. Islets are partially separated from an acinar cell parenchyma by an incomplete capsule infiltrated by connective tissue originating from the interstitial septa of the exocrine tissue. Islets consist of insulin producing β cells, glucagon producing α cells, somatostatin producing δ cells, and pancreatic polypeptide producing PP cells. In rodents the islet core is a uniform homocellular medulla of β cells, with α, δ, and PP cells located around the periphery. In human islets the different cell types are more heterogeneously distributed. The ratio of β:δ:α:PP cells is 68:10:20:2 in human adults. The function of the islets is mediated by a multitude of stimuli which regulate the secretions of the various cell types. The tissue is rich in vascular supply and nervous inputs which allow it to respond to metabolic, endocrine, and neuronal signalling.

Islets contain a capillary network which is approximately 10 times denser than that of the surrounding exocrine tissue (Henderson and Moss, 1985, Kuroda et al., 1995). Accordingly the islets receive 20% of the total pancreatic blood flow yet constitute only 2-3% of the total gland mass (Lifson et al., 1985). Consequently the blood perfusion of the islets is approximately 10 fold higher than that of the exocrine pancreatic tissue. The efficient blood perfusion in the islets provides a rich supply of oxygen and nutrients and allows for rapid response to blood glucose dynamics and dispersion of secreted hormones. Dense glomerulus-like anastomosing capillaries with fenestrated endothelium for rapid peptide release infiltrate the islet. The blood flow within islets is organised centrifugally which results in blood supplying β, then α, then δ cells (Samols et al., 1988). This would indicate that metabolic and hormonal signals are carried in this orientation. This also suggests that glucagon and somatostatin act on β cells through the systemic circulation. However gap junctions connecting islet cells are thought to provide a more direct communication. Blood flow to the islets can be altered in response to changes in blood glucose concentration by innervation of sympathetic adrenergic neurons supplying the blood vessels of the islets (Jansson, 1994). The nervous network in islets consists of cholinergic parasympathetic, adrenergic sympathetic and peptidergic neurons. Parasympathetic innervation from the vagus stimulates insulin release whereas adrenergic sympathetic innervation inhibits insulin and stimulates glucagon secretion. Nerves extending from outside the pancreas into the pancreatic tissue also secrete peptides such as enkephalin, substance P, cholecystokinin (CCK), calcitonin gene related peptide (CGRP), and neuropeptide Y (NPY). Vasoactive intestinal polypeptide (VIP) containing nerves are the most abundant of this type and are known to stimulate all islet hormones by paracrine mechanisms.

1.1.4 Physiological Insulin Secretion

In order to meet the metabolic needs of the body the β cell has complex and sensitive mechanisms for detecting the metabolic demands of the body and secreting insulin appropriately. Blood glucose levels are maintained at 4-7 mM by the balance between glucose entry into the circulation from the liver, intestinal absorption, and insulin-mediated glucose uptake into peripheral tissues such as muscle and adipose tissue. Glucose is the principle regulator of insulin secretion either directly through glucose transporter 2 (GLUT-2) transporters on the β cell or by amplifying the release of other insulin secretagogues.

Insulin is synthesised in the β cells firstly by the expression of the preproinsulin gene product comprising an N-terminal sequence attached to proinsulin. Preproinsulin is cleaved by signal peptidases to yield proinsulin which comprises A and B chains connected by the connecting

peptide chain (C-peptide). Proinsulin is packaged at the Golgi apparatus into secretory granules within which prohormone convertase 2 and 3 (PC2/3), and carboxyl peptidase H break down proinsulin to insulin and C peptide to from mature granules consisting of insulin and C-peptide. The mature insulin molecule consists of the 21 amino acid A chain and the 30 amino acid B chain linked by two disulphide bonds to give monomeric protein of 6 kDa. The cleavage of proinsulin is accompanied by a fall in pH suggesting that maturation of the granules requires a critical pH level. Insulin biosynthesis is regulated to meet the metabolic demands of the body. An acute rise in blood glucose concentration stimulates rapid upregulation and processing of preproinsulin, but does not affect the rate of conversion from proinsulin to insulin in the granules. Secreted insulin can be reabsorbed back into β cells in an autocrine manner which suggests a negative feedback mechanisms exists to self-regulate insulin secretion (Persaud et al., 2002).

Insulin is secreted in a biphasic manner with the first phase lasting a few minutes followed by a sustained second phase. Glucose enters the β cell via the GLUT-2 transporter and becomes phosphorylated by glucokinase. GLUT-2 which is also found on liver, intestine, and kidney cells forms the glucose sensor together with glucokinase. Due to its high K_m the rate of glucose entry into the β cell is proportional to the extra cellular glucose concentration. Glycolysis and mitochondrial metabolism produce adenosine triphosphate (ATP) from the glucose which closes ATP-sensitive potassium channels (K_{ATP}). This results in depolarisation and consequent opening of voltage-dependent calcium channels which triggers insulin granule translocation and exocytosis. Secretory granules are translocated on a network of tubulin microtubules by a combination of contractile proteins including actin microfilaments and myosin. Exocytosis of the granules occurs by a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex mediation. Vesicle-SNARE proteins on the granule dock with target-SNARE proteins on the plasma membrane in conjunction with elevated intracellular Ca^{2+} and calcium-binding granule proteins such as synaptotagmin to permit fusion of the granule and plasma membranes. Released insulin stimulates the uptake of glucose from the circulation via GLUT-4 transporters expressed in adipose and skeletal muscle tissues, and GLUT-2 transporters expressed in the liver tissue, thereby reducing the blood glucose concentration. Insulin also activates glycolysis, glycogen and fat synthesis, and inhibits glycogenolysis. Glucagon acts to counter-balance the effects of insulin by increasing blood glucose concentration by promoting glycogenolysis and gluconeogenesis predominantly in the liver. Somatostatin has an inhibitory effect on α and β cells and pancreatic polypeptide has systemic effects which inhibit food intake and gastric emptying.

1.1.5 Modulation of Insulin Secretion

The translocation and exocytosis of insulin secretory granules is a Ca^{2+} dependent process. An increase in intracellular Ca^{2+} can therefore stimulate the release of insulin. Glucose and amino acids cause an increase in intracellular Ca^{2+} by depolarising the membrane either directly by carrying positive charge into the cell or indirectly by generating ATP from metabolism which closes K_{ATP} channels. Insulin secretion can be modulated by hormones, peptides, and neurotransmitters. These molecules have an effect on either ion channels that regulate membrane potential, mobilization of intracellular Ca^{2+} stores, or modification of contractile protein interactions which sensitize translocation and exocytosis. Glucagon can potentiate insulin secretion by generating cyclic adenosine monophosphate (cAMP) in the β cell which ultimately leads to sensitization of the secretory machinery to Ca^{2+} (Samols et al., 1965). Somatostatin is associated with a decreased production of cAMP in β cells and as such acts as a paracrine inhibitor of insulin secretion (Samols and Stagner, 1990, Hauge-Evans et al., 2009). Preptin is derived from pro-insulin-like growth factor II and has been located to β cells where it can amplify glucose mediated insulin secretion (Buchanan et al., 2001). Parasympathetic nerve neurotransmitters acetylcholine (Ach), VIP, and gastrin-releasing peptide (GRP) along with pituitary adenylate cyclase-activating polypeptide (PACAP) all induce insulin secretion. Sympathetic nerve neurotransmitters norepinephrine, NPY, and galanin all inhibit insulin secretion. Putative sympathetic nerve neurotransmitters include CCK and neurotensin which have both been shown to stimulate insulin release. Other peptides such as bradykinin and oxytocin and various hormones such as growth hormone, parathyroid hormone and cortisol have also been shown to stimulate insulin secretion (Nielsen et al., 2001). Finally, incretin hormones secreted from intestinal endocrine cells play a major role in stimulating insulin secretion. These will be described in detail elsewhere in the general introduction (1.5.1). The organisation and physiology of the islet provides a multifaceted and complex regulation system for insulin secretion. Therapeutic approaches should aim to accurately mimic or directly provide physiological insulin secretion in order to provide an accurate and dynamic treatment for insulin deficiency.

1.2 Therapeutic Approaches for Type 1 Diabetes

1.2.1 Insulin Replacement Therapy

Since the discovery of insulin in the 1920's insulin therapy has been developed to treat T1DM. Insulin replacement therapy is effective in controlling hyperglycaemia by administration of exogenous insulin by subcutaneous injections. Patients using insulin therapy are expected to lead a near to normal lifestyle. The treatment is demanding for patients and requires the

assistance of a medical team to educate the patient in self management. This involves attention to eating and exercise, and appropriate blood glucose monitoring and insulin administration. The treatment regimen attempts to mimic normal physiological insulin secretion patterns. Administration of intermediate or long-acting insulin simulates basal secretion throughout the entire day, and rapid-acting insulin administered before meals simulates prandial insulin secretion. However it is not possible to achieve constant normoglycaemia as exogenous insulin administration can not accurately and continuously mimic the pulsatile and ultradian oscillatory nature of physiological insulin secretion. Rapid-acting insulin is absorbed too slowly and lasts too long to mimic normal prandial peaks, while long-acting insulin does not provide the steady low concentrations required between meals. To further the complexity, insulin must be administered to compensate for subjectively predicted variables such as food intake, exercise, and stress. Subcutaneous injections of identical doses of insulin can result in variable metabolic effects from day to day. This variability of insulin action can prevent optimal glycaemic control from being achieved and is a source of uncertainty and frustration for patients. Absorption rate is not the only factor as identical plasma concentrations can even cause variable glycaemic control (Heinemann et al., 1998). Over the lifetime of a patient there is therefore a significant accumulation of time in hyper- and hypo-glycaemic states. Persistently aberrant blood glucose levels are associated with secondary complications in the long term such as microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (cardiovascular, cerebrovascular, and peripheral vascular disease) disorders. The major cause of mortality for patients with disease duration under 20 years is renal pathology and over 30 years is cardiovascular pathology (Orchard, 1994). Despite the efficacy of insulin therapy it has been estimated that a 10-year-old boy or girl developing diabetes in the year 2000 would lose 18.7 and 19.0 life years on average, respectively, compared with non-diabetic peers (Narayan et al., 2003).

An influential study by the diabetes control and complications trial (DCCT) reported that the use of intensive insulin therapy to achieve consistent blood glucose control was effective in preventing secondary complications (DCCT, 2003). However the DCCT reported that hypoglycaemic episodes were three times as likely using intensive insulin treatment verses conventional treatment. Some patients suffer from episodes of hypoglycaemia as a result of incorrect dosing of insulin or other factors. Such episodes can be unpredictable and highly disruptive to day-to-day quality of life. Severe hypoglycaemic episodes present with confusion, convulsion or unconsciousness and frequent episodes can lead to cognitive impairment. Fear of hypoglycaemic episodes can also interfere with the patients' ability to correctly administer insulin therapy. It is estimated that T1DM patients are hypoglycaemic (>3.3 mM) 10% of the

time (Boland et al., 2001) suffering an average of two symptomatic episodes per week and one occurrence of severe disabling hypoglycaemia per year (MacLeod et al., 1993). Additionally it is estimated that 2-4% of deaths of people with T1DM are due to hypoglycaemia (Laing et al., 1999). Hypoglycaemia usually induces a stress response by epinephrine release which the patient can recognise and act upon. However for some patients with hypoglycaemia unawareness the stress response is attenuated, resulting in patient inaction and therefore an increased chance of progression to a severe hypoglycaemic episode. Brittle diabetes patients are described as having erratic glucose levels that are unpredictable and interfere with patient lifestyle. Labile diabetes is characterised by a gradual decline in glucose levels over the day even if they are not extremely erratic. For a subset of around 10% of diabetic patients with brittle or labile diabetes, frequent hypoglycaemic episodes, hypoglycaemic unawareness, or a consistent failure of insulin based management due to clinical or emotional problems, the application of islet transplantation has shown to be beneficial compared to insulin therapy (Ryan et al., 2004a).

Islet transplantation is the focus of this thesis, however other approaches are also being developed as alternatives to conventional insulin therapy. Insulin pumps can be used alone or coupled with continuous glucose monitoring to direct the infusion of insulin in what is referred to as a closed loop system or artificial pancreas. Continuous glucose monitoring has been achieved although it does not have the accuracy of conventional blood glucose meters (De Block et al., 2008). Insulin pumps alone offer continuous insulin infusion which can be adjusted for optimal basal insulin replacement. Adequate algorithms are yet to be developed to link continuous glucose monitoring with insulin pump action to fulfil the dynamic specific insulin requirements of individual patients. Whole pancreas transplantation is another alternative approach to insulin therapy. Pancreas transplantation is usually performed as a multi-organ transplantation in conjunction with kidney transplantation. As diabetic patients in need of kidney transplantations will undergo invasive surgery and lifetime immunosuppression, pancreas transplantation can be performed simultaneously without greatly increasing the existing risk. Previously pancreas transplantation resulted in better outcome compared to allogeneic islet transplantation as measured by insulin independence. However there is now evidence that islet transplantation provides comparable transplantation outcome to pancreas transplantation (Maffi et al., 2011). However islet transplantation has the advantages of being less surgically invasive and has reduced chance of surgical complications.

1.2.2 Islet Transplantation

Islet transplantation for T1DM aims to replace the islet tissue in order to restore normal physiological glycaemic control to patients. It is hoped that restoring physiological glycaemic control would eliminate hypoglycaemic episodes, reduce the risk of progression of secondary complications, and also free patients from the burdens of injections, glucose testing, and dietary restrictions. The clinical procedure for islet transplantation involves the infusion of islets from cadaveric donors through a percutaneous cannulation of the portal vein and engraftment of the islets in the venules of the liver. Before the turn of the century less than 10% of islet graft recipients achieved insulin independence. The first major breakthrough in clinical islet transplantation was the Edmonton protocol which was reported to achieve insulin independence in 7/7 patients at 1 year post-transplantation (Shapiro et al., 2000). The success of the study was due to using large islet grafts involving multiple infusions from multiple donors. The approach used a glucocorticoid-free immunosuppressive regimen, fresh rather than cultured islets, and reduced cold ischemia time. Although this result was a major improvement on previous clinical islet transplantation outcomes the longevity of graft function was limited with only 10% remaining insulin independent after 5 years. The Edmonton protocol has since provided the basis of clinical islet transplantation at centres worldwide. The most recent report from the clinical islet transplantation registry analysed the outcome of 677 islet grafts. The success of islet transplantation has gradually improved with 3 year insulin independence rates of 27%, 37%, and 44% in the early, middle, and late 00's (Barton et al., 2012). Recently there has been a drive towards achieving insulin independence with grafts derived from single donors (Shapiro, 2011). If graft success can be achieved with a single donor, islet transplantation will be comparable with pancreas transplantation which requires a single pancreas. In islet graft recipients without insulin independence some degree of physiological insulin production and hypoglycaemic awareness seems to persist. Consequently the risk of hypoglycaemia can be greatly reduced over the long term for islet transplantation recipients (Ryan et al., 2004b). Although islet transplantation does not assure complete insulin independence in the long term, patients report the reduction in hypoglycaemic episodes to be a major benefit. With some degree of physiological insulin control restored the necessity for larger and potentially inaccurate doses of exogenous insulin is obviated. In addition to the physiological benefits of a reduced frequency of hypoglycaemia, patients have also reported an increase in their quality of life due to a reduction in anxiety associated with hypoglycaemia. There is also evidence that islet transplantation can reduce the progression of secondary complications (Fiorina et al., 2003).

There is a donor shortage for islet transplantation such that with the current protocol and source of islets from heart-beating brain-dead donors it is estimated that only 0.1% of T1DM patients could be treated (Shapiro et al., 2001). This is exacerbated as donor pancreases are also considered for whole pancreas transplantation and multiple donors are required for islet transplantation. Isolation of islets causes vascular connections to be severed leaving the islet reliant on diffusion to obtain sufficient oxygen and nutrients until revascularisation has taken place after transplantation. Islets may also be damaged from collagenase digestion, mechanical force, and oxygen and nutrient deficiency during islet isolation. Isolation disrupts the complex architecture of the islet and causes loss of support from surrounding ductal cells (Ilieva et al., 1999) extracellular matrix (ECM) proteins (Wang and Rosenberg, 1999) and endothelial cells (Johansson et al., 2009), which all provide trophic factors for islet viability and function. Culture of islets has also been reported to cause hypoxic stress (Lau et al., 2009). Transplantation of islets rapidly after isolation was thought to be optimal, however current islet transplantation strategies emphasise the importance of a short culture period pre-transplantation. Short term culture removes nonviable islets and decreases tissue factor expression which can cause inflammation and consequent islet loss (Johansson et al., 2005a). The culture period also allows time to perform microbiological screening and initiate immunosuppression in the recipient.

Optimal engraftment of islets should prevent further islet damage and stress to that endured during islet isolation, culture, and transplantation. Ideally, islet morphology should be maintained and the islet architecture should be reconstituted with revascularisation and reinnervation, and the restoration of paracrine and ECM interactions. As clinical islet transplantation is at the intraportal site the restoration of neuronal innervation and paracrine interaction similar to that of the native pancreatic site are not possible. Islets entrapped in ECM scaffolds have demonstrated improved islet viability and glucose-stimulated insulin secretion (GSIS) (Zhang et al., 2012), and improved graft outcome in transplantation models (Salvay et al., 2008). However revascularisation is considered to be the most important factor in islet engraftment (Davalli et al., 1996). Following transplantation revascularisation is generated by both donor and recipient endothelial cells (Brissova et al., 2004). Revascularisation progresses with observable sprouting at 2 days post transplantation and takes up to 10-14 days to complete revascularisation (Menger et al., 2001). Delayed and insufficient vascularisation results in deprivation of oxygen and nutrients resulting in cell death which can lead to graft failure (Linn et al., 2006). Indeed hypoxia has been suggested to be a major factor in the early loss of islets (Davalli et al., 1995, Jirak et al., 2009).

Upon transplantation, loss of graft function over time is due to both acute and chronic host challenges. The acute loss of cells in the first week is due to damage from the transplantation procedure and the instant blood-mediated inflammatory reaction (IBMIR). The IBMIR involves platelet deposition, coagulation, secretion of harmful cytokines such as IL-1β, TNF-α, and IFN-γ and leukocyte infiltration resulting in the immediate loss of up to half the islet mass (Eich et al., 2007). The gradual loss of surviving islets over time is thought to be due to the host immune rejection and the toxic effect of the immunosuppressive agents. The immune response to islet grafts is similar in mechanism to the autoimmune response (1.1.2) except it is initiated predominantly by MHC alloantigens as opposed to autoantigens. In addition to the alloimmune response which is present in any type of allograft, the autoimmune challenge to β cell antigens which is particular to T1DM patients may also contribute to the continued destruction of the implanted islets. Immunosuppressants are able to inhibit the acute rejection to some degree but chronic rejection continues resulting in fibrosis, tissue damage, and graft failure, despite the immunosuppressive regimen. Indeed it has been demonstrated that under immunosuppressive regimens immunological parameters such as T cell alloreactivity correlates with clinical outcome (Roelen et al., 2009)

The transplantation of allogeneic islets necessitates the use of immunosuppression in order to prevent graft rejection. Immunosuppression naturally results in an increased risk of infection, and also malignancy (Riminton et al., 2011). Long-term follow-up studies from islet transplantation are forthcoming but indications from renal transplant patients under similar immunosuppressive regimes suggest a 42-fold increase in risk of infection (Abbott et al., 2002) and a 10-15% risk of cancer over a 10 year period post transplantation (Euvrard et al., 2003). Nephrotoxicity also occurs with an estimated 16.5% of patients developing chronic renal impairment. This is particularly unfavourable as recipients are already at risk of kidney disease as a secondary complication of diabetes. Other complications for immunosuppressed islet transplant patients include mouth ulcers (96%), diarrhoea (67%), acne (35%) and some individuals require granulocyte colony-stimulating factor (GCSF) due to severely low mean white cell counts. Immunosuppressive agents are also known to be harmful to islet viability and function (Kaestner, 2007). The advances in islet transplantation in the last decade have mainly been due to improvements in strategies to prevent islet rejection. The Edmonton protocol established the use of IL-2 receptor antagonist for induction and mammalian target of rapamycin (mTOR) inhibitor and calcineurin inhibitor for maintenance of immunosuppression. Current protocols include induction with a T cell deleting antibody, with or without a TNF-α inhibitor, and maintenance with an mTOR inhibitor or an inosine monophosphate dehydrogenase inhibitor combined with a calcineurin inhibitor (Barton et al., 2012).

Several approaches aiming to improve islet transplantation outcome are in development. The process can be optimised at every stage including optimisation of islet isolation for greater yields and islet viability, maintenance of islet viability and function during culture, and enhancing islet engraftment and revascularisation. However, arguably the most important factor in the outcome of islet transplantation is the host immune response. Islet transplantation presents a challenge whereby it is not only necessary to provide for the islets to maintain islet viability and function but also to protect the islets from a dynamic and complex system which seeks to destroy the islets. For T1DM patients able to achieve relatively good normoglycaemia through insulin therapy and in the absence of renal disease, it is difficult to justify islet transplantation given the risks of immunosuppression. Overall islet transplantation results in stabilization of glucose control, better quality of life and may improve secondary diabetic complications. The risks are acute morbidity from the procedure, chronic morbidity from immunosuppression, and increased relative risk of mortality. In effect the patient is exchanging insulin therapy for immunosuppressive therapy. Induction of graft tolerance would provide a solution to the problem of chronic graft rejection. There are several strategies to confer immune tolerance including donor-reactive T cell depletion, co-stimulatory blockade, immune deviation, and generation of regulatory T cells (Bhatt et al., 2012). T cell depletion (Silveira et al., 2006) and co-stimulatory blockade (Posselt et al., 2010) have been used in clinical islet transplantation however they do not induce complete tolerance. Co-transplantation of cells can confer immune deviation and will be discussed in this thesis (1.6.3). Finally regulatory T cell therapy is problematic due to the necessity to produce large numbers of antigen specific T cells and simultaneously restrain them to a regulatory T cell lineage. It would therefore be desirable to be able to implant islets in an allogeneic setting without any manipulation of the host immune system.

1.2.3 Immunoisolation

The aim of immunoisolation is to encapsulate the islets in a permselective biomaterial which allows the passage of small vital molecules such as oxygen and insulin but prevents the entry of large molecules and cells. This physical barrier should prevent cellular contact and recognition of MHC by host lymphocytes and therefore abolish cell mediated cytotoxicity. In addition antibodies should ideally be excluded by the barrier in order to prevent antibody-mediated cytotoxicity and complement activation. The islets can therefore function effectively whilst preventing the destruction caused by host immunity and hence obviate the need for an immunosuppressive regimen. Encapsulation devices can broadly be categorised into three types. Intravascular macrocapsules which act as perifusion chambers implanted into blood

vessels. Extravascular macrocapsules which are diffusion chambers in the shape of a tube or disk which are implanted in body cavities such as the peritoneal cavity. And finally microcapsules which attempt to encapsulate islets singularly in spherical capsules. Both forms of macrocapsule have been largely unsuccessful due to the large size of the devices resulting in large diffusion distances for vital molecules. In addition large intravascular devices are difficult to surgically implant and promote blood clotting which increases the risk of thrombosis. Microcapsules have been the most widely used immunoisolation device for islet transplantation. The small size and spherical shape of microcapsules is designed to provide adequate cover of the islet without having unnecessary biomaterial, therefore minimising the diffusion distance and also limiting the interaction with surrounding tissues.

1.2.4 Alginate Microencapsulation

Microencapsulation of islets was first described in 1980 by Lim and Sun using alginate as the encapsulation material (Lim and Sun, 1980). Alginate is a component of the ECM of brown algae consisting of the polysaccharides β-D-mannuronic acid (M) and 1,4-linked α-L-guluronic acid (G). Raw alginate from seaweed is purified to remove polyphenols, proteins, and endotoxins to improve the biocompatibility (Dusseault et al., 2006). Islets are suspended in dissolved alginate and using air, mechanical or electrostatic force, or a combination of these, droplets are drawn into a divalent cationic solution containing Ca^{2+} and/or Ba^{2+} which causes gelification by cross-linking the alginate molecules to form a hydrogel. Hydrogels are hydrophilic and negatively charged, resulting in minimised protein and cell adhesion. Additionally the pliable gel formed from alginate hydrogels minimises mechanical or frictional forces to the surrounding tissue. Materials other than alginate have been used for microencapsulation, however alginate has several advantages. Alginate does not interfere with islet function, and can be cross-linked at physiological temperature, pH, and osmotic potential. Indeed insulin secretion from alginate microencapsulated islets is proportionally responsive to variable concentrations of glucose and insulinotropic agents (Figure 1). Alginate encapsulation of islets has also been shown to improve the survival of islets in culture (Sandler et al., 1997, Lopez-Avalos et al., 2001), and is stable *in vivo*. Alginate molecules are linear block co-polymers of M and/or G which vary in composition depending on the source. Blocks of G and blocks of M and G are able to cross-link with divalent cations (Donati et al., 2005). Alginates with specific M:G ratios can be selected to optimise the stability and binding efficiency to form microcapsules with specific properties. Due to the cross-linking ability of G blocks, high-G alginate is more stable and resistant to mechanical stress although it is also more permeable than high-M alginate (Martinsen et al., 1989). The most commonly used microcapsules were traditionally composed of alginate with an outer layer of polycation usually of poly-L-lysine

(PLL) and an alginate covering to mask immunoreactive unbound polycation groups (APA). Polycation is used to provide stability which is essential for Ca^{2+} gelified alginate and also decreases the permeability of the capsules. M rich alginate is able to bind PLL better which results in greater control over permeability and better coverage of PLL. Barium-alginate hydrogel has a stronger gel than calcium-alginate hydrogel and consequently is often used without a polycation coating (Thu et al., 1996). Barium is toxic, however using low concentrations and adequate microcapsule washing and culture, barium leakage from capsules is lower than established clinically tolerable levels (Morch et al., 2012). The benefit of cross-linking with Ba^{2+} is only observed in alginates with high-G content (>60%).

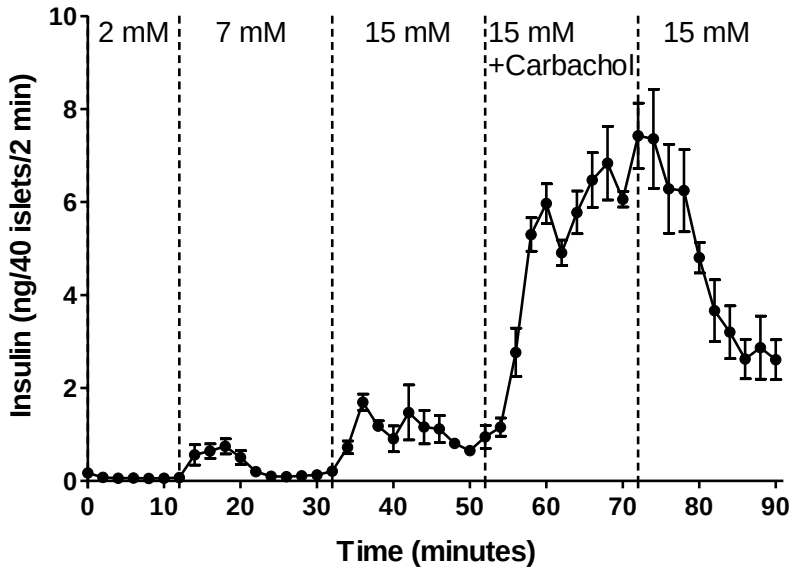


Figure 1. Perifusion of alginate microencapsulated islets. The insulin secretion of Institute for Cancer Research (ICR) mouse islets was assessed in response to glucose/glucose and carbachol challenge in a temperature-controlled islet perifusion system. Islets were exposed to concentrations of glucose/glucose and carbachol as indicated over 90 min (*n*=4(1)).

It is challenging to assess the microencapsulated islet transplantation field as capsule composition is highly varied between groups and within groups over time. There is often a lack of "head to head" data comparing different capsule compositions *in vivo*. Additionally the source of alginate and purification is often variable which further complicates analysis. There are many reports of long term blood glucose correction by transplanting microencapsulated islets into rodent models of diabetes. Transplantation of high-M barium-alginate microencapsulated allogeneic islets have been shown to maintain normoglycaemia for >350 days in non-obese diabetic (NOD) mice (Duvivier-Kali et al., 2001). In a follow up study it was found that capsule stability leading to capsule breakage was problematic when high-M barium-alginate capsules were transplanted into rats. Consequently blood glucose was maintained for only 53±6 days (Omer et al., 2005). High-G barium-alginate capsules offer similar biocompatibility to high-M barium-alginate capsules (Zimmermann et al., 1992), have higher capsule stability, and have demonstrated efficacy in long-term allogeneic transplantation

studies in NOD diabetic mice (Bohman and King, 2008). High-G barium-alginate capsules offer an attractive capsule composition and have been used for the studies in this thesis. Microencapsulated islet transplantation has also been reported in large animals although with less success. Transplantation of syngenic islets in APA capsules into diabetic dogs resulted in maintenance of normoglycaemia for 6 months (Soon-Shiong et al., 1993). The insulin requirements of diabetic cynomolgus monkeys were reduced for 6 months by transplantation of microencapsulated neonatal porcine islet cell clusters (Elliott et al., 2005). An ongoing clinical trial is investigating the use of barium-alginate microencapsulated islets transplanted into the peritoneal cavity of non-immunosuppressed diabetic patients (Tuch et al., 2009). Preliminary results of two patients indicate only a transient reduction in insulin requirements and undetectable C-peptide by 1-4 weeks. Capsules recovered at 16 months contained necrotic islet cells and were surrounded by fibrous tissue indicating poor biocompatibility. Another study using alginate-poly-L-ornithine (PLO)-alginate microencapsulated allogeneic islets in 4 human patients resulted in a reduction in insulin requirements and abolishment of hypoglycaemic episodes for at least 3 years and minimal biocompatibility issues (Basta et al., 2011). The frequency of hypoglycaemic episodes is a major factor in determining if non-encapsulated islet transplantation should be administered. If microencapsulated islet transplantation can be shown to abolish hypoglycaemic episodes it may stimulate rapid development of microencapsulated islet transplantation protocols in the clinic. However it is clear that in large animals and humans, biocompatibility of the capsules remains a critical issue determining graft outcome.

1.2.5 Biocompatibility

Pericapsular overgrowth of microcapsules is generated by non-specific absorption of proteins onto the material surface and chemotactic signals from the islets allowing adhesion and recruitment of macrophages and fibroblasts to the capsule surface. This process takes place for the most part in the first few weeks following transplantation and does not increase thereafter (De Vos et al., 1999). Overgrowth has been found to directly correlate with graft failure in part due to the overgrowth inhibiting adequate diffusion of oxygen and nutrients to the islet (de Groot et al., 2004). Indeed it has been shown that cells overgrowing capsules are metabolically very active with high glucose oxidation rates (King et al, 2003). Overgrowth occurs in approximately 10% of capsules, however it has an impact on neighbouring capsules (de Groot et al., 2003) and has been estimated to cause approximately 40% of the loss of microencapsulated islets (De Vos et al., 1999). The composition, purity, and integrity of the biomaterial including imperfections in the capsule surface have an effect on overgrowth. PLL is used to provide strength to capsules, however it is immunogenic which can result in capsule

overgrowth if the PLL is inadequately bound with the alginate (Clayton et al., 1991). The use of Ba^{2+} solution for gelification has addressed this problem as Ba^{2+} cross-linked capsules have high enough stability without the use of PLL (Zimmermann et al., 2000). However, microcapsules with PLL have been shown to exclude cytokines more effectively (de Groot et al., 2001).

The islet inside a microcapsule also has an impact on the biocompatibility of microencapsulated islet grafts. Studies have reported that islets can protrude outside of the capsule, particularly if small sized capsules are used (Halle et al., 1994, De Vos et al., 1996b, De Vos et al., 1996a). However there is evidence suggesting that this may be avoidable using specific capsule compositions (Strand et al., 2002). Adequately encapsulated islets also play a role in recruitment of host immune cells by mechanisms which can be described as chemotaxis. Passive shedding of islet antigens can potentially generate either an auto- or alloimmune reaction resulting in activation of macrophages and resultant release of cytokines such as IL-1β, TNF-α, and IFN-γ, NO and oxygen free radicals (Gill, 1999). These agents are small enough to penetrate the semipermeable membrane of some microcapsules and affect the function and vitality of islet cells (Eizirik and Mandrup-Poulsen, 2001). Islets under stress are known to secrete monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP), NO and IL-6. All these factors are known to contribute to the recruitment and activation of inflammatory cells (de Vos et al., 2004). For example, MCP-1 is produced in response to hypoxic conditions in order to stimulate angiogenesis. MCP-1 also recruits macrophages and consequently may be involved in graft failure (Paule et al., 2000). In addition to microencapsulated islet biocompatibility an inflammatory response is generated immediately post-transplantation to facilitate normal wound healing after surgery (Mikos et al., 1998). The surgical procedure causes minor tissue damage however the release of inflammatory chemotactic factors in the intraperitoneal cavity after surgery such as fibrinogen, thrombin, histamine, and fibronectin has been reported (de Vos et al., 2002).

1.2.6 Diffusion

It has been shown that microencapsulated grafts fail in the absence of overgrowth which suggests that biocompatibility is not the only factor concerning graft outcome (De Vos et al., 1999). Microencapsulation offers better diffusion than macroencapsulation due to the increased surface:volume ratio, although it may still be suboptimal to support islet viability and function. In the majority of tissues the maximum diffusion distance for vital molecules such as oxygen and nutrients from capillaries is around 200 μm (Orive et al., 2006). Microencapsulated islets free-floating in the peritoneal cavity lack any vascular connection and must rely predominantly on passive diffusion from the peritoneal fluid. This is exacerbated by the

capsule creating a relatively large diffusional distance through the alginate to and from the islet. The absence of convection movement surrounding the capsule and within a capsule can create a gradient of vital molecules from the surface of the capsule to the core (Schrezenmeir et al., 1994). Consequently diffusion of vital molecules to and from the islet is inhibited which is detrimental to islet viability and function. It has been demonstrated that the oxygen tension of islets in small 180 µm diameter capsules is reduced by 55-73% compared to the oxygen tension outside of the capsule (Chen et al., 2012). In addition, mathematical modelling studies have suggested the oxygen tension in the core of islets in 500 µm alginate microcapsules in a 40 mmHg environment such as the intraperitoneal cavity may be as low as <2 mmHg (Avgoustiniatos and Colton, 1997, Johnson et al., 2009). It has been suggested that the most likely limiting factor for islets implanted in bioartificial devices is the diffusion distance to and from blood capillaries (Cotton, 1996). Even partial hypoxia can inhibit ATP-dependent cell functions such as insulin secretion and may induce inflammatory mediators (Dionne et al., 1993). Autologous microencapsulated islets grafted in the peritoneal cavity often have a necrotic core. The cells at the core are furthest away from the source of oxygen and nutrients outside of the capsule which suggests that poor diffusion is responsible. Microencapsulated islets receive glucose by passive diffusion, and secreted insulin enters systemic circulation by passive diffusion through the peritoneal fluid. Consequently insulin secretion is delayed due to poor diffusion (Trivedi et al., 2001) although a reduction in capsule size has been shown to minimize the lag in insulin secretion. Even so, it remains uncertain if the suboptimal metabolic control from insulin secretion into the peritoneal cavity will be sufficiently robust to minimise secondary complications. Due to the challenges presented to microencapsulated islet grafts the efficacy of the graft is limited, and consequently a >2 fold higher islet mass is necessary achieve stable normoglycaemia when compared with non-encapsulated grafts under the kidney capsule (Siebers et al., 1993).

1.3 The Subcutaneous Site for Islet Transplantation

Biocompatibility and adequate diffusion of vital molecules are both multifaceted challenges to the success of microencapsulated islet grafts. The site of implantation can have an effect on both of these factors. The optimal transplantation site for islet grafts will permit maximum survival and function of islets, maximum effectiveness of secreted hormones, and safety to the patient (Kemp et al., 1973). For microencapsulated grafts the intraperitoneal site has been used for the majority of studies predominantly due to the large capacity of the site and also as non-invasive surgery is required. However the site is suboptimal as microcapsules do not have

close contact with capillaries to supply vital molecules for islet survival. It has also been suggested that the peritoneum is a preferential site for inflammation and immunological reaction (Hall et al., 1998). Additionally removal of microencapsulated grafts from the intraperitoneal site is difficult and highly invasive. The accumulation of capsules on the pelvic floor may also be an issue in humans. Consequently the investigation of alternative sites which have a large enough capacity for microencapsulated grafts is valid. The subcutaneous site provides a large and easily accessible site which requires minimally invasive surgery to transplant islet grafts. Islet grafts from the subcutaneous site can be biopsied, easily removed, and can be easily re-implanted. Secreted insulin from subcutaneous islet grafts can only reach the liver and fat tissues through adsorption into the systemic circulation which causes a lag in response to blood glucose changes. However, injected exogenous insulin reaches the circulation through the subcutaneous site. As an alternative therapy to insulin therapy a subcutaneous islet graft would potentially be superior as insulin would be delivered to the same site but in a physiologically responsive manner. Taken together the subcutaneous site is an attractive option for islet transplantation. However there are challenges to implanting islet grafts subcutaneously and research is ongoing to optimise islet transplantation at the site.

1.3.1 Transplantation of Non-Encapsulated Islets at the Subcutaneous Site

Transplantation of islets at the subcutaneous site was first reported by Paul Lacy's group in the early 1970's (Kemp et al., 1973). The study compared the subcutaneous, intraperitoneal, and intraportal sites for syngeneic islet transplantation into diabetic rats. At the time intraperitoneal and intramuscular implantation of islets had already been reported. The authors reasoned that as islets in their native site secrete insulin into the portal venous system the intraportal site may be appropriate. They also suggested that in terms of safety the least hazardous implantation site may be the subcutaneous site. Transplantation of 890 islets subcutaneously had no impact on blood glucose. Transplantation of 770 islets intraperitoneally reduced the blood glucose to between 11.1-22.2 mM. Finally, transplantation of 550 islets intraportally resulted in reversal of the diabetic state and maintenance of normoglycaemia for 50 days. This landmark study stimulated research into the intraportal site for islet transplantation which eventually lead to the adoption of the site for clinical islet transplantation. The failure of the graft at the subcutaneous site was suggested to be due to poor perfusion of the islet graft.

There are reports of graft success after transplantation of non-encapsulated islets without any matrix or device to the unmodified subcutaneous site. Transplantation of 1,200 rat islets into immunodeficient mice resulting in maintenance of normoglycaemia for 28 days (Tatarkiewicz

et al., 1999), and transplantation of 600 mouse islets into syngeneic mice resulting in maintenance of normoglycaemia for 91 days have been reported (Juang et al., 2005). Conversely, the vast majority of studies with use of up to 3000 rodent islets implanted into mice (Perez-Basterrechea et al., 2009) and 5000 rodent islets implanted into rats (Kawakami et al., 2000) report graft failure of non-encapsulated islets at the unmodified subcutaneous site (Yang et al., 2002b, Fumimoto et al., 2009, Kawakami et al., 2001, Kemp et al., 1973, Juang et al., 1996, Halberstadt et al., 2005, Bharat et al., 2005).

1.3.2 Transplantation of Immunoisolation Devices at the Subcutaneous Site

Due to the overwhelmingly poor transplantation outcomes at the unmodified subcutaneous site the use of implantable devices, biomaterials, and matrices have been employed to alter the site to enhance the viability and function of subcutaneously transplanted islets. Following almost two decades since Paul Lacy's group first attempted islet transplantation at the subcutaneous site, his group were the first to describe the use of an implantable device to improve graft success at the subcutaneous site (Lacy et al., 1991). 500 rat islets were immobilised in cross-linked alginate inside poly(acrylonitrile-co-vinyl chloride) hollow fibers of 600 µm diameter. When implanted at the subcutaneous site of mice 4/5 maintained normoglycaemia for over 60 days. The authors do not discuss possible mechanisms but the hollow fibre may have provided a protective casing from mechanical force. It is also possible that the material stimulated angiogenesis thereby improving the supply of oxygen and nutrients to the islets and the diffusion of insulin from islets to the circulation. Another implantable immunoisolation device called the "Theracyte" composed of an inner immunoisolation membrane, an outer angiogenic membrane, and a mesh for stability has been described (Tatarkiewicz et al., 1999). A Theracyte device loaded with 1,500 rat islets was implanted subcutaneously into diabetic mice resulting in maintenance of normoglycaemia for an average of 94 days (Yang et al., 2002b). The authors suggested the vascularisation surrounding the device improved the graft outcome.

One group have focused on the development of a pre-vascularisation approach by delivering basic fibroblast growth factor (bFGF) to the subcutaneous site to promote angiogenesis before implantation of the islets. Studies were carried out in syngeneic (Kawakami et al., 2000, Kawakami et al., 2001) allogeneic (Gu et al., 2001) non-discordant xenogeneic (Wang et al., 2002) and discordant xenogeneic (Wang et al., 2003) transplantation models. All the studies involved the pre-vascularisation of a subcutaneous site for 1-2 weeks by sustained release of bFGF from an implanted device or gelatine microspheres. Devices were either removed before implantation of the islets or islets were implanted into the pre-vascularised devices.

Xenogeneic islets transplanted in an agarose based macrocapsule resulted in graft success of up to 101 days. It was demonstrated that non-encapsulated islets failed due to rejection (Wang et al., 2003), suggesting the macroencapsulation provided immunoprotection. Islets implanted after pre-implantation of devices or microspheres containing no bFGF resulted in graft failure (Kawakami et al., 2001, Wang et al., 2002). This suggests that the bFGF-mediated neovascularisation observed was critical to graft success at the subcutaneous site in their model.

One study has reported xenogeneic transplantation of pig islets into a subcutaneous site in humans without the use of immunosuppression or immunoisolation (Valdes-Gonzalez et al., 2005). Sertoli cells which are immunoprivileged cells derived from the testes were co-transplanted with the islets to confer immunoprivilege to the graft site. Approximately 17,000 islet equivalents (IEQs) and 30-100 sertoli cells per IEQ were delivered into stainless steel mesh tubes pre-vascularised for 2 months at the subcutaneous site. This resulted in 50% of recipients with reduced insulin requirements and two patients achieving insulin independence for up to 2 months. The study was controversial as no non-human primate studies were carried out prior to the human studies.

1.3.3 Transplantation of Microencapsulated Islets at the Subcutaneous Site

Xenogeneic islets immunoisolated in alginate microcapsules or macrocapsules have been transplanted to the subcutaneous site in a series of studies by the laboratory of Dennis Dufrane. Islets were microencapsulated in 1% high viscosity, high-M, calcium-alginate capsules and transplanted at the subcutaneous site of rats to determine if it provides better biocompatibility, islet survival, and islet functionality than the intraperitoneal site (Dufrane et al., 2006). Capsule breakage and overgrowth of empty capsules was lower at the subcutaneous site. Viability, insulin content and *in vivo* insulin secretion (as measured by C-peptide levels) from microencapsulated pig islets was better at the subcutaneous site. The authors suggest that due to the reduced macrophage recruitment and therefore pericapsular overgrowth, maintenance of islet viability and function is better at the subcutaneous site.

In a follow up study, a variety of alginates were tested for suitability for transplantation of microcapsules at the subcutaneous site (Veriter et al., 2010). The study compared 3% high viscosity, high-M calcium-alginate with 3% high viscosity, high-G calcium-alginate. High-G alginate capsules without islets were found to be permeable to 150 kDa molecules, induced fibrosis, and induced angiogenesis creating an oxygen tension of up to 25 mmHg. In contrast high-M alginate capsules without islets were impermeable to 150 kDa molecules, did not

induce fibrosis, and induced angiogenesis creating an oxygen tension of up to 40 mmHg. Due to the advantages of high-M alginate it was selected for transplantation studies. Intraperitoneal transplantation of 8000 encapsulated pig islets into rats resulted in graft failure by day 10 whereas equivalent transplantations to the subcutaneous site resulted in maintenance of normoglycaemia for 60 days. This suggests that the high-M microcapsules are specifically suited to implantation at the subcutaneous site.

Having established the optimal alginate for application at the subcutaneous site further experiments were carried out in non-human primates (Dufrane et al., 2010). 30,000 IEQs per Kg microencapsulated in 1% high-M alginate were implanted subcutaneously into diabetic cynomolgus monkeys resulting in blood glucose normalisation for only 2 weeks. A macroencapsulation device consisting of a decellularised collagen layer sandwiched between layers of 3% high-M alginate was also tested. Macroencapsulation of 50,000 IEQs implanted at a subcutaneous site resulted in maintenance of normoglycaemia for an average of 161 days. The authors do not offer a direct explanation of why the microcapsules failed and the macrocapsule succeeded. The inclusion of the collagen layer in the macrocapsule is suggested to provide mechanical support and promote islet cell adhesion. Perhaps of more significance is the use of 1.6 times the number of IEQs and the higher concentration of alginate in the macrocapsule. It was reported that 1% alginate was permeable to 150 kDa molecules but 3% alginate was not. The increased permeability in the microcapsules may have resulted in increased permeability to cytotoxic molecules and islet antigens leading to cell destruction and recruitment and activation of immune cells respectively. It is not possible to determine from this study if the geometric properties of the microcapsule or macrocapsule are superior at the subcutaneous site.

1.3.4 Implantation of Angiogenic Devices at the Subcutaneous Site

The proximity of the vasculature to islet grafts is thought to be a fundamental aspect of graft success at the subcutaneous site due to the relative availability of oxygen and nutrients to sustain graft viability and the diffusion of insulin into the circulation to confer graft efficacy. Several studies have been reported with the aim of promoting angiogenesis to provide a suitable environment for islet grafts. These do not attempt to immunoisolate islet grafts and are carried out with either syngeneic or immunodeficient animal models. Promotion of neovascularisation has been attempted using biomaterials alone such as steel mesh enclosures (Halberstadt et al., 2005, Pileggi et al., 2006) polymer sheets (Juang et al., 1996) or Thercyte devices (Tatarkiewicz et al., 1999). 3000 islets transplanted into a steel mesh enclosure pre-vascularised for 40 days resulted in maintenance of normoglycaemia for 160 days in diabetic

rat recipients (Pileggi et al., 2006). Inside the steel mesh was a removable stopper which was removed before implanting the islets. The authors suggested that using a device to pre-vascularise the subcutaneous site which is completely removed before implanting the islets may not be optimal. Removal of the whole device may result in damage to the integrity of the vascular structures and cause inflammation at the site. Using their device the vascularised steel mesh stays in place and only the stopper is removed, thereby creating a space for the islet tissue without damaging the newly formed vasculature.

1.3.5 Other Approaches for the Subcutaneous Site

Islets have been transplanted to the subcutaneous site in basement membrane extract (Bharat et al., 2005, Xu et al., 2011, Golocheikine et al., 2010). Subcutaneous transplantation of 250 mouse islets in matrigel basement membrane extract supplemented with vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) into mice resulted in maintenance of normoglycaemia up to the end of the experiment at 30 days. Equivalent transplantation without supplementation of VEGF and HGF resulted in graft failure. Fibrin glue has also been used as a carrier solution for islet transplantation to promote revascularisation and islet functionality (Andrades et al., 2007, Kim et al., 2012). Co-transplantation with cells at the subcutaneous site such as adipose cells (Fumimoto et al., 2009) and fibroblasts in blood plasma (Perez-Basterrechea et al., 2009) have also been reported to improve graft efficacy due to enhanced vascularisation. Dispersed islets grown into a "cell sheet" and transplanted subcutaneously have also been described (Saito et al., 2011). The authors suggest that diffusion of oxygen and nutrients to a sheet of cells only a few cells thick may be better than that of cells in an islet. However a control group with an equivalent number of islets was not included in this study.

1.3.6 Pre-Vascularisation of the Subcutaneous Site

Revascularisation of islets takes 7-14 days (Merchant et al., 1997, Mendola et al., 1994). During this period islets are dependent on diffusion of oxygen and nutrients from the surrounding tissue to maintain viability and function (Olsson and Carlsson, 2005). When islets are co-transplanted simultaneously with angiogenic devices, neovascularisation of the subcutaneous site is initiated upon transplantation and so there is a lag before a fully vascularised site is available to the islets. Alternatively when islets are transplanted into a pre-vascularised site the fully vascularised site is immediately available and therefore the graft is likely to maintain viability and become efficacious in a shorter time period. In a study using polyglycolide sheets it was found that pre-vascularised sheets cured a mouse at 3 weeks whereas islets transplanted simultaneously with the sheets cured two mice at weeks 4 and 5 (Juang et al.,

1996). This study suggests that simultaneous co-transplantation with angiogenic devices may be sufficient for graft success but pre-vascularisation may accelerate the rate of blood glucose normalisation. Conversely, in a study using the Theracyte device there was no difference in the time to normoglycaemia with islets transplanted into pre-vascularised devices or simultaneously co-transplanted in naïve devices (Tatarkiewicz et al., 1999). Several other studies using a single simultaneous co-transplantation report robust graft success without pre-vascularisation (Dufrane et al., 2010, Lacy et al., 1991, Golocheikine et al., 2010). Although many studies report the advantage of a pre-vascularised site compared to the unmodified subcutaneous site there is only anecdotal evidence that pre-vascularisation is more efficacious than simultaneous co-transplantation. This is of interest as pre-vascularisation followed by islet transplantation requires two separate surgeries where the second surgery is into a highly vascularised site prone to bleeding and inflammation. The pre-vascularisation approach may also impose timescale limits which may be problematic due to donor islet availability.

1.4 Helper Cells for Islet Transplantation

1.4.1 Islet Co-Transplantation

The use of helper cells to improve islet transplantation outcome has been explored with several cell types. The most commonly co-transplanted cell type with islets are sertoli cells (Ramji et al., 2011, Li et al., 2010c, Yin et al., 2009, Korbutt et al., 1997). Sertoli cells are immunoprivileged cells derived from the testes which produce immunomodulatory factors including Fas ligand and transforming growth factor beta (TGF-β). Co-transplantation of sertoli cells with islets has been demonstrated to provide some protection against the alloimmune response. Hepatic stellate cells have also been used for co-transplantation due to their impact on revascularisation (Yin et al., 2007) and immunomodulatory capability (Chou et al., 2011, Yang et al., 2009, Yang et al., 2010a, Jara-Albarran et al., 2001). Hepatic stellate cells are able to induce apoptosis in T cells and stimulate regulatory T cell production. Co-transplantation with dendritic cells also provides immunomodulatory capacity by altering antigen presentation and T cell priming (Li et al., 2010b, Yang et al., 2008, Ali et al., 2000). Endothelial cells have been co-transplanted to enhance revascularisation (Kang et al., 2012, Pan et al., 2011) and have also been used as a cell coating to protect islets from the IBMIR (Kim et al., 2011a, Kim et al., 2009a, Johansson et al., 2005b). As a group, stem cells are the most commonly used cell type for co-transplantation with islets. Neural stem cells (Melzi et al., 2010, Olerud et al., 2009), adipose tissue-derived stem cells (Ohmura et al., 2010, Cavallari et al., 2012), bone marrow cells (Sakata et al., 2010b, Sakata et al., 2010a, Figliuzzi et al., 2009, Lee et al., 2005), and mesenchymal stem cells (MSCs) (Rackham et al., 2011, Huang et al., 2010, Berman et al.,

2010, Jacobson et al., 2008) have all been used in co-transplantation studies. Stem cells have been shown to have several functions which are advantageous for islet transplantation which will be discussed later in this thesis (1.6).

1.4.2 Islet Co-Encapsulation

Encapsulation of islets in biomaterial or scaffolds provides the possibility of integrating molecules or cells localised to the immediate environment of the islet to provide support and function to the graft. In comparison to non-encapsulated co-transplantation, co-encapsulation has the advantage that molecules and cells can be held in close proximity and are not able to disperse away from the islet. Integrating molecules into islet encapsulation devices typically requires modification of the molecule or the use of sustained release devices such as microspheres so that the agent does not diffuse out in an instant. There have been several attempts to incorporate immunosuppressants into encapsulation devices (Song et al., 2012, Pinto et al., 2010, Buchwald et al., 2010). Alginate has been simply mixed with unmodified anti-inflammatory molecules and cross-liked to create functionalised microcapsules. However this simple approach results in rapid diffusion out of the capsules (Bunger et al., 2005). Co-encapsulation with degradable microspheres allows the release of molecules over a period of time and has been demonstrated with the antioxidant vitamin D3 (Luca et al., 2000) and the non-steroidal anti-inflammatory drug ketoprofen (Ricci et al., 2005). Bioactive devices have been used to enhance vascularisation by incorporation of VEGF (Vernon et al., 2012, Sigrist et al., 2003a, Sigrist et al., 2003b) and fibroblast growth factor (FGF) (McQuilling et al., 2011, Opara et al., 2010). Molecules have also been co-encapsulated with islets to protect against NO generated from pericapsular macrophages (Yang et al., 2005, Chae et al., 2004b, Wiegand et al., 1993). Islets have been co-encapsulated with immobilised haemoglobin to improve oxygen availability to the islets, resulting in improved graft outcome (Kim et al., 2005b, Chae et al., 2004a, Chae et al., 2002). Similarly, oxygen generating molecules (Pedraza et al., 2012, Wu et al., 1999) and even algal cells (Bloch et al., 2006a, Bloch et al., 2006b) have been co-encapsulated with islets. The most commonly co-encapsulated cell type are sertoli cell cells which confer localised immunoprivilege to attenuate the overgrowth reaction (Yang et al., 2002a, Calafiore et al., 2001, Yang and Wright, 1999, Korbutt et al., 1998, Luca et al., 2010). Co-encapsulating with cells may be more advantageous than co-encapsulation with molecules as a continuous, unlimited and even physiological augmentation to the graft may be possible with cells. The integration of insulinotropic peptide glucagon-like peptide-1 (GLP-1) into microcapsules with islet has also been explored (Kim and Bae, 2004, Kim et al., 2005a). Some *in vitro* efficacy was demonstrated with this strategy however the full potential of GLP-1 treatment for microencapsulated islet grafts is yet to be explored.

1.5 Glucagon-Like Peptide-1 and Islet Transplantation

1.5.1 Glucagon-Like Peptide-1 and Islets

Incretin hormones are rapidly released into the circulation within minutes of nutrient ingestion to potentiate the release of insulin. It is estimated that incretin release is responsible for 50-70% of the total insulin secreted after oral glucose administration (Elrick et al., 1964). The incretin hormones are gastric inhibitory polypeptide (GIP) secreted from K cells and GLP-1 secreted from L cells. The proglucagon gene is transcribed in L cells, α cells, and neurons in the caudal brainstem and hypothalamus (Mojsov et al., 1986, Drucker and Asa, 1988). Differential processing of proglucagon messenger ribonucleic acid (mRNA) by PC1/3 or PC2 results in the production of GLP-1 and glucagon respectively. In L cells PC1/3 is prevalent, leading to the production of GLP-1 and also other products of the proglucagon gene including glicentin, oxyntomodulin, intervening peptide-2, and GLP-2. Release of GLP-1 is stimulated by direct contact with nutrients in the intestine, neuronal stimulation, and endocrine factors. GLP-1 is inactivated by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DPP-IV) located on cell surfaces and in soluble form. It is estimated that half of the active GLP-1 is inactivated by DPP-IV before entry to the circulation (Hansen et al., 1999) and once in the circulation a further 50% is inactivated within 2 minutes. Fasting levels of circulatory GLP-1 are 5-10 pM and increase approximately 2-3 fold postprandially (Elliott et al., 1993).

The GLP-1 receptor (GLP-1R) is a transmembrane G-protein-coupled receptor (GPCR) which is expressed in a wide range of tissues including α, β, and δ cells of the islet. GLP-1R agonists stimulate glucose-dependent insulin secretion in islets (Mojsov et al., 1987). The primary effector of GLP-1 induced insulin secretion is cAMP but GLP-1 also has direct effects on ion channels, intracellular Ca^{2+}, ATP synthesis, and insulin granule exocytosis. Unlike sulphonylurea insulinotropic agents GLP-1 not only enhances insulin secretion but also mediates the replenishment of insulin stores. GLP-1 promotes insulin gene transcription, mRNA stability and synthesis resulting in replenishment of insulin stores (Alarcon et al., 2006). Other effects of GLP-1 signalling on islets include inducing increased pancreatic duodenal homeobox-1 (PDX-1) expression (Wang et al., 1999), glucose-dependent inhibition of glucagon (Nauck et al., 2002) and stimulation of somatostatin secretion (Fehmann and Habener, 1991). GLP-1R agonism stimulates β cell proliferation (Buteau et al., 2003) and neogenesis (Xu et al., 2006), and inhibits apoptosis (Li et al., 2003) resulting in increased β cell mass. PDX-1 has been suggested as a mediator for these processes as GLP-1R agonism in β-cell-specific PDX-$1^{-/-}$ mice is ineffective (Li et al., 2005). GLP-1 also has effects on non-islet cells including effects which impact blood glucose homeostasis. GLP-1 has been shown to reduce hepatic glucose

production (Hvidberg et al., 1994), increase insulin sensitivity (Sandhu et al., 1999), reduce appetite (Toft-Nielsen et al., 1999), and reduce gastric emptying (Imeryuz et al., 1997). GLP-1 also has cardioprotective (Read et al., 2012) and neuroprotective (During et al., 2003) effects. Similarly to GLP-1, GIP also stimulates glucose-dependent insulin secretion, insulin synthesis, β cell proliferation, and inhibits β cell apoptosis.

There is evidence that GLP-1 is central to β cell development as GLP-1R $^{-/-}$ mice exhibit abnormal islet architecture, glucose intolerance, and impaired recovery from β cell damage (Ling et al., 2001). α cells predominantly express PC2, however under certain conditions PC1/3 is expressed resulting in GLP-1 production α cells. The earliest pancreatic progenitor cells produce both PC2 and PC1/3 which suggests transient expression of GLP-1 is involved in islet development (Wilson et al., 2002). GLP-1 production in islets has been observed in response to partial pancreatectomy (De Leon et al., 2003), induction of streptozotocin (STZ) diabetes (Thyssen et al., 2006), islet isolation, and culture (Masur et al., 2005, Heller and Aponte, 1995). The production of GLP-1 in α cells is therefore a natural phenomenon which is observed in response to tissue damage. It is therefore likely that GLP-1 can be produced in the islet in order to promote regeneration and inhibit apoptosis. There is some evidence that GLP-1R agonists have anti-inflammatory properties. Islets cultured in GLP-1R agonist exendin-4 were reported to have reduced production of pro-inflammatory cytokines such as IFN-γ, IL-17, IL-1β, and IL-2 (Cechin et al., 2011). In addition it was found that serine protease inhibitor 9 (PI-9) expression was induced in islets by exposure to exendin-4. PI-9 is able to inhibit the activity of granzyme B which is involved in cytotoxic T cell destruction. The presence of PI-9 therefore may inhibit T cell mediated autoimmune and alloimmune islet destruction.

1.5.2 L Cells

Due to the rarity of L cells and the complex anatomy of the intestinal epithelium, isolation of unmodified, homogeneous, primary L cells has not been established. Primary preparations containing L cells are heterogeneous and typically unsustainable. The foetal rat intestinal cell (FRIC) protocol isolates primary epithelial intestinal cells although it produces a heterogeneous and largely uncharacterised cell population (Brubaker and Vranic, 1987). Isolation of homogenous L cell populations is possible, however it requires using genetically modified mice. Transgenic mice with L cell specific expression of a fluorescent protein allow isolation of L cells from intestinal epithelium isolates by fluorescence-activated cell sorting (FACS) (Reimann et al., 2008). Immortalized cell lines have been developed for studying L cells. The human cecal carcinoma cell line NCI-H716 has been used to study L cells, however it has been suggested it is not an optimal model due aberrant regulation of the proglucagon promoter.

The STC-1 line was derived from intestinal tumours of transgenic mice expressing the rat insulin II promoter linked to both the simian vacuolating virus 40 large T antigen (SV40Tag) oncogene and the polyoma small T antigen oncogene (Rindi et al., 1990). Secretion of GLP-1 (Abello et al., 1994) and GIP (Kieffer et al., 1995) from STC-1 cells has been confirmed suggesting the STC-1 line is heterogeneous, consisting of both L cells and K cells. STC-1 cells modified to express insulin under the GIP promoter have been shown to correct glycaemia in diabetic rodents (Han et al., 2007, Zhang et al., 2008). The GLUTag cell line was derived from intestinal tumours of transgenic mice expressing the SV40Tag oncogene under the rat glucagon promoter (Drucker et al., 1992). It has been suggested that the GLUTag cell line provides a good L cell model as GLUTag cells respond to regulatory mechanisms controlling GLP-1 secretion in accordance with primary L cells and *in vivo* models (Brubaker et al., 1998). GLUTag cells are considered to be homogenous L cells and have been demonstrated to secrete GLP-1 in response to several stimuli including glucose (Gribble et al., 2003) and insulin (Lim et al., 2009). GLUTag cells modified to express insulin under the GLP-1 promoter have been shown to express insulin but could not correct glycaemia in diabetic rodents (Bara et al., 2009).

1.5.3 Glucagon-Like Peptide-1 for Diabetes Therapy

In animal models of T2DM, GLP-1 therapy has been shown to promote β cell neogenesis (Xu et al., 1999), proliferation, and differentiation (Perfetti et al., 2000), inhibit apoptosis (Farilla et al., 2002), and delay diabetes onset (Wang and Brubaker, 2002). A β cell is said to be glucose competent if the insulin secretory response is responsive and proportional to dynamic glucose levels (Kulkarni et al., 2002). GLP-1 has been shown to restore glucose competence to glucose resistant β cells (Holz et al., 1993). Several GLP-1 receptor agonists are in clinical development and exendin-4 (Byetta®) has been approved for treatment in T2DM patients (Furman, 2012). Exendin-4 is a GLP-1R agonist with a 50% amino acid homology to GLP-1 and has a longer half-life of 6 h compared to the 1-2 minute half-life of GLP-1. Exendin-4 is recommended as an adjunctive therapy for patients unable to achieve adequate blood glucose control with metformin, sulfonylurea, thiazolidinediones or a combination of these. Therapy with such agents has been demonstrated to improve glycaemic control and also to induce weight loss due to GLP-1R agonist-mediated suppression of appetite (Zander et al., 2002). The use of GLP-1R agonists could be relevant to specific strategies for treating T1DM. If β cell destruction could be halted before completion GLP-1R agonists may prove useful to exploit the full insulin secretory capacity of β cells, encourage β cell regeneration, and prevent β cell apoptosis. GLP-1 has been shown to induce expression of anti-apoptotic proteins in β cells resulting in inhibition of apoptosis. Stimulation of the GLP-1 receptor has been shown to attenuate β cell apoptosis in STZ treated rodents (Tourrel et al., 2001, Li et al., 2003). At diagnosis T1DM patients typically

have 20-30% of normal insulin secretory capacity (Madsbad et al., 1980) which suggests intervention could be beneficial at this stage. GLP-1R agonism has been shown to delay diabetes onset in NOD mice (Zhang et al., 2007, Hadjiyanni et al., 2008). A study using combination therapy of GLP-1 and β cell neogenesis factor gastrin demonstrated that restoration of normoglycaemia and increase in β cell mass could be achieved in NOD mice with combination therapy but not with either GLP-1 or gastrin alone (Suarez-Pinzon et al., 2008). The authors also suggest that GLP-1 contributes to regulating the autoimmune response against β cells as autoantibodies were undetectable in some mice treated with GLP-1.

1.5.4 Glucagon-Like Peptide-1 and Islet Transplantation

The cohort of beneficial effects GLP-1 induces in β cells can be harnessed to improve islet transplantation. Pre-transplantation culture of islets in exendin-4 supplemented media can improve the rate of reversal of hyperglycaemia in mice (King et al., 2005). This suggests GLP-1 may offer a protective advantage to islets in short term culture. It has been shown that transplanted foetal islet-like cell clusters have greater revascularisation, insulin producing cells (IPCs), and curative potential when recipients are treated with exendin-4 (Suen et al., 2006). This suggests GLP-1R agonism permits the growth, differentiation, and maturation of transplanted islet precursor cells. There are mixed reports concerning the capacity of GLP-1R agonists to induce β cell proliferation in transplanted islets. Induction of β cell proliferation has been demonstrated in islet grafts using a high dose of 100 $mg.kg^{-1}.day^{-1}$ (Tian et al., 2011). Another report has suggested administration of approximately 85 $mg.kg^{-1}.day^{-1}$ of GLP-1R agonist does not induce β cell proliferation (Crutchlow et al., 2008). Exendin-4 treatment in mouse islet graft recipients has also been reported to reduce the number of apoptotic β cells in the early post transplantation period (Sharma et al., 2006, Toyoda et al., 2008).

Marginal mass islet transplantation in conjunction with long acting GLP-1R agonist liraglutide was reported to improve time to normoglycaemia, glucose tolerance and graft outcome in mice (Merani et al., 2008). Interestingly discontinuation of liraglutide resulted in reversal of the positive effect on glucose tolerance and delayed initiation of liraglutide treatment was ineffective. Reduced β cell apoptosis was observed 48 h post-transplantation and liraglutide attenuated the detrimental effect on graft outcome mediated by an immunosuppressive agent. Liraglutide has been suggested to have anti-apoptotic effects in human islets in culture (Toso et al., 2010). Islets pre-cultured with liraglutide transplanted into immunodeficient mice had no impact on graft outcome whereas systemic administration initiated upon transplantation resulted in improved graft outcome. The authors suggest that pre-treatment alone is insufficient to confer an advantage post-transplantation and continuation of

treatment *in vivo* offers better utility of liraglutide treatment. It has also been shown that administering DPP-IV inhibitor in the diet of islet graft recipients can improve graft outcome (Kim et al., 2008). In a study with NOD mice transplanted with islets and treated with DPP-IV inhibitor it was found that continuous treatment initiated before transplantation prolonged graft survival whereas initiation of treatment after transplantation did not (Kim et al., 2009b). The authors demonstrate that the DPP-VI inhibitor used also decreases the homing of T cells which is likely to contribute to the findings. Some studies have reported no impact on long term blood glucose with exendin-4 treatment for islet graft recipients (Crutchlow et al., 2008, King et al., 2005). However in these studies the islet number in the grafts were not stringent enough to detect an effect as the control grafts also maintained normoglycaemia.

1.5.5 Glucagon-Like Peptide-1 Therapy for Clinical Islet Transplantation

GLP-1R agonist treatment may be an alternative to exogenous insulin for clinical islet transplantation recipients with insulin requirements. Therapy with GLP-1R agonists have the advantage that the insulin response is potentiated but is still physiologically responsive resulting in more accurate glycaemic control than exogenous insulin therapy can achieve. As GLP-1 induces glucose-dependent stimulation of insulin secretion the risk of hypoglycaemia is reduced in comparison to drugs such as sulfonylureas. It has been established that transplanted islets are able to respond to GLP-1R agonism in humans although the response may be less than that of native islets in non-diabetic patients (Fung et al., 2006). GLP-1 has been used in clinical islet transplantation patients with allograft dysfunction and exogenous insulin requirements (Froud et al., 2008). It was found that insulin requirements could be reduced by a third and 3/16 patients became insulin independent. However 4 patients discontinued treatment due to side effects. Systemic administration of GLP-1 is associated with side effects such as cramping and nausea (Elbrond et al., 2002, Kolterman et al., 2003). Another clinical study reported 39% reduction in insulin requirements over the treatment period (Ghofaili et al., 2007). However the effect was reversed once treatment ceased. This suggests GLP-1R agonism may have conferred a transient enhancement of GSIS but did not increase β cell mass which would have a lasting impact on glycaemic control. It has been reported that GLP-1R agonist treatment following clinical islet transplantation resulted in 5/5 insulin independent patients compared to 1/5 without treatment at 18 months (Faradji et al., 2008). This suggests that GLP-1R agonist treatment immediately post-transplantation may have a lasting effect as islets are most vulnerable in the early post-transplantation period.

Due to the abundance of DPP-IV in the circulation which mediates the breakdown and clearance of GLP-1, systemic administration of GLP-1 is inefficient. The use of GLP-1 to

promote β cell proliferation in rodents uses doses in the range of 50-100 $\mu g.Kg^{-1}$.day (Li et al., 2003, Ogawa et al., 2004). This dose range far exceeds the dose GLP-1R agonist dose used clinically for T2DM patients of 10 $\mu g.day^{-1}$ (Calara et al., 2005). Additionally it has been reported that clinical islet transplantation recipients are more susceptible to the side effects of systemic GLP-1R agonist therapy (Froud et al., 2008). As a result it is unlikely that systemic administration of GLP-1R agonists could be used safely at doses high enough to stimulate β cell proliferation.

1.5.6 Ectopic Expression of Glucagon-Like Peptide-1

Genetically engineering islet cells to produce GLP-1 would provide the islets with a direct source of GLP-1. It may be possible to achieve highly stimulatory concentrations of GLP-1 by production of GLP-1 localised to the islets. The degradation of endogenous GLP-1 caused by the exposure to DPP-IV in the circulation would be greatly reduced or avoided completely. Ectopic expression of GLP-1 in β cells would result in production of the protein at the site of action requiring only intercellular transport to the site of action. It has been shown that mouse insulinoma (MIN6) cell line β cells ectopically expressing GLP-1 are resistant to the toxic effects of immunosuppressive drugs (D'Amico et al., 2005). GLP-1 expressing β cells had improved viability and functionality, and reduced apoptosis compared to controls when exposed to a toxic immunosuppressive agent. GLP-1 expression resulted in constitutive expression of anti-apoptotic protein B-cell lymphoma 2 (Bcl-2). In accordance ectopic expression anti-apoptotic proteins X-linked inhibitor of apoptosis protein (XIAP) (Emamaullee et al., 2005, Plesner et al., 2005) and Bcl-2 (Sutherland et al., 2004, Contreras et al., 2001) in islets has been shown to improve islet graft outcome. Systemic delivery of viral vectors has been used to deliver the GLP-1 gene into T1DM animal models. An adenoviral vector administered into the tail vein reversed the diabetic state of STZ diabetic mice and temporarily reversed the diabetic state of NOD mice (Liu et al., 2007). The vector was non-specific and probably expressed GLP-1 in indiscriminately infected cells. Another study used a specific adeno-associated vector which efficiently infects β cells and expressed GLP-1 under a β cell specific promoter (Riedel et al., 2010). Intraperitoneal administration of the vector followed by STZ treatment resulted in protection against the development of diabetes. β cell specific expression of GLP-1 did not have an impact on circulating GLP-1 levels (Riedel et al., 2010). This suggests localised production of GLP-1 in islets results in higher enough concentrations of GLP-1 locally to impact β cell survival but without risk of increasing systemic GLP-1 to levels which may induce side effects.

Transduction of genes into isolated islets to confer expression of GLP-1 for islet transplantation has been reported. GLP-1 has also been expressed specifically in α cells in whole islets for transplantation studies (Wideman et al., 2006). α cells produce proglucagon which is converted into glucagon by PC2. Ectopic expression of PC1/3 in α cells therefore resulted in conversion of proglucagon into GLP-1. It was found that α cell production of GLP-1 resulted in improved GSIS, reduced apoptosis, and enhanced graft performance over 20 days. The authors suggest that GLP-1 secreted from α cells was able to diffuse across the interstitial space to stimulate GLP-1R on β cells. PDX-1 staining was reported to be increased in transduced grafts. Islets transduced to ectopically express GLP-1 have also demonstrated increased insulin secretion, protection from hydrogen peroxide induced cell damage, increased β cell proliferation in transplanted islets, and improved graft efficacy for at least 35 days (Chae et al., 2012). The study used adenovirus vectors which typically have a short duration of gene expression of less than 14 days (Michou et al., 1997). The authors suggest that the short term GLP-1 expression is likely to confer an advantage in the early post-transplantation period when the islets are vulnerable. Gene transduction to confer the production of GLP-1 in diabetic animal models has shown some efficacy. However vectors can only express GLP-1 for a short period and there are several concerns about the safety of gene therapy. Isolation of islets allows the specific infection of only the graft tissue therefore limiting any off target expression in the host which could have unforeseen side effects. However safety problems associated with gene therapy such as insertional mutagenesis (Hacein-Bey-Abina et al., 2003) and immune reactivity (Raper et al., 2003) are still a concern for grafted islets. These issues are likely to hinder the translation of gene therapy for islet transplantation into the clinic.

1.5.7 Co-Encapsulation with Glucagon-Like Peptide-1

The incorporation of insulinotropic agents into polymers could be used to improve the insulin productivity of islets in encapsulation strategies for transplantation. The incorporation of sulphonylurea into polymers to enhance islet or β cell insulin secretion has been investigated (Park and Akaike, 2004, Kim et al., 2003, Park et al., 2002, Na et al., 2001, Park et al., 2001, Hwang et al., 1998). Improved insulin secretion has been reported by interacting sulphonylurea co-polymers with islets. One study described the co-encapsulation of a sulphonylurea co-polymer with islets in alginate microcapsules (Kim et al., 2003). A carboxylated moiety of the sulphonylurea glibenclamide was conjugated to a polysaccharide to confer water solubility to increase the bioavailability of agent. The co-polymer was shown to increase insulin secretion although not as much as would be expected from glibenclamide, suggesting the conjugation to the polymer inhibited the action of the glibenclamide moiety. It was also shown that the improvement in insulin secretion was sustained for at least 4 weeks.

The incorporation of GLP-1 into islet encapsulation devices has been investigated. Unmodified GLP-1 encapsulated in commonly used biomaterials for islet encapsulation would readily diffuse out due to its low molecular weight. Consequently GLP-1 in zinc crystallized form was developed so that it could be encapsulated and provide slow release of GLP-1 by monomers shedding from the crystals. Zinc-crystallized GLP-1 was incorporated with islets in a poly(N-isopropylacrylamide-co-acrylic acid) matrix in a hollow fiber macrocapsule. Insulin secretion was found to slowly increase over at least 7 days demonstrating the insulinotropic action of GLP-1 and the slow release of GLP-1 from the crystals. In a follow up study the crystallised form of GLP-1 was compared with a GLP-1-polymer conjugate in alginate microcapsules (Kim and Bae, 2004). Incorporation of free GLP-1 into alginate microcapsules resulted in poor capsule formation which the authors suggest may be due to the positive charges on the GLP-1 molecules increasing the viscosity of the alginate. It was found that the stimulation observed with crystallised GLP-1 was further enhanced with GLP-1-polymer conjugate and could be sustained for up to 8 weeks. The authors suggest that the greater solubility of the GLP-1 polymer conjugate enhanced the availability of GLP-1 for action on β cells. It was found however that after 5 weeks the increased insulin secretion dropped rapidly. It was suggested that exhaustion due to overstimulation of the islets may have caused the sudden drop in insulin secretion. Another experiment showed that the effect of the GLP-1-polymer conjugate gradually declined in response to glucose over an 8 week period. This suggests that the GLP-1 was either being degraded or metabolised and therefore had limited longevity (Kim et al., 2005a).

GLP-1 has been immobilised in PEG hydrogel for co-encapsulation with islets (Lin and Anseth, 2009). A photopolymerised cross-linked PEG hydrogel network provided a platform for incorporation of cysteine modified GLP-1. Islets or β cells encapsulated in GLP-1 functionalised PEG had higher viability, insulin secretion, and reduced apoptosis. However the production of free radicals during the photopolymerisation process can cause damage to cells, and the immunoprotective capability of photopolymerised PEG has not been fully elucidated (Cruise et al., 1999). Another conformal coating approach has been reported using a layer-by-layer approach with layers of PEG and GLP-1 conjugated to biotin, and held together with streptavidin (Kizilel et al., 2010). The coating was found to completely cover islets and also increase GSIS. Again the immunoprotective capability of this approach has not been fully validated *in vivo*. Other reports conceptualise the same approach as a transiently functional coating to limit islet damage in culture and from the IBMIR (Cabric et al., 2010, Wilson et al., 2010, Teramura and Iwata, 2008). GLP-1 mimetic peptide amphiphiles which self-assemble

into nanofibers and can be cross-linked to encapsulate cells have been developed (Khan et al., 2012). Encapsulated rat insulinoma cells were found to have improved GSIS and proliferation, and were resistant to cytokine mediated cell death. The encapsulation is readily degradable and therefore is intended to act as a scaffold as opposed to an immunoprotective device.

1.6 Mesenchymal Stem Cells and Islet Transplantation

1.6.1 Mesenchymal Stem Cells

MSCs are adult pluripotent stromal cells which are characterised by their ability to differentiate into mesodermal lineages including osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006). The capacity for self-renewal, ability to adhere to plastic, and the expression or absence of specific surface antigens also defines MSC characterisation. When adhered to plastic, MSCs have a spindle-shaped, fibroblast-like morphology. MSCs are most commonly derived from bone marrow, however they can be found in the stroma of almost all vascularised adult tissues and organs (da Silva Meirelles et al., 2006). Stromal cells are the connective tissue cells of tissues and organs which are commonly lymphoid in origin. MSCs however are non-lymphoid stromal cells which have the capacity to secrete stromal components in order to support adhesion, proliferation, and survival of distinct cell subsets (Uccelli et al., 2008). As a result MSCs play an important role in tissue repair and regeneration.

1.6.2 Mesenchymal Stem Cell Therapy for Type 1 Diabetes

MSCs are amenable to therapeutic utility as they are easily accessible, expandable *in vitro*, and can be preserved without significant loss of function (Parekkadan and Milwid, 2010). MSCs have been considered safe to transplant for some therapeutic applications and have been used in clinical trials for cardiovascular (Ripa et al., 2007), neurological (Lee et al., 2008), and immunological disease (Lazarus et al., 2005). There are safety concerns about the use of MSCs for therapeutic use due to the potential of self-renewing cell types to form cancer cells (Reya et al., 2001). However there have been no reports of tumour formation using human MSCs in clinical trials (Abdi et al., 2008). In the context of T1DM, MSCs have been experimentally applied therapeutically in a few different ways. The potential of MSCs to differentiate into other cell types makes them attractive for cell replacement therapy. *In vitro* differentiation of MSCs into IPCs has been reported by using specific culture medium enriched with insulin-promoting factors. MSC derived IPCs have been transplanted to control blood glucose in diabetic animal models (Gabr et al., 2012). Several problems prevent the translation of MSC derived IPCs to clinical use, including the low proportion of differentiation to IPCs, restricted proliferation of IPCs, and lack of glucose responsively.

MSCs are known to home to damaged tissue and have been shown to migrate to the islets in diabetic animal models (Sordi et al., 2005, Lee et al., 2006d). Chemokine receptors on MSCs enable then to migrate towards chemokines secreted from the islets (Sordi et al., 2005). The ability of MSCs to migrate is advantageous as they can be delivered into the circulation with minimal surgery. Systemic administration of MSCs has been reported to delay onset of diabetes, improve glycaemic levels, reduce pancreatic insulitis, stimulate β cell regeneration and inhibit autoimmune destruction of β cells (Bassi et al., 2012, Ezquer et al., 2008, Fiorina et al., 2009). Experiments transplanting MSCs into NOD mice resulted in delay of onset in pre-diabetic animals (Fiorina et al., 2009), temporary reversal of hyperglycaemia (Fiorina et al., 2009), induction of T regulatory cells (Madec et al., 2009), and suppression of diabetogenic T cells and dendritic cell proliferation (Jurewicz et al., 2010). These studies suggest that due to the immunomodulatory function of MSCs, they are able to attenuate the autoimmune response in a T1DM model and therefore permit the survival and regeneration of β cells. MSCs have also been reported to induce β cell regeneration in STZ treated animals (Dong et al., 2008, Lee et al., 2006d, Xu et al., 2012). This demonstrates that MSCs are not only able to prevent autoimmune β cell destruction but also actively promote regeneration of β cells. There is some evidence that surviving β cells in STZ treated rats can spontaneously regenerate (Jorns et al., 2001). It has also been suggested that α cells may be able to transdifferentiate into β cells after β cell ablation (Thorel et al., 2010). MSCs may facilitate these β cell regeneration processes. One study has reported MSC homing to the islets and differentiating into IPCs, although this remains unconfirmed (Ianus et al., 2003). However other studies have reported no impact on blood glucose by transplanting MSCs alone into diabetic animals (Longoni et al., 2010, Urban et al., 2008, Rackham et al., 2011).

1.6.3 Immunomodulation

The immunomodulatory properties of MSCs make them an attractive candidate for therapeutic use in autoimmune diseases such as rheumatoid arthritis, lupus erythematosus, multiple sclerosis, and T1DM. The use of MSCs to inhibit rejection of allogeneic tissue such as skin grafts, graft-versus-host disease (GVHD), and islet transplantation is also being explored. In addition to suppression of the alloimmune response, suppression of the autoimmune response is also beneficial for islet transplantation as the autoimmune response can also target transplanted islet tissue (Prowse et al., 1986). The mechanism of immunomodulation by MSCs is thought to involve an initial cell contact phase and also the paracrine secretion of mediators which inhibit immune cell proliferation and responses. MSCs have been shown to induce cell cycle arrest in activated T cells (Glennie et al., 2005), stimulate the production of regulatory T cells (Djouad et al., 2003), inhibit dendritic cell differentiation (Jiang et al., 2005),

and inhibit B cell proliferation, activation, differentiation, and antibody production (Corcione et al., 2006).

Rejection of islets is a predominantly T cell mediated process (Gill et al., 1989) and pro-inflammatory cytokines such as of IFN-γ, TNF-α, and IL-1β contribute to loss of islets (Satoh et al., 2007). Several studies have demonstrated that co-transplantation of MSCs with islets can improve graft outcome by suppressing immune rejection in allogeneic models (Xu et al., 2012, Kim et al., 2011b, Huang et al., 2010, Berman et al., 2010, Longoni et al., 2010, Li et al., 2010a, Ding et al., 2009, Solari et al., 2009, Jacobson et al., 2008). MSC mediated effects on T cells in islet and MSC co-transplantation experiments have been reported. MSCs have been shown to suppress the secretion of pro-inflammatory cytokines IFN-γ (Longoni et al., 2010; Solari et al., 2009) and TNF-α (Solari et al., 2009) from T cells in islet co-transplantation studies. It has been reported that matrix metalloproteases (MMPs) secreted by MSCs reduce surface expression of CD25 on responding T cells leading to inhibition of T cell proliferation and responsivity to activating factors such as IL-2 (Ding et al., 2009). MSCs have also been reported to increase regulatory T cell numbers (Li et al., 2010a, Xu et al., 2012, Solari et al., 2009, Berman et al., 2010) and alter the composition of T cell subsets. MSCs specifically reduce CD25 expression on T effector cells resulting in apoptosis and impaired proliferative response. However MSCs do not reduce expression of CD25 on regulatory T cells but foster their survival and expansion (Xu et al., 2012). The maturation and antigen presenting capability of dendritic cells have been shown to be inhibited by MSCs (Li et al., 2010a). MSCs have been co-cultured with allogeneic dendritic cells to alter the dendritic cell phenotype (Huang et al., 2010). When the MSC-conditioned dendritic cells alone were co-transplanted with islets a delay in graft rejection was observed compared to islets transplanted alone or with non-conditioned dendritic cells. Soluble factors released from MSCs can reduce MHC class II and increase IL-10 expression in dendritic cells and enhance the ability of dendritic cells to suppress T cell responses.

The immunomodulatory capabilities of MSCs in islet co-transplantation are multifaceted and provide some protection from the inflammatory and immune response. However some reports suggest MSCs are unlikely to be used as a standalone therapy and are more likely to be applied with the goal of reducing immunosuppressant doses (Xu et al., 2012). The synergistic effect of sub-therapeutic levels of immunosuppressants and MSCs has been demonstrated (Kim et al., 2011b).

1.6.4 Revascularisation

Currently 2-4 pancreata are often needed to isolate enough islets for a single recipient for clinical islet transplantation. Therefore there is a need to improve the efficiency of islet

transplantation. Syngeneic transplantations in mice have been used to demonstrate the impact of co-transplanting MSCs in the absence of immune rejection. Minimal mass transplantations with as low as half the number of islets required for graft success in control grafts have been used to demonstrate improved graft efficacy by co-transplantation with MSCs (Rackham et al., 2011, Figliuzzi et al., 2009). The reduction of up to half of the islets equates to a large improvement in efficiency which could help improve the donor shortage in the clinic. Several studies have implicated MSC-mediated enhancement of revascularisation to the graft success of islet and MSC co-transplantations (Figliuzzi et al., 2009, Park et al., 2010a, Davis et al., 2012, Cavallari et al., 2012, Ohmura et al., 2010, Ito et al., 2010, Rackham et al., 2011). These studies have all reported increased vascular density in the islet graft when MSCs are co-cultured or co-transplanted with islets. Upon transplantation islets are completely free from any vascular connection to the circulation and are therefore reliant on diffusion for oxygen and nutrients to maintain viability. In order to gain a sufficient supply of oxygen throughout the islet it is necessary for the islet to revascularise. Evidence of revascularisation has been observed as early as 2 days post transplantation, although it takes 10-14 days to complete the process (Menger et al., 2001). Delayed and insufficient vascularisation results in deprivation of oxygen and nutrients resulting in cell death which can lead to graft failure (Linn et al., 2006). MSCs have been shown to express angiogenic factors including VEGF, HGF, IL6, IL-8, and platelet-derived growth factor (PDGF) (Figliuzzi et al., 2009, Ito et al., 2010, Park et al., 2010a, Sordi et al., 2010a, Park et al., 2009, Golocheikine et al., 2010). Co-culture also been shown to increase angiogenic VEGF and angiopoietin receptor expression in islets (Park et al., 2010a). It has been demonstrated that ectopic expression of VEGF from transplanted islets can improve graft outcome by enhancing vascularisation (Cheng et al., 2007). MMP secreted from MSCs can facilitate the migration of endothelial cells, which may also enhance islet revascularisation (Ghajar et al., 2006).

1.6.5 Immunological Discordance

By the nature of MSC characterisation MSCs derived from different tissues share similar morphology and immunophenotype, however there is some evidence that MSCs derived from different tissues have different differentiation capacities (da Silva Meirelles et al., 2006) and secrete differing levels of trophic factors (Veriter et al., 2011). Although, it has been demonstrated that MSCs from various sources including kidney (Rackham et al., 2011), pancreas (Sordi et al., 2010a) bone marrow (Figliuzzi et al., 2009), and adipose tissue (Ohmura et al., 2010, Cavallari et al., 2012) improve graft outcome when co-transplanted with islets. This suggests that the beneficial properties of MSCs for islet co-transplantation may be shared among a variety of MSC sources. For islet transplantation the individual from whom the MSCs

are derived could be the recipient, donor, or a third party. Acquisition of allogeneic MSCs could be relatively easy compared with islets as cells are readily available from living donors in the case of bone marrow or adipose tissue-derived cells. However recipient tissue is typically preferable for transplantation as immunosuppression or immunoprotection are not necessary. MSCs derived from diabetic patients have been reported to have similar morphology, phenotype, and differentiation potential as those from non-diabetic controls (Phadnis et al., 2009). This suggests that deriving MSCs from diabetic patients for co-transplantation therapy may be a viable option. There is some evidence that allogeneic MSCs are immunogenic (Nauta et al., 2006). If this is the case then rejection of the MSCs would probably inhibit any MSC-mediated enhancement of islet graft survival. If MSCs are immunogenic autologous MSCs may be the preferred source. However mice infused with autologous MSCs from mice harbouring the NOD autoimmune condition caused visceral tumours in mice. This was not observed in mice infused with allogeneic MSCs from healthy donors. This suggests that MSCs from individuals with autoimmune conditions may not be appropriate for autografts (Fiorina et al., 2009).

There are mixed reports concerning the respective ability of allogeneic or syngeneic MSCs to modulate the immune response. Allogeneic but not autologous MSCs were able to delay the onset of diabetes in NOD mice. Similarly allogeneic but not autologous MSCs used to pre-treat dendritic cells were able to attenuate graft rejection (Huang et al., 2010). MSCs have been described as hypo-immunogenic as they lack MHC class II molecules and other classical co-stimulatory molecules (Liechty et al., 2000, Devine et al., 2001, Pochampally et al., 2004, Tse et al., 2003). This may explain why allogeneic transplantation of MSCs is tolerated and maintains efficacy in some studies. Clinical studies of hematopoietic stem cells co-transplanted with MSCs to attenuate graft-versus-host disease (GVHD) have typically come from third party HLA mismatched donors (Le Blanc et al., 2008). One study found that syngeneic and allogeneic MSCs were equally efficacious for immunomodulation in islet co-transplantation (Longoni et al., 2010). Conversely, other studies have shown that syngeneic but not allogeneic MSCs co-transplanted with allogeneic islets were able to improve graft outcome (Solari et al., 2009, Kim et al., 2011b).

It has been demonstrated that co-transplantation with MSCs improves both syngeneic and allogeneic islet grafts (Longoni et al., 2010). The improvement in syngeneic islet grafts is likely to be related to the enhanced viability, vascularisation, and graft organisation. Whereas in allogeneic islet grafts MSCs are likely to provide the same advantages but also modulate the immune response. In grafts of syngenic islets which do not require the immunomodulatory

capabilities of MSCs, syngeneic rodent MSCs (Rackham et al., 2011, Ito et al., 2010, Solari et al., 2009) and xenogeneic human MSCs (Cavallari et al., 2012, Park et al., 2010a) have been used to improve islet graft outcome. This suggests the immunological discordance of the MSC source may not be critical to MSC mediated enhancement of islet graft outcome in the absence of an alloimmune response.

1.6.6 Co-Culture of Islets with Mesenchymal Stem Cells

Isolation of islets removes the islets from an environment with a unique and responsive supply of factors to support islet viability and function. Islet vasculature and neuronal connections are severed removing the blood supply and neuronal innervations. Additionally damage can occur during isolation, including the effects of collagenase, mechanical force, and deprivation of oxygen, nutrients and survival factors. It has been proposed that a lack of trophic agents account for poor islet survival in culture conditions (Ilieva et al., 1999). Ideally islet culture should contain factors which sustain islet viability and function and also restrain further tissue damage and promote tissue regeneration. Studies have reported increased islet viability and function by co-culturing with factors (Ling et al., 1994) or other cell types (Miki et al., 2006). Co-culture with MSCs has also been shown to improve islet viability and function. Co-culture of islets with MSCs or with cell free MSC conditioned medium has been shown to lower ADP/ATP ratios and increase GSIS and viability (Park et al., 2010a). This suggests MSCs are able to act through paracrine mechanisms to enhance islet functionality.

MSC-conditioned medium has been found to contain the growth factors HGF and TGF-β. Islets transduced with the HGF gene have improved preservation of β cell mass and graft efficacy when transplanted into diabetic animals (Dai et al., 2003, Garcia-Ocana et al., 2003, Lopez-Talavera et al., 2004). TGF-β1 plays a regulatory role in β cell development (Sanvito et al., 1994) and has been shown to enhance β cell function when administered systemically in NOD mice (Luo et al., 2005). PDX-1 has been shown to be upregulated in islets by co-culture with MSCs (Park et al., 2010a). PDX-1 is known to increase β cell regeneration and proliferation (McKinnon and Docherty, 2001, Taniguchi et al., 2003). Co-culture with MSCs also increases expression of heme oxygenase-1 (HO) which is known to have cytoprotective effect on islets by suppressing oxidative stress (Lee et al., 2006a, Pileggi et al., 2001). Co-culture also been shown to increase anti-apoptotic signalling molecules XIAP, Bcl-2, and B-cell lymphoma-extra large (Bcl-xL), and also VEGF receptor expression (Park et al., 2010a). Besides its angiogenic properties, VEGF has also been implicated as a survival factor for islets (Cross et al., 2007).

Another aspect of the application of MSCs for improving islet transplantation is the influence of the microenvironment on MSC function. The differentiation potential of stem cells has been shown to be influenced by the microenvironment (Li and Xie, 2005). It has also been shown that pro-inflammatory cytokines stimulate the immunosuppressive capacity of MSCs (Krampera et al., 2006, Polchert et al., 2008), and hypoxic conditions stimulate the angiogenic capacity of MSCs (Efimenko et al., 2011, Hung et al., 2007). In a study with non-human primates failing grafts could be rescued by infusion of MSCs. Interestingly the authors claim MSCs infused in stabilised grafts were ineffective and suggest the inflammatory environment of grafts undergoing rejection may be necessary to activate the immunomodulatory functions of MSCs. Treatment of MSCs with hyaluronic, butyric, and retinoic acid has been shown to enhance MSC secretion conferring improved islet co-transplantation outcome compared to co-transplantation with non-treated MSCs (Cavallari et al., 2012). The secretion profile of MSCs is likely to be dependent on the stimuli in the microenvironment of transplanted MSCs or in the culture conditions of cultured MSCs. This is also likely to affect the impact MSCs have on islets in co-transplantation or co-culture.

1.6.7 Mesenchymal Stem Cell Location and Differentiation

There are conflicting reports about the location and phenotype of MSCs as the post-transplantation period progresses. MSCs tagged with nanocrystal markers could be identified up to 3 weeks after transplantation with islets under the kidney capsule (Ito et al., 2010). It was found that some tagged MSCs also stained for an endothelial cell marker suggesting differentiation of MSCs into endothelial cells. There are other reports of the capacity for MSCs to differentiate into endothelial cells *in vitro* (Oswald et al., 2004) and *in vivo* (Silva et al., 2005). Homing of green fluorescent protein (GFP)-tagged MSCs to the islets and differentiation into IPCs has been reported (Ianus et al., 2003). Conversely it has been reported that 1 month after infusion of GFP-expressing MSCs into cynomolgus monkeys, MSCs could not be detected in the islets but some signal could be found in the liver and lungs (Berman et al., 2010). Indeed in another study MSCs could not be detected under the kidney capsule of mice at 1 month but were detectable at 3 days (Rackham et al., 2011). Collagen fibres were identified at the site at 1 month which may be beneficial to graft function (Jalili et al., 2011).There is some evidence that MSCs do not need to be administered locally with the islets. An islet co-transplantation study found MSCs could be administered either locally with the islets in the portal vein or systemically in the tail vein with equal efficacy (Longoni et al., 2010). It has also been reported that infusions of MSCs several days post-transplantation into the circulation can prolong graft survival (Berman et al., 2010). However these studies were with allogeneic islets, therefore the

main advantage of MSCs in this case were related to their immunomodulatory properties which can be applied systemically.

Direct contact between islets and MSCs is not necessary to utilise some beneficial effect from MSCs. Indirect co-culture such as transwell systems prevent direct contact between the islets and MSCs by separation with a permeable membrane. MSC-induced improvement in islet viability and function by indirect co-culture (Jung et al., 2011, Karaoz et al., 2010) and with MSC conditioned medium (Park et al., 2010a) have been reported. One report suggests that co-transplantation with MSCs is not necessary as pre-transplantation treatment alone can improve graft outcome. Islets exposed to MSC-conditioned medium for 48 h enhanced graft outcome when transplanted in diabetic animals (Park et al., 2010a). In agreement with other reports (Robertson, 2004, Boker et al., 2001) the authors suggest that the preservation of islet viability in culture results in better quality islets and therefore improved graft outcome. It has also been shown that MSC co-culture can induce intra islet endothelial cell proliferation (Cavallari et al., 2012). Islet endothelial cell proliferation is likely to accelerate the rate of revascularisation post-transplantation. There is also some evidence to suggest direct co-culture is superior to indirect co-culture with islet and MSCs (Jung et al., 2011).

Islet transplantation to extra vascular sites such as under the kidney capsule has been reported to result in aggregation of the islets into an amorphous mass (Davalli et al., 1996, Biarnes et al., 2002). MCS have also been reported to attenuate this process, enhancing the maintenance of islet morphology more similar to that of islets in their native site, and preserving endocrine and endothelial cell distribution (Rackham et al., 2011). The distribution of α and δ cells were found to be more similar to that of native islets in MSC co-transplanted grafts, which may facilitate the normal regulation of insulin secretion (Bosco et al., 1989, Hauge-Evans et al., 2009).

1.7 Minimilisation of Immunoisolation Devices

Microencapsulation is the most commonly used islet immunoisolation method. However the diffusion distance to and from the islet and the increase in graft volume using microcapsules limit graft success and the choice of implantation site respectively. Based on an average islet diameter of 150 µm and a microcapsule diameter of 500 µm the diffusion distance is 250-175 µm to and from the islet cells, assuming the islet is in the centre of the microcapsule. Using mathematical modelling the oxygen tension has been predicted to be 25 mmHg at the islet

surface and as low as 0.15 mmHg at the centre of a microencapsulated islet in a 40 mmHg environment such as the intraperitoneal cavity (Johnson et al., 2009). Assuming the same islet and microcapsule dimensions, a microencapsulated islet graft would be approximately 37 times the volume of a non-encapsulated islet graft. Consequently a 70 kg human recipient implanted with 10,000 islets per kg would need to receive around 45.8 mL of microencapsulated islets compared to 1.2 mL of non-encapsulated islet tissue. Infusion of immunoisolated islet grafts into the clinically preferred intraportal site requires the development of islet immunoisolation strategies which minimise the immunoisolation device and graft volume.

1.7.1 Smaller Microcapsules

Conventional methods of microcapsule production such as using an electrostatic microcapsule generator can typically produce capsules in the range of 150-1500 µm. One way to combat the limitations imposed by the size of microcapsules is to make them smaller. The diffusion of insulin secreted from glucose stimulated microencapsulated islets out of microcapsules has been shown to be more efficient using smaller capsules (Chicheportiche and Reach, 1988). Also a study using empty capsules reported that smaller capsules were more biocompatible than large capsules in relation to pericapsular overgrowth (Robitaille et al., 1999). However other studies have reported that reducing the size of the capsule can result in protrusion of the islets (Halle et al., 1994, De Vos et al., 1996b, De Vos et al., 1996a). Islet protrusion was reported to be found in 24% of 500 µm capsules and correlated with increased graft failure compared to 700 µm capsules (De Vos et al., 1996a). Conversely, a study using the same protrusion detection method reported no evidence of islet protrusion in 500 µm capsules (Strand et al., 2002). The authors suggested that the difference may be due to the inhomogeneous composition of their microcapsules. The use of mannitol as the osmolyte in the alginate and a low concentration of cations in the gelification solution produces capsules 10 times more concentrated at the perimeter of the capsule (Thu et al., 2000). This may position the islets in the centre of the capsule upon gelification, thereby preventing islet protrusion.

The safety of infusing small microcapsules intraportally has been explored. Infusion of 10,000 capsules of 315 µm resulted in only a transient increase in intraportal pressure, whereas infusion of 10,000 capsules of 420 µm resulted in the death of 2/3 of recipient rats (Leblond et al., 1999). A study in pigs reported that the infusion of 10,000 400 µm microcapsules per kg induced a small and transient increase in intraportal pressure. Analysis of the liver gave similar hemodynamic, biological and radiological results as those observed in clinical non-

encapsulated islet transplantation. In a study using high-M alginate microcapsules transplanted intraportally increasing overgrowth up to the end point of the experiment at week 6 was reported (Mathe et al., 2004). The biocompatibility of a range of alginate microcapsules implanted intraportally into rats has been described (Toso et al., 2005). It was found that capsules incorporating a polycation layer were the least biocompatible, Ba^{2+} cross-linked capsules were more biocompatible than calcium cross-linked capsules, and there was no difference in biocompatibility between high-M or high-G alginate capsules. It has been shown that short term immunosuppression can attenuate capsule overgrowth at the intraportal site leading to a reduction in overall overgrowth in the long term (Toso et al., 2005, Mathe et al., 2004).

Rat islets immunoisolated with APA capsules of 250-350 µm have been transplanted into the peritoneal cavity of diabetic mice resulting in maintenance of normoglycaemia for 7 months (Lum et al., 1992). Pig islets immunoisolated in "small barium alginate microcapsules" and transplanted into the peritoneal cavity of diabetic rats resulted in maintenance of normoglycaemia for 9-385 days (Cappai et al., 1995). Dog islets immunoisolated in 300-400 µm alginate-PLO-alginate capsules were transplanted into the peritoneal cavity of diabetic dogs, resulting in maintenance of normoglycaemia in 2/3 of the dogs for 140-200 days (Calafiore et al., 1999). None of the transplantation studies into diabetic animals had a control group with larger microcapsules. Consequently it is impossible to draw any conclusions about the relative efficacy of smaller microcapsules. Allogeneic rat islets encapsulated in 350 µm alginate microcapsules have been implanted into the intraportal site of diabetic rats (Schneider et al., 2003). This resulted into only a transient reduction in blood glucose at day 1. The authors proposed that the capsules occluded the blood vessels leading to hypoxia and islet death.

1.7.2 Conformal Coating

Conformal coating of islets involves applying a coating which conforms to the shape of the individual islet, thereby providing an immunobarrier or functional coating with minimal dead space. The potential advantages of conformal coating compared to conventional immunoisolation approaches such as microencapsulation are related to the reduced scale of the coating. Conformal coatings should permit better diffusion of oxygen, nutrients, glucose, and insulin by reducing the diffusion distance to and from the islet, therefore improving islet viability and functionality. Conformal coating of islets also leads to a negligible increase in graft volume, therefore conferring suitability for optimal sites such as the intraportal site. Several methods of conformal coating for islets have been described with coating thickness ranging from 10 nm to 50 µm.

1.7.2.1 Interfacial Photopolymerisation

Conformal coatings of cross-linked polyethylene glycol (PEG) can be formed on the islet surface by interfacial photopolymerisation (Sawhney et al., 1994, Cruise et al., 1998, Weber et al., 2006). A fluorescent dye photoinitiator such as eosin Y is absorbed onto the islet surface and the islets are then suspended in acrylated PEG solution. Using an argon laser the fluorescent dye is photoexcited generating a free radical which initiates polymerisation of the PEG prepolymers via the acrylate termini. This forms a conformal coating of cross-linked PEG hydrogel around the islet in the order of 10-50 µm thickness (Sawhney et al., 1994). Functionalisation of the coating has been described with cell-matrix moieties incorporated into photopolymerised PEG hydrogel layers (Weber et al., 2008). Islets coated by interfacial PEG photopolymerisation have been implanted to diabetic animal models (Hill et al., 1997, Cruise et al., 1999). Maintenance of normoglycaemia was reported in diabetic athymic mice for approximately 70 days when transplanted intraperitoneally with 5,000-8,000 coated pig islets (Cruise et al., 1999).

1.7.2.2 Emulsification

A conformal coating of alginate has been achieved by co-incubation of islets with a two-phase aqueous emulsification with alginate, ficoll, and PEG (Calafiore et al., 1999). The emulsion droplets engulf the islets in alginate which can then be cross-linked with gelification solution. The alginate coated islets were exposed to PLO then alginate to complete the coating. Wistar-Furth rat islets transplanted intraperitoneally into diabetic Lobund-Wistar rats or CD-1 mice resulted in maintenance of normoglycaemia for 140 days using an unreported number of islets.

1.7.2.3 Reactive Polyethylene Glycol

PEG molecules with a reactive group have been used to immobilise PEG molecules onto the islet surface. PEG with an amine reactive end group such as isocyanate (Panza et al., 2000) or *N*-hydroxysuccinimide (Lee et al., 2002) react with the amine groups on the islet cell surface proteins or collagen layer to immobilise PEG onto the islet surface. Immobilisation of PEG has been shown to prevent splenocyte activation (Lee et al., 2004) and lymphocyte infiltration (Jang et al., 2004) but could not prevent infiltration of cytotoxic molecules. Reactive PEG has been the most widely investigated conformal coating in diabetic animal models (Contreras et al., 2004, Lee et al., 2006b, Lee et al., 2006c, Lee et al., 2006a, Lee et al., 2007, Yun Lee et al., 2007, Wee et al., 2008, Jeong et al., 2011). Allogeneic transplantation of 1,200 activated PEG coated rat islets under the kidney capsule resulted in maintenance of normoglycaemia for 1

year in diabetic rats when coupled with a minimal dose of cyclosporine (Yun Lee et al., 2007). Under the same conditions non-coated islets rejected at day 12. Reactive PEG coating appears to be able to block direct cellular immune attack but does not provide protection from cytotoxic molecules released from recruited lymphocytes. The low dose of immunosuppressant may prevent the release of cytotoxic molecules from the recruited lymphocytes permitting survival of the graft. A similar coating has also been applied in an allogeneic transplantation model without immunosuppression (Lee et al., 2007). 1,200 rat islets coated 3 times with activated PEG transplanted under the kidney capsule of diabetic rats resulted in maintenance of normoglycaemia for 100 days in 3/7 of the recipients. Comparing the studies, increasing the thickness of the coating improved the immunoprotective capability of the immunobarrier. However complete immunoprotection may require a thicker or less permeable immunobarrier which can protect against cytotoxic molecules. Reactive PEG coated islets have also been implanted intraportally (Wee et al., 2008). Allogeneic transplantation of 4,000 activated PEG coated rat islets resulted in maintenance of normoglycaemia for 28 days in diabetic rats with short term cyclosporine administration. The reduction in efficacy compared with similar studies using the kidney capsule site may be related to challenges at the intraportal site such as the IBMIR and the lymphocyte rich environment.

1.7.2.4 Hydrophobic Polyethylene Glycol

Amphiphilic molecules composed of a hydrophilic PEG molecule conjugated to a hydrophobic phospholipid molecule can be used to create a conformal coating. The hydrophobic portion of the PEG-lipid spontaneously integrates with the cell membrane of the islet surface, anchoring the PEG molecule. Hydrophobic PEG coating has been applied to diabetic animal model studies. Xenogeneic transplantation of 1,000 hydrophobic PEG coated hamster islets intraportally resulted in maintenance of normoglycaemia for only 6 days in diabetic mice (Teramura and Iwata, 2009b). In a syngeneic model, intraportal transplantation of 125 hydrophobic PEG coated mouse islets resulted in maintenance of normoglycaemia for 100 days in diabetic mice. Non-coated islets failed at 40 days which indicates that the coating may provide protection from acute challenges at the intraportal site such as the IBMIR. Conjugation of the thrombolytic protein urokinase to the PEG by deoxyribonucleic acid (DNA) hybridisation also improved the blood glucose stability in the first 20 days in the study.

1.7.2.5 Functionalised Conformal Coatings

Conformal coatings are amenable to functionalisation as a small amount of functional protein can be applied in immediate contact with the islet. PEG attached by amine reactive groups or

anchored into cell membranes by hydrophobic interaction can provide a base for the addition of linkers for attaching functional proteins or for building a layer-by-layer structures.

One form of linker is an amine reactive group on the unbound end of the PEG molecule. This can be used to bind functional proteins engineered with thiol groups. Using a PEG-lipid, human soluble complement factor (Luan et al., 2011a), and urokinase and thrombomodulin (Chen et al., 2011a) have been immobilised onto the islet surface using this method. DNA hybridisation has also been used as a linker method and involves using PEG bound or anchored to the cell surface which is conjugated with a poly-adenosine (poly-A) DNA sequence. Attachments can then be made with a poly-thymine (poly-T) complementary DNA sequence conjugated to the molecule of choice. Functional structures or proteins such as anticoagulant-loaded liposomes (Chen et al., 2011b) and urokinase (Takemoto et al., 2011) have been immobilised on the islet surface using DNA hybridisation.

It may be possible to encapsulate allogeneic islets in the recipients' cells to prevent recognition of the foreign tissue. The immobilisation of cells onto the islet surface to create a cell encapsulation barrier has been demonstrated using a DNA hybridisation method (Teramura et al., 2010) and a streptavidin-biotin based conjugation method (Teramura and Iwata, 2009a, Marek et al., 2011). The biotin-streptavidin/avidin linker method involves using PEG bound or anchored to the cell surface which is conjugated with biotin. Attachments can then be made with streptavidin/avidin conjugated to the molecule of choice. Biotin-streptavidin/avidin based conjugation methods have been used to immobilise molecules such as urokinase and heparin (Teramura and Iwata, 2008), VEGF and heparin (Cabric et al., 2010), thrombomodulin (Wilson et al., 2010), and GLP-1 (Kizilel et al., 2010) onto the islet surface. The immobilisation of heparin onto islets has been reported to reduce thrombi, infarct, and thrombin antithrombin levels when transplanted intraportally into pigs (Cabric et al., 2007).

The majority of the studies describing conformal coating functionalisation focus on anti-coagulation and anti-complement factors which could be applied to attenuate the IBMIR for intraportal grafts. It has not yet been established if these approaches can be designed to provide long-term immunoprotection as well as short term post-transplantation functionality. Immobilisation methods such as PEG-lipid or DNA hybridisation rely on anchorage to proteins or lipids in the cell membrane to form a coating. Turnover of cell membrane components is likely to limit the longevity of such coatings. Layer-by-layer techniques involving multiple layers of polymer coatings may provide longer-term function and/or immunoprotection.

1.7.2.6 Layer-by-Layer Membranes

A layer-by-layer membrane involves the deposition of sequential layers of polymers or proteins to yield an islet coating of desired thickness and characteristics. The layer-by-layer approach to immunoisolation allows for specialisation of the immunobarrier by altering the number of layers and the composition. For example, in a layer-by-layer coating the first layer in contact with the islet could contain ECM moieties, survival factors, growth factors, or insulinotropic factors. Several intermediate layers could be added to provide an immunobarrier with specific permeability. The outer layer might then be composed of anti-inflammatory, anti-rejection, or angiogenic factors.

Functional groups or conjugates on the exposed end of a reactive or anchored PEG molecule can be used to seed a layer-by-layer membrane such as alginate and PLL (Miura et al., 2006) or poly(vinyl alcohol) (PVA) (Teramura et al., 2007). Hydrophobic PEG has been used to seed a functional layer-by-layer coating using human soluble form complement receptor 1 (sCR1) and heparin to confer anti-complement activation and anti-coagulation activity respectively (Luan et al., 2011b). A layer-by-layer coating seeded on reactive PEG with multiple layers of biotin-PLL-PEG conjugate and streptavidin has been tested *in vivo* (Wilson et al., 2008). Allogeneic transplantation of a “minimal mass” of 250 coated mouse islets intraportally resulted in maintenance of normoglycaemia for only 1 day. The same laboratory did a similar experiment with multiple layers of PEG-PLL conjugate and alginate (Wilson et al., 2011) resulting in maintenance of normoglycaemia in recipients for only 1 day. A higher proportion (47% vs. 25%) became temporarily normoglycaemic compared to recipients of uncoated islets.

Layer-by-layer polyion complex nanofilms have been studied extensively for the purpose of coating implantable biodevices (Tang et al., 2006). The principle of this method is to exploit the weak electrostatic forces in oppositely charged polymers in order sequentially build a multilayered film onto a surface. This approach has been adopted for conformal coating of islets by seeding directly onto the islet surface. Applying a polyion layer-by-layer coating to the islet surface can be problematic as most cationic polymers such as PLL are cytotoxic and if not effectively isolated from the cell membrane can cause damage. A layer-by-layer coating of reactive PEG and either PLL, poly(ethyleneimine), or poly(allylamine) were all found to decrease islet cell viability (Lee et al., 2007). Polyallylamine hydrochloride with poly(styrene sulfonate) (PSS) (Krol et al., 2006) have been used as materials for a layer-by-layer coating of islets, but have not been tested in diabetic animal models.

1.8 Aims and Objectives

Microencapsulated islet transplantation into the intraperitoneal site is the most common approach for immunoisolated islet transplantation. However it is suboptimal and hence there is a need to develop novel approaches to enhance immunoisolated islet graft outcome. The overall aim of the thesis was to improve the efficacy of immunoisolated islets. This was achieved by a variety of strategies as outlined below.

- Site

The subcutaneous site may offer superior graft outcome for microencapsulated islet grafts. The relative ability of equivalent microencapsulated islet grafts implanted intraperitoneally and subcutaneously to maintain normoglycaemia in diabetic mice will be explored.

- Co-encapsulation with helper cells

Cells which secrete factors which are beneficial to islet viability and function are likely to improve graft outcome when co-encapsulated with islets for transplantation.

  - L cells

L cells secrete GLP-1 which has been shown to enhance β cell insulin secretion, insulin synthesis, and survival. The impact of incorporating L cells with islets in microcapsules on graft outcome will be explored using a minimal mass diabetic transplantation model.

  - MSCs

MSCs co-transplanted with islets have been shown to improve graft outcome in non-encapsulated islet grafts. The impact of incorporating MSCs with islets in microcapsules on graft outcome will be explored using a minimal mass diabetic transplantation model.

- Nano-scale encapsulation

Microcapsules are too large to be implanted into smaller capacity sites such as the kidney subcapsular site which enables better support of islet graft viability and efficacy. A novel conformal coating will be examined for its ability to permit islet graft viability and function in syngeneic transplantations and providing immunoprotection in allogeneic transplantations.

2 Materials and Methods

2.1 Animals

2.1.1 Mice

All procedures were carried out in the UK in accordance with the UK Animals Scientific Procedures Act 1986. All animals were procured from Harlan (Huntingdon, UK) or Charles River (Margate, UK), housed on a 12 h light/dark cycle and had access to food and water *ad libitum* except when fasted. All surgeries were carried out under isofluorane anaesthesia (1-5% isofluorane, 95% oxygen, 1 $L.min^{-1}$) and 30 $\mu g.kg^{-1}$ buprenorphine (Vetergesic, Reckitt Benckiser Healthcare, Hull) was administered subcutaneously before surgery. Surgical tools were cleaned and autoclaved for all procedures and the surgical area shaved and sanitised with a chlorhexidine gluconate based antimicrobial skin cleanser (Hibiscrub, Molnlycke Health Care, Dunstable). Surgery was completed by suturing incisions with 4-0 VICRYL (Ethicon, Johnson and Johnson), ear marking, and transferring mice to a 37°C incubator to recover. ICR mice are an outbred strain which were used for *in vitro* studies. C57BL/6J and BALB/C mice are inbred strains with defined immunological discordance which were used in combination for syngeneic and allogeneic transplantation studies as appropriate.

2.1.2 Isolation of Mouse Islets

Mice were killed by cervical dislocation, the abdomen exposed by laparotomy, and the liver was inverted to reveal the common bile duct. The ampulla of vater was clamped to close the route from the common bile duct to the duodenum. 2.5 mL collagenase (1 $mg.mL^{-1}$ type XI from clostridium histolyticum in modified Eagle's medium (MEM) (Sigma, Pool, UK)) was injected into the common bile duct to distend the pancreatic tissue. Pancreata were excised and stored in groups of 2-3 per 50 mL falcon tube, on ice for <60 min before incubation in a water bath at 37°C for 10 min. Following the enzymatic digestion the tissue was washed and centrifuged (340 g, 10°C, 75 sec) 3x in supplemented MEM (10% Newborn Calf Serum (NCS), 100 $U.mL^{-1}$ penicillin, 100 $ng.mL^{-1}$ streptomycin (Sigma)). The tissue was resuspended in supplemented MEM and filtered in a 450 µm mesh to remove the large pieces of exocrine tissue and undigested tissue. Following centrifugation (365 g, 10°C, 90 sec) the tissue was resuspended in 15 mL Histopaque 1077 (polysucrose solution at a density of 1.077 $g.mL^{-1}$ (Sigma)) and 10 mL supplemented MEM was gently added to create a density interface. Centrifugation (1900 g, 10°C, 24 min) was carried out with slow acceleration and no brake so not to disturb the density interface, and resulted in the accumulation of islets at the density

interface and exocrine fragments in the pellet. Islets were collected, washed and centrifuged (365 g, 10°C, 90 sec) 3x in supplemented MEM. To further purify the islets sedimentation was carried out 6x by resuspending the islets in 25 mL supplemented MEM and allowing the islets to settle for 4 min on ice, then discarding the top 10 mL containing the remaining small exocrine fragments. Islets were cultured overnight (37°C, 5% CO_2) in supplemented Roswell Park Memorial Institute (RPMI) medium (10% foetal bovine serum (FBS) (Sigma) and 100 $U.mL^{-1}$ penicillin, 100 $ng.mL^{-1}$ streptomycin) at a density of 3-4 pancreata of islets per 2 x 9 cm Petri dishes before either encapsulation or culture.

2.1.3 Models of Type 1 Diabetes

A diabetic state was induced in mice by glucose mimetic toxins which are selectively taken up through the GLUT-2 transporter on β cells. Streptozotocin (STZ) is an alkylating agent which causes DNA damage and was used unless stated otherwise. 180 $mg.kg^{-1}$ STZ (Sigma) carried in sterile citrate buffer (10 mM citric acid, 150 mM NaCl, pH 4.5) was administered intraperitoneally. Alloxan administration results in the accumulation of reactive oxygen species and β cell death. 75 $mg.kg^{-1}$ alloxan tetrahydrate (Sigma) carried in phosphate buffered saline (PBS) (Sigma) was administered through the tail vein. For tail vein injections mice were anaesthetised by isofluorane and the tail was warmed in 37°C water to enlarge the tail vein. The tail was dried and the alloxan tetrahydrate was injected using a 1 mL syringe with a 29G needle. Induction of diabetes with STZ or alloxan was 4-7 days before transplantation and animals with blood glucose of >20 mM were used for transplantation.

2.1.4 Animal Monitoring

Diabetic mice were monitored for weight and non-fasting blood glucose with a blood glucose meter (Accucheck aviva, Roche, Burgess Hill, UK) 3x per week. Measurements were taken at 10:00 am on Monday's, Wednesday's, and Friday's. Blood was drawn from a needle prick in the extremity of the tail with a 30G needle. Mice exceeding 15% loss of weight in 3 days or 20% loss of weight overall were killed. 1 unit of insulin (Caninsulin, Intervet, Walton, UK) was administered with an insulin syringe (Terumo MYINJECTOR 29G x ½", Somerset, NJ, USA) to prevent excessive weight loss.

2.2 Tissue Culture

2.2.1 Culture of Mouse Islets

Mouse islets or microencapsulated islets were picked and counted with a pipette into groups of 150 islets in 6 cm Petri dishes or 400 islets in 9 cm dishes (≈7 islets per cm^2). Islets/microencapsulated islets were cultured in supplemented RPMI (11.1 mM glucose) in a tissue culture incubator (37°C, 5% CO_2). The media was changed 3x per week.

2.2.2 Cryopreservation of Cells

Cells were cryopreserved to make cell stocks for long term storage. Cells were resuspended in 4°C cryopreservation solution (90% FBS, 10% dimethyl sulfoxide (DMSO) (Sigma)) to yield a cell density of $1x10^6$ cells.mL^{-1}. 1 mL volumes were transferred to 2 mL screw-top cryotubes and these were transferred to an isopropanol freezing container which was incubated at -80°C overnight. Cryotubes were then transferred to liquid nitrogen (-196°C) for long term storage. Cells were recovered from cryopreservation in order to reinitiate cell culture. Cryotubes were removed from liquid nitrogen and defrosted in a water bath for 3 min at 37°C. Cells were washed in culture media twice and then seeded into a T25 culture flask (Nunc, Thermo Scientific, Roskilde, Denmark).

2.2.3 Passage of Cells

Cells were passaged in order to keep cells within a density range for normal cell growth. Adherent cells were passaged every 2-4 days or when 80-100% confluent. Culture media was removed, cells were washed in calcium and magnesium free PBS (Sigma) and then enough trypsin solution (0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma) to cover the cells was added. Cells were incubated in a tissue culture incubator for 3-5 min to disrupt cell adhesion and then the flask was agitated by hand to detach the remaining cells. Culture media was added to stop the reaction and the cells were centrifuged for 3 min at 300g. The supernatant was discarded and the cells were triturated in fresh culture media before a proportion was re-seeded back into the culture flask supplemented with fresh media. The proportion of cells re-seeded was between 1/3 and 1/5 depending on the growth characteristics of the cell type.

2.2.4 Estimation of Cell Number

Detached and triturated cells were filtered through a sterile 40 µm cell strainer (BD Biosciences, Oxford) to yield a single cell population. A 10 µl sample was loaded into a

Neubauer haemocytometer chamber enclosed with a cover slip. Cells were counted in 4x 1 mm^2 areas and the average was used to estimate the number of cells per mL in the original population.

2.3 Immunoisolated Islet Transplantation

2.3.1 Microencapsulation Materials

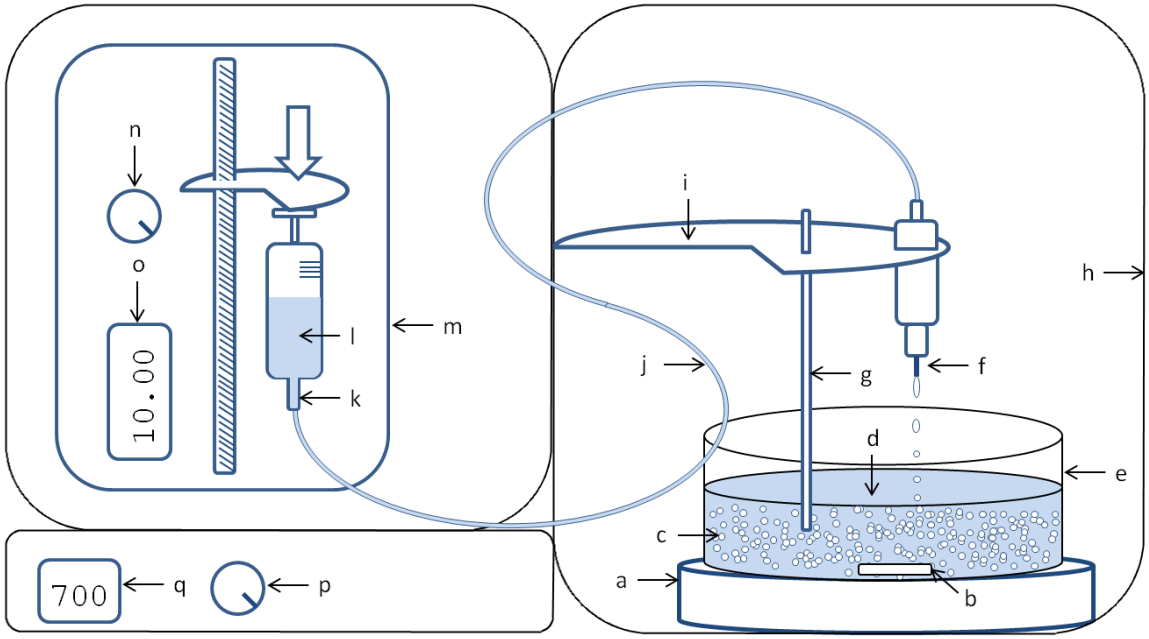
Materials for microencapsulation were made with sterile and endotoxin-free materials and where possible sterile conditions. All solutions were made with deionised, distilled, 0.1 µm sterile filtered, endotoxin-free water (<0.005 Endotoxin Units.mL^{-1}) (Hyclone, Logan, UT, USA) and all compounds excluding the alginate were "BioUltra" grade from Sigma. Ultrapure (UP) saline (154 mM NaCl, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) and gelification solution (0.15 M mannitol, 1 mM HEPES, 1 mM $BaCl_2$, 50 mM $CaCl_2$) were made by adding the compounds to 1 L of water and then adding endotoxin-free sodium hydroxide (NaOH) (Sigma) to attain pH 7.35-7.45 by sampling. 2% alginate was made by adding 1 g ultrapure low viscosity high-G (≥60%) sodium alginate (PRONOVA™ UP LVG, FMC corp., Drammen, Norway) to 25 mL water (4% alginate) and mixing at 4°C for >16 h in a 50 mL falcon tube. Mannitol solution (600 mM mannitol, 2 mM HEPES) was made in 40 mL water and adjusted to pH 7.35-7.45 with endotoxin-free NaOH by sampling. Mannitol solution was then added to the alginate to make up to 50 mL (2% alginate, 300 mM mannitol, 1 mM HEPES) and the solutions were mixed at 4°C for >1 h and the pH was measured and adjusted to pH 7.35-7.45 by sampling. Saline and gelification solution were sterile filtered in 1 L 0.22 µm polyethersulfone (PES) vacuum filters (Merck Millipore, Billerica, MA, USA) and the alginate was filtered in a 50 mL Steriflip™ 0.22 µm PES vacuum filter (Millipore). Alginate was aliquoted in volumes of ≈1 mL into endotoxin-free, sterile 2 mL microcentrifuge tubes. All solutions were stored at 4°C.

The day before microencapsulation the nozzle was checked for blockage and then the glassware, arm, rod, and nozzle parts of the bead generator were soaked for >16 h in 10 $g.L^{-1}$ E-toxa clean (Sigma) in a 2 L beaker to remove residual endotoxin. The equipment and beaker were then rinsed thoroughly in tap water, then deionised water, and finally autoclaved and dry cycled in an autoclave.

2.3.2 Microencapsulation

Microcapsules were made in an electrostatic bead generator which was custom built at the Norwegian University of Science and Technology, Norway (Figure 2). Islets and/or cells were

transferred to a sterile and endotoxin-free microcentrifuge tube, vortexed, centrifuged (300 g, 5 min), and put on ice. The islets and/or cells were washed 2x in 1 mL 4°C UP saline and then the saline was removed to leave the islets in 100 µl. To prevent islets attaching to the inside of the pipette tip a 200 µl pipette tip was exposed to RPMI supplemented with FBS and then washed 2x in UP saline. The islets were then taken up in the 100 µl with the pipette tip and transferred into a 2 mL syringe containing 900 µl 2% alginate. The islets were mixed with the alginate to create a homogenous solution of 1.8% alginate with dispersed islets throughout. Some leading 1.8% alginate without islets or cells was injected into the delivery tubing to prevent the islets from gathering at the alginate meniscus. The alginate-islet and/or cell suspension syringe was then attached to the polythene delivery tubing (0.58 inner diameter, Portex, Smiths Medical, Kent) to create a continuous solution with the leading alginate. The suspension was delivered to the electrostatic bead generator at 10 $mL.h^{-1}$ by a syringe pump (WPI, Florida, USA) through the delivery tubing to a 0.2 mm nozzle (Mars Matic 750 M07 Drafting Point, Staedtler, Bridgend, UK). Droplets were electrostatically pulled with a differential charge of 7 kV into the gelification solution causing gelification by cross-linking of the alginate with Ba^{2+} and Ca^{2+} ions to create microcapsules. Microcapsules were approximately 500 µm in diameter and were inhomogeneous with higher density towards the outside of the capsule. The capsules were cultured overnight in serum-free RPMI-1640 (100 $units.mL^{-1}$ penicillin, 100 $ng.mL^{-1}$ streptomycin).



a. Magnetic stirrer
b. Stir bar
c. Microcapsules
d. Gelification solution
e. Glass beaker
f. Nozzle
g. Electrostatic rod
h. Microencapsulation chamber
i. Arm
j. Delivery tubing
k. Syringe
l. Alginate
m. Syringe pump
n. Syringe pump controller
o. Syringe pump display
p. Bead generator controller
q. Bead generator display

Figure 2. Schematic of electrostatic bead generator producing alginate microcapsules.

2.3.3 Microencapsulated Islet Transplantation

Mice were anaesthetised, administered analgesic, and the surgical area was shaved and sanitised. For intraperitoneal transplantation a small incision was made in the skin and then the *linea alba* of the peritoneum. For subcutaneous transplantation a small incision was made in the skin above the proximal femur and a subcutaneous pocket was fashioned down to the knee joint. The graft was delivered in <500 µl serum-free RPMI with a sterile, endotoxin-free 1000 µl pipette. Incisions were sutured and the animal was placed in a recovery incubator.

2.3.4 Microencapsulated Islet Recovery

Mice were killed by cervical dislocation and the skin was dissected laterally all the way around the abdomen. The skin was pulled back to reveal the entire thorax and abdomen and 5 mL saline was injected to distend the peritoneal cavity. The mouse was placed in a 9 cm Petri dish and the peritoneum was cut longitudinally from the sternum allowing the saline to carry the capsules into the Petri dish. The peritoneal cavity was flushed with saline and then inspected under a microscope where the remaining capsules were picked with forceps. Capsules recovered from the subcutaneous site were picked with forceps. Capsules were washed in saline and cultured in 2 mM glucose supplemented Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) for >1 h in a tissue culture incubator before further assessment.

2.3.5 Nano-Scale Islet Encapsulation

The layer-by-layer coating exploits the charge of the cell membranes of the islet perimeter and the charge of the polysaccharide materials. The negatively charged cell membrane serves as a bed for electrostatic interaction with positively charged chitosan-phosphorylcholine (PC). The negatively charged alginate then interacts with the deposited chitosan-PC to form a chitosan-PC-alginate bilayer. The required number of chitosan-PC-alginate bilayers could be built up sequentially. The outmost bilayer was composed of chitosan-PC-chondroitin-4-sulfate-PC. PC groups were linked to chitosan (low molecular weight, 75% deacetylation (Sigma)) and chondroitin-4-sulfate (Sigma) by reductive amination of PC glyceraldehyde as described by Miyazawa and Winnik (Miyazawa and Winnik, 2002). Solutions of 1 $mg.mL^{-1}$ chitosan-PC, sodium alginate (low viscosity alginic acid sodium salt from brown algae (Sigma)), and chondroitin-4-sulfate-PC were prepared in Hank's buffered salt solution (HBSS) supplemented with 2 mM $CaCl_2$ and 0.2 µm sterile filtered. The nano-scale encapsulation was carried out with the islets in a 1.5 mL microcentrifuge tube on ice. Islets were suspended in the polysaccharide solutions for 5 min at a time and washed with supplemented HBSS by sedimentation 2x after each exposure to remove unabsorbed polysaccharide.

2.3.6 Non-Encapsulated and Nano-Scale Encapsulated Islet Transplantation

Islets or nano-scale encapsulated islets were kept on ice before centrifuging into a pellet within PE50 polyethylene tubing (Becton Dickinson, Sparks, MD) coated with a siliconising reagent (Sigmacote, Sigma) to prevent islet attachment. To centrifuge the islets the tubing was bent in half and the islets were delivered into one end of the tubing. The tubing was placed in a centrifuge tube with the bend at the bottom. The tube was then centrifuged (200g, 2 min) so that the islets gathered in a pellet at the bend. The tubing was then attached to a Hamilton syringe (Fisher, Pittsburg, PA) at one end without allowing any air bubbles to form and the tubing was cut at the pellet at the other end, ready for transplantation. Sequential lumbar incisions were made through the skin then the peritoneum of the anaesthetised recipient, then the left kidney was externalised and kept moist with PBS. An incision in the kidney capsule was made by dragging a needle across the surface, the end of the tubing was inserted under the capsule and the islets were delivered. The incision in the capsule was heat-sealed with a cauteriser (Bovie, Clearwater, FL, USA), the kidney internalised, the peritoneum and skin sutured, and the mouse put in the recovery incubator.

2.3.7 Glucose Tolerance Test

Mice were fasted for 16 h and the basal blood glucose was measured. 2 $g.kg^{-1}$ sterile glucose solution (1.67 M glucose in PBS) was administered intraperitoneally and blood glucose measurements were taken after 15, 30, 60, 90, and 120 min post-administration.

2.3.8 Nephrectomy

Nephrectomy of the graft bearing kidney of cured mice was carried out to confirm the underlying diabetic state and the function of the graft. Sequential lumbar incisions were made through the skin and the peritoneum of the anaesthetised recipient, then the left kidney was externalised and kept moist with PBS. Connective tissue around the kidney was dissected away to expose the renal artery, renal vein, and the ureter. The ureters and the vessels were clamped and tied off below the clamp using a tied suture. Using a sterile disposable scalpel (Swann morton limited, Sheffield, UK) the vessels were cut above the clamp to remove the kidney. The tie was internalised and the peritoneum and skin sutured.

2.4 Immunohistochemistry and Cell Staining

2.4.1 Fixation of Tissues

Fixation of tissues preserves the tissue structure and protein composition by cross-linking with paraformaldehyde. Tissues were fixed in fixation solution (4% paraformaldehyde in PBS) for 2 h, washed in PBS and then exposed sequentially for 1 h at a time to 30%, 50%, and 70% ethanol. Samples could be stored at this stage or immediately paraffin-embedded.

2.4.2 Paraffin-Embedding of Microencapsulated Islets

Paraffin-embedding integrates fixed tissue into paraffin blocks ready for sectioning. Fixed microencapsulated islets in 70% ethanol in 1.5 mL tubes were first exposed to 80% ethanol with 0.75% eosin to stain the sample. The sample was further dehydrated in 95% then 100% ethanol for 5 min each, and then exposed to xylene for 3 min. After removing the xylene 60°C molten paraffin wax (Paraplast, Sigma) was poured over the sample to fill the tube. Tubes were incubated at 60°C for 4-16 h to allow full penetration of the wax into the sample. Tubes were incubated at 4°C for 4-16 h to solidify the wax, then using a corkscrew the entire paraffin mould was removed from the tube with the capsules at the tip. The tip was cut off and was re-melted in a block mould on a 60°C hot plate. Molten wax was poured over the mould and a cassette was placed on top. More molten wax was added on top of the cassette lid and the block was incubated at 4°C for 4-16 h before removing the mould.

2.4.3 Paraffin-Embedding of Graft-Bearing Kidneys

The area of tissue of the kidney containing the graft was dissected under a microscope and then processed using a Leica TP1020 tissue processor. The tissue was placed in a cassette and then exposed to 90% industrial methylated spirit (IMS), 3x 100% IMS, 6.6% IMS in xylene, 2x xylene, and molten paraffin wax on an automated program for 120 min for each treatment. The tissue was then embedded in a paraffin wax block using a mound and cassette.

2.4.4 Sectioning of Embedded Tissues

Blocks were stored at 4°C for >4 h prior to sectioning. Blocks were mounted onto a microtome (Leica Microsystems, Milton Keynes) by the cassette and cut with a 34° microtome blade (MB DynaSharp, Thermo Scientific) held at 5° by progressing the block forward by 5 µm to produce each section. Sections were transferred to a 40°C water bath and then mounted onto slides (Superfrost Plus, Thermo Scientific).

2.4.5 Haematoxylin and Eosin Staining

Haematoxylin and eosin stain for the nucleus and cytoplasm respectively to aid the visualisation of cell morphology. Slides were put on a 60°C heat plate for 5 min to melt the wax, de-waxed by exposure to xylene for 2x 5 min, rehydrated by sequential exposure to 100%, 95%, and 70% ethanol for 5 min each and then rinsed in tap water. To stain for haematoxylin, slides were exposed to haematoxylin (Sigma) for 5 min, rinsed in tap water for 5 min, de-differentiated by dunking the slides 4x in 1% acid alcohol and rinsed again in tap water for 5 min. Slides were then exposed to eosin for 5 min and then rinsed in tap water for 5 min. Slides were then dehydrated in 70%, 95%, and 100% ethanol, then xylene 2x for 5 min each. A cover glass was mounted onto each slide using DPX mountant (Sigma).

2.4.6 Immunostaining

Slides were de-waxed and rehydrated as described above. Slides were exposed to 3% hydrogen peroxide (Sigma) for 10 min to block endogenous peroxidise activity, and then rinsed in tap water for 5 min. Slides were transferred to a humidified chamber and individual sections were circled with a hydrophobic barrier pen (Invitrogen). The following immunostaining steps were separated by rinsing steps with Tris-buffered saline (TBS) (50 mM Tris (Sigma), 150 mM NaCl). As kidney sections were used in some experiments which contains high levels of endogenous biotin a blocking stage was carried out with a biotin blocking system (Dako Cytomation, Ely) as standard. Sections were exposed to avidin for 10 min to bind endogenous

biotin and then with biotin for 10 min to saturate the avidin. Blocking buffer (10mg.mL^{-1} bovine serum albumin (BSA), 15 mM NaN_3, in TBS) was added for 10 min to prevent non-specific antibody binding, and then poured off the slide before adding the primary antibody for 1 h at room temperature or for 16 h at 4°C. Biotin conjugated secondary antibody was added for 40 min then the slides were washed in TBS for 10 min. To detect antibody binding, sections were exposed to streptavidin horseradish peroxidise (Streptavidin-HRP) (Dako) to bind biotin on the secondary antibody. Sections were exposed to 3,3'-diaminobenzidine (DAB) and hydrogen peroxide (Dako) causing oxidation of DAB by HRP resulting in brown staining. Sections were monitored for staining development and the reaction was stopped by rinsing with tap water. Sections were then counterstained with haematoxylin, dehydrated, and mounted with cover glass.

2.4.7 Fluorescent Microscopy

Capsules were washed in PBS then incubated for 5 min at room temperature in 10 pg.mL^{-1} fluorescein diacetate (FDA) (Sigma) in PBS. The capsules were washed twice in PBS then viewed under a fluorescence microscope. Recorded images were focused at the largest cross-section of the microcapsule with 478−495 nm excitation and 510−555 nm emission filters to visualise live FDA stained cells.

2.4.8 Confocal Microscopy

Microcapsules were stained with live/dead (FDA/propidium iodide (PI)) staining and visualised using a Zeiss LSM 510 confocal microscope with a C-Apochromat 10x/45W objective and software by LSM 510, release 2.02 (Carl Zeiss, Oberkochen, Germany). Recorded images were focused at the largest cross-section of the microcapsule through 478−495 nm and 530−560 nm excitation filters and 510−555 nm and 573−648 nm emission filters to visualise FDA and PI staining respectively.

2.5 Cell Content and Secretion

2.5.1 Adenosine Triphosphate Assay

The CellTiter-Glo® luminescent cell viability assay (Promega, Southampton, UK) was used to measure the ATP content of microencapsulated islets. The assay is based on the principle that in the presence of ATP, recombinant luciferase catalyses the conversion of luciferin to oxyluciferin which generates luminescence. Detecting the intensity of the luminescence gives a relative measure of the ATP concentration. Combined with simultaneous measurements from

a range of samples with known ATP concentration to make a standard curve, the ATP concentration of unknown samples can be deduced.

CellTiter-Glo® substrate was diluted in CellTiter-Glo® buffer and added in 50 µl volumes to the wells of a black walled, transparent bottomed 96-well plate. ATP standards of 0, 1, 10, and 100 pM ATP (Sigma) were added to the reagent in duplicate. Unknown samples were added and after shaking for 2 minutes the luminescence was repeatedly recorded every 2 min for 1 h using a luminometer (Vertitas, Promega Biosystems, Southampton, UK). The maximum luminescence for each well was included for analysis and values were converted from relative light units (RLU) to ATP values using the standard curve.

2.5.1 Deoxyribonucleic Acid Content Assay

The Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) was used to estimate the number of cells in microcapsules by measuring the DNA content of the microcapsules. The reagent is an ultrasensitive stain which fluoresces at specific excitation and emission wavelengths when bond to double stranded DNA. The DNA content of unknown samples is determined by the intensity of the fluorescence relative to a range of samples with known DNA content.

Samples were prepared by picking 50 capsules then washing and resuspending in 200 µl of ice cold tris-EDTA buffer (10 mM tris-HCl, 1mM EDTA, pH 7.5). The capsules were ruptured with a 29G insulin syringe, homogenised by sonication, and incubated at 4°C for >16h. Unknown samples and standards of 0, 0.1, 1, 10, 100 ng DNA were added in duplicate in 100 µl volumes into the wells of a black walled transparent bottomed 96-well plate. 100 µl of reagent was added to each well and after 2 min the fluorescence in each well was measured with 480 nm excitation and 520 nm emission filters using a microplate reader (Hidex Chameleon, Turku, Finland). Samples containing microcapsules without cells were used as a baseline and the number of cells calculated from the DNA content assuming each cell contains 3.5 pg DNA.

2.5.2 Glucose-Stimulated Insulin Secretion

Islets were pre-incubated in 2 mM supplemented DMEM for >1 h. Groups of 10 islets were picked into 1.5 mL tubes and the residual medium was removed using an insulin syringe. Samples were incubated in a water bath at 37°C in 600 µl sub-stimulatory glucose solution (2 mM glucose in Gey & Gey buffer (5 mM KCl, 111 mM NaCl, 27 mM $NaHCO_3$ 220 µM KH_2PO_4, 50 mM $CaCl_2$, 1 mM $MgCl_26H_2O$, 0.5 $mg.mL^{-1}$ BSA (Sigma) bubbled with 95% O_2, 5% CO_2 for 5 min)). After 1 h tubes were agitated, pulse centrifuged, and 300 µl sub-stimulatory samples were collected and 300 µl 38 mM glucose Gey & Grey buffer was added resulting in a

stimulatory glucose concentration of 20 mM. After 1 h tubes were agitated, pulse centrifuged, and 500 µl stimulatory glucose samples were collected and both sample set were stored at -20°C.

2.5.3 Insulin Content

Insulin content samples were collected following GSIS and processed on ice. 100 µl acid alcohol (1% HCL, 70% ethanol) was added to the islets in 100 µl Gey & Gey buffer. The islets in solution were pushed through a 29G insulin syringe to rupture the capsules and homogenise the samples. The samples were sonicated for 10 sec and incubated at 4°C for 16 h before being stored at -20°C.

2.5.4 Insulin Perifusion

The dynamic rate of insulin secretion from islets was assessed by an in-house multi-channel perifusion system. Perifusions were carried out in a 37°C heated room with 37°C buffers to maintain physiological temperature. Each channel consists of a chamber in which the islets are placed on 1 µm nylon filters in filter holders (Swinnex, Millipore, Cork, Ireland). Test substances in supplemented (0.5 $mg.mL^{-1}$ BSA) Gey & Gey buffer were perifused over the islets through the camber by peristaltic pumps. Perifused samples were collected every 2 min and stored at -20°C.

2.5.5 Insulin Radioimmunoassay

The insulin concentration of GSIS, insulin content and perifusion samples was determined by an in-house radioimmunoassay. The assay is based on the competitive binding of radiolabeled insulin and sample insulin to an insulin antibody. A greater concentration of sample insulin results in less radiolabeled insulin binding to the antibody and visa versa. Detecting the amount of radioactivity bound to the precipitated antibody gives a relative measure of the insulin concentration of the sample insulin. Combined with simultaneous measurements from a range of samples of known concentration to make a standard curve, the insulin concentration of unknown samples can be deduced.

Standard samples (0.04, 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5.0, 10.0 $ng.mL^{-1}$) were prepared by serial dilution of 10 $ng.mL^{-1}$ insulin (Sigma) in borate buffer (133 mM boric acid, 68 mM NaOH, 10 mM EDTA (Sigma), 0.5 $mg.mL^{-1}$ BSA). Unknown samples were diluted in borate buffer to an estimated insulin concentration of 0.5 $ng.mL^{-1}$, and then standards in triplicate and unknown samples in duplicate were added to LP3 tubes. I^{125}-labelled insulin was diluted to give a count

per minute of 10,000 per 100 µl. Insulin primary antibody was derived from Hartley guinea pigs challenged with bovine insulin and was used at a concentration of 1:60,000. I^{125}-labelled insulin and insulin antibody were added to all standards, samples and relevant controls (Table 1). Following 48 h of incubation at 4°C γ-globulin solution (γ-globulin (Sigma), 30% PEG, Tween 20 (Sigma)) was used to precipitate the antibody and the tubes were centrifuged at 15,000 g for 15 minutes at 4°C. Following aspiration of the supernatant to remove unbound insulin, samples were read in a γ counter (WIZARD^2, Perkin Elmer, Waltham, USA).

	Buffer (µl)	Antibody (µl)	Tracer (µl)	Standard (µl)	Sample (µl)
Total Radioactivity			100		
Non-specific binding	200		100		
Maximum binding	100	100	100		
Standards		100	100	100	
Samples		100	100		100

Table 1. Preparation of the controls, unknown samples and standards for the insulin radioimmunoassay.

2.5.6 Glucagon-Like Peptide-1 Enzyme-Linked Immunosorbent Assay

The GLP-1 peptide enzyme-linked immunosorbent assay (ELISA) (Millipore) is based on the capture of active GLP-1 from the sample by a monoclonal antibody immobilised in the wells of a microplate, and the subsequent binding of an anti-GLP-1 alkaline phosphatase conjugate. Exposing the conjugate to methyl umbelliferyl phosphatase generates the fluorescent product umbelliferone. By simultaneously assaying known concentrations of GLP-1 to create a standard curve, the concentration of GLP-1 from unknown samples can be interpolated. On the day of the assay, samples and standards were defrosted on ice. The assay was carried out according to the manufacturer's protocol. Briefly, wells were washed, then non-specific binding or assay buffer were added to the wells. Standards, quality control samples and unknown samples in duplicates were added to wells with assay buffer, and the plate was sealed and incubated (4°C, 24 h). Following incubation and washing, anti-GLP-1 alkaline phosphatase conjugate was added and incubated (20°C, 2 h). After washing, methyl umbelliferyl phosphatase substrate was added and incubated in the dark (20°C, 20 min). Stop solution was added to arrest phosphatase activity and the plate was read in a fluorescence plate reader (Hidex Chameleon) with an excitation/emission wavelength of 355 nm/460 nm.

2.6 Statistical Analysis

The statistical analyses were carried out using SigmaPlot 12.0 software (Systat Software Inc., Hounslow, UK). For parametric data comparing two groups the t-test was used and for >2 groups analysis of variance (ANOVA) using the Holm-Sidak post hoc test was used. For non-parametric data comparing two groups the Mann-Whitney Rank Sum Test was used and for >2 groups the Kruskal-Wallis ANOVA on Ranks (Dunn's method) was used. For survival curves the Kaplan-Meier Survival Analysis (Log-Rank) was used. *n* indicates the number of data points the statistical analysis was performed on. Unless stated, *n* is the number of experiments or the number of mice as appropriate. Where statistics were performed on pooled observations the number of experiments or mice are also stated in brackets (e.g. *n*=18(3) indicates 18 pooled data points from 3 experiments/mice were used for statistical analysis). All data are expressed as mean ± standard error of the mean.

3 Immunoisolation of Islets in High Guluronic Acid Barium-Alginate Microcapsules does not Improve Graft Outcome at the Subcutaneous Site.

3.1 Introduction

The conventional implantation site for microencapsulated islets is the intraperitoneal site. The site has a large capacity and implantation requires a simple procedure. However microencapsulated islet grafts require over double the number of islets compared with non-encapsulated islet grafts at sites such as under the kidney capsule. Additionally, retrieval of microencapsulated grafts would involve a highly invasive procedure. Taken together the intraperitoneal site is suboptimal for microencapsulated islet transplantation and alternative sites may offer superior graft outcomes. Due to its large capacity, the subcutaneous site is suitable for microcapsules and also benefits from minimally invasive implantation and easy graft retrieval, which would be advantageous for clinical use. The stability of some microcapsules has been reported to be greater in the subcutaneous site than the intraperitoneal site (Dufrane et al., 2006, Thanos et al., 2007) and transplantation of microcapsules into the subcutaneous site of diabetic animals was reported to be more efficacious than transplantation to the intraperitoneal site (Veriter et al., 2009). Microcapsules of low viscosity and high guluronic acid (G) alginate cross-linked with $BaCl_2$ without a polycation coating have demonstrated high stability (Martinsen et al., 1989), low immunogenicity (Kulseng et al., 1999), and graft success when transplanted to the intraperitoneal site in mouse allograft models (Bohman and King, 2008). However, large numbers are required to reverse hyperglycaemia when transplanted to the intraperitoneal site. In this study we investigated whether the subcutaneous site could provide a viable alternative using microcapsules which have previously proven successful at the intraperitoneal site.

3.2 Materials and Methods

3.2.1 Animals

Male inbred mice of the C57 black 6J strain (C57BL/6J) aged 6-8 weeks were used as islet donors and male C57BL/6J mice weighing 25g (8-10 weeks) were used as recipients. Diabetes was induced by tail vein injection of alloxan and blood glucose measurements were made over 28 days following transplantation.

3.2.2 Islet Transplantation

Isolated islets from several mice were pooled and half were used for non-encapsulated transplantation and half for microencapsulated transplantation. Diabetic animals received 700 non-encapsulated or microencapsulated syngeneic islets either subcutaneously or intraperitoneally. In addition, one individual diabetic mouse received 1750 microencapsulated syngeneic islets subcutaneously. To investigate islet viability, function, and morphology at each site without the confounding factor of hyperglycaemia, microencapsulated transplantations were also carried out in normoglycaemic mice. Simultaneous transplantations of 200 microencapsulated syngeneic islets to the intraperitoneal site and 100 to the subcutaneous site were carried out. 100 microencapsulated islets were also cultured *in vitro* as controls. After 7 or 28 days the microcapsules were recovered and analysed.

3.2.3 Insulin Measurements

Insulin secretion and insulin content samples were from groups of 10 encapsulated islets explanted from each mouse or picked from each culture dish.

3.2.4 Adenosine Triphosphate Assay

Samples for the ATP content assay were from groups of 10 microencapsulated islets explanted from each mouse or picked from each culture dish. Samples were prepared by homogenising groups of 10 microencapsulated islets in 50 µl volumes of media using a fine gauge insulin syringe. The homogenate was added directly to the assay buffer and then measured using a luminometer according to the standard protocol.

3.2.5 Histological Scoring

Microencapsulated islets were fixed, embedded, sectioned, and haematoxylin and eosin stained. Islet sections were evaluated by blinded scoring of 168±28 islets per graft. Islets were scored on a scale of 1-4 as follows; 1: 0-25% loss of islet shape and structure with no fragmented cells; 2: 25-50% loss of islet shape and structure with some fragmented cells; 3: 50-75% loss of islet shape and structure with many fragmented cells; 4: 75-100% loss of shape and structure with mostly fragmented cells.

3.3 Results

3.3.1 Efficacy of Microencapsulated Islets at the Subcutaneous and Intraperitoneal Sites

There was no improvement in the hyperglycaemic state of alloxan-diabetic mice implanted subcutaneously with 700 non-encapsulated islets or microencapsulated islets (Figure 3). Microencapsulated islets implanted intraperitoneally reduced the blood glucose for up to 28 days with mean values consistently less than 11 mM (Day 0 vs. day 1, 3, 7, 14, 21 and 28 (P<0.001 Repeated measures ANOVA, n=4-7). In the non-encapsulated intraperitoneal group, 25% of the transplantations resulted in blood glucose concentrations of less than 11 mM, but the mean values for the group were consistently above 17 mM with 75% of the mice remaining overtly hyperglycaemic. The mouse transplanted with 1750 microencapsulated islets subcutaneously failed to maintain normoglycaemia.

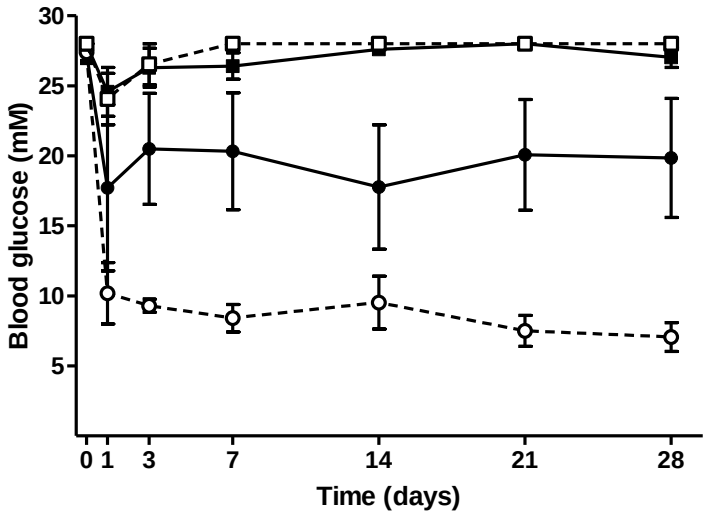


Figure 3. Blood glucose measurements of diabetic mice with intraperitoneal and subcutaneous grafts. Subcutaneous non-encapsulated (black squares, solid line, n=7), subcutaneous microencapsulated (white squares, dotted line, n=5), intraperitoneal non-encapsulated (black circles, solid line, n=4), and intraperitoneal microencapsulated (white circles, dotted line, n=5) islet grafts.

3.3.2 Function and Viability of Recovered Microencapsulated Islets

To establish how rapidly islet function failed and to remove potential secondary effects of hyperglycaemia on islet function, microencapsulated islets were transplanted into normoglycaemic mice and recovered to determine the functionality and viability of the islets. Grafts were recovered at 7 days at which point the grafts from the diabetic mice had stabilised the recipients' blood glucose.

3.3.2.1 Insulin Secretion

There were no differences between basal insulin secretion between the islet treatment groups (P=0.228, ANOVA on Ranks, n=5) (Figure 4). However, stimulated insulin secretion was lower in islets recovered from the subcutaneous site than either cultured islets or islets recovered from

the intraperitoneal cavity. There was no difference between the stimulated insulin secretion from cultured islets and those recovered from the intraperitoneal site.

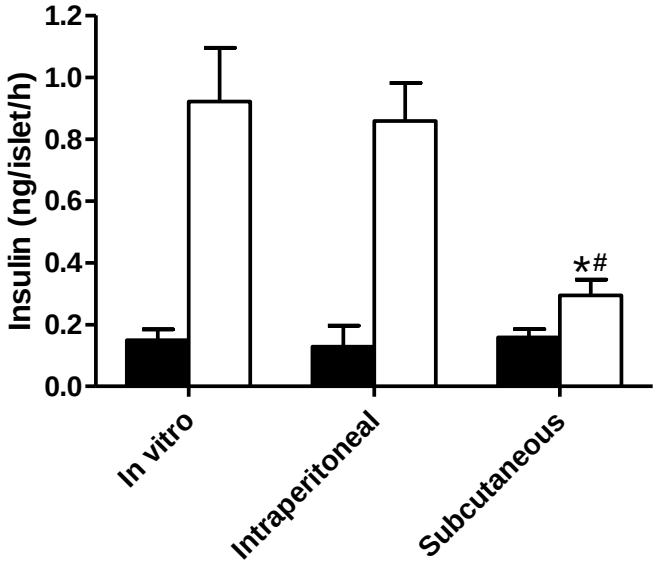


Figure 4. Insulin secretion of microencapsulated islets recovered from the intraperitoneal and subcutaneous sites.
Islets were exposed to 2 mM (black) and 20 mM (white) glucose 7 days following culture or transplantation. **P*<0.05 vs. *in vitro*, $^{\#}$*P*<0.05 vs. intraperitoneal (One way analysis of variance, *n*=4-5).

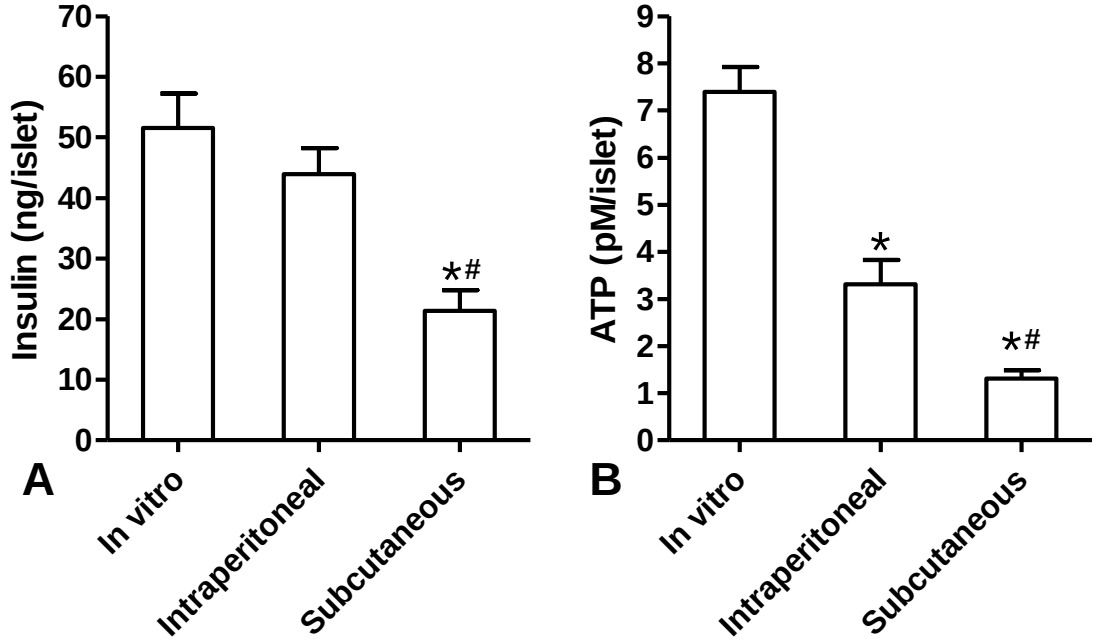


Figure 5. Insulin and adenosine triphosphate content of microencapsulated islets recovered from the intraperitoneal and subcutaneous sites.
A. Insulin content of microencapsulated islets cultured or transplanted for 7 days. **P*<0.05 vs. *in vitro*, $^{\#}$*P*<0.05 vs. intraperitoneal (One way analysis of variance, *n*=8-11). **B.** Adenosine triphosphate content of microencapsulated islets recovered after 7 days of culture or transplantation. **P*<0.05 vs. *in vitro*, $^{\#}$*P*<0.05 vs. intraperitoneal (One way analysis of variance, *n*=4-6).

3.3.2.2 Insulin and Adenosine Triphosphate Content

Islets recovered from the subcutaneous site had lower insulin content than both cultured islets and intraperitoneal graft islets (Figure 5).The intraperitoneal graft islets had lower ATP content than cultured islets, and subcutaneous graft islets had lower ATP content than both cultured islets and intraperitoneal graft islets.

3.3.3 Morphological Scoring of Microencapsulated Islets

The morphology of microencapsulated islets recovered from normoglycaemic animals after 28 days was histological evaluated by blinded scoring of islet sections (Figure 6). There was no difference between the frequencies of each score for the intraperitoneal islets. In contrast islets from the subcutaneous site had significantly fewer scores of 1 (near normal morphology) compared to scores of 3 and 4 (poor morphology), suggesting a greater frequency of cell death in islets implanted in the subcutaneous site.

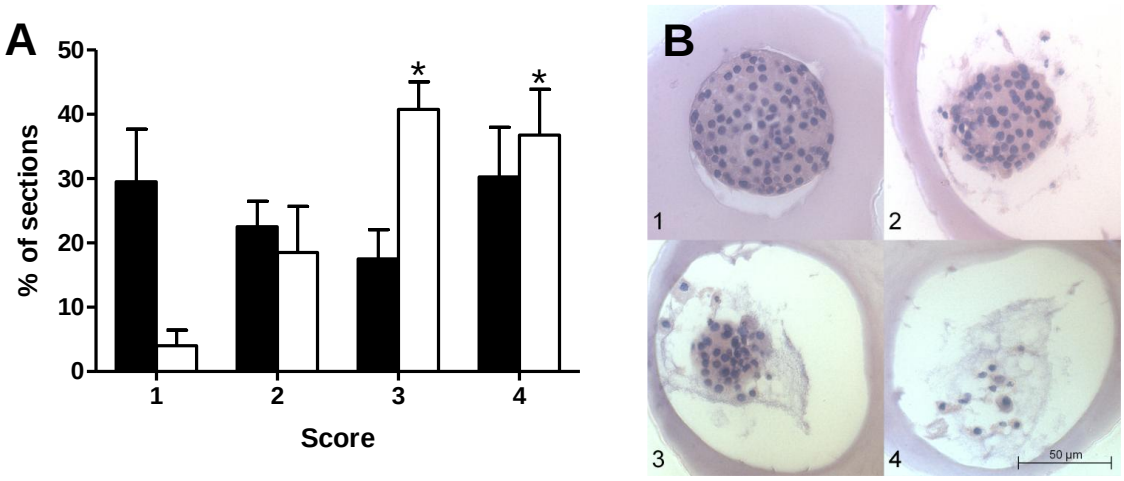


Figure 6. Histological scoring of microencapsulated islet sections recovered from intraperitoneal and subcutaneous grafts.
A. Histological scoring of sections from intraperitoneal (black) and subcutaneous (white) microencapsulated islets recovered after 28 days. Islets were scored for cell death; 0-25% (1), 25-50% (2), 50-75% (3), 75-100% (4). *P<0.05 vs. (1) (n=4). **B.** Examples of haematoxylin and eosin stained microencapsulated islet sections scored for cell death on the scale of 1-4.

3.4 Discussion

The subcutaneous site offers a number of potential advantages for clinical transplantation of encapsulated islets, including ease of implantation, sufficient capacity for the encapsulated graft material, and the opportunity to retrieve or replace the graft with ease. In the present study we used a mouse model of diabetes to compare transplantation outcomes and graft survival between the intraperitoneal and subcutaneous implantation sites.

Intraperitoneal transplantation of 700 microencapsulated islets restored normoglycaemia for up to 28 days, confirming our previous studies using the same capsule composition in which hyperglycaemia was reversed in syngeneic (Bohman et al., 2006), allogeneic, and spontaneously diabetic NOD mice (Bohman and King, 2008). In contrast, non-encapsulated

intraperitoneal grafts did not restore normoglycaemia, demonstrating that microencapsulation is advantageous at the site. Similar findings have been reported previously (Bohman and King, 2008, Korbutt et al., 2004) and it has been suggested that microencapsulation protects the graft from exposure to the mechanical stress subjected to free-floating islets in the peritoneal cavity. The presence of a high degree of mechanical stress in the intraperitoneal site is consistent with reports of a higher proportion of broken capsules compared to capsules implanted at the subcutaneous site (Dufrane et al., 2006, Thanos et al., 2007). In the present study we did not observe any broken capsules, indicating our capsules are stable upon implantation into the site. The microcapsule may also improve islet survival and function by providing a stable matrix for the islet which is more similar to that of the native islet environment than in the intraperitoneal environment. In accordance, the application of a matrix surrounding islets has previously been shown to benefit islet viability and function (Schrezenmeir et al., 1993, De Carlo et al., 2010).

There are a number of possible causes for the failure of non-encapsulated subcutaneous islet grafts including immune destruction, mechanical stress, and a poor supply of oxygen and nutrients at the site. Subcutaneous transplantation of both encapsulated and non-encapsulated islets failed to reduce the blood glucose of diabetic mice, indicating graft failure from the outset. The transplantations in the current study were syngeneic and additionally capsules recovered from mice at both sites were free from overgrowth when observed by eye, indicating a lack of immunological interference. Studies using non-encapsulated grafts at the unmodified subcutaneous site have reported graft failure in syngeneic (Fumimoto et al., 2009, Juang et al., 1996, Kawakami et al., 2000) or immune-deficient (Tatarkiewicz et al., 1999) animal models, indicating that the host immune response is not the limiting factor in graft success. Several studies have suggested that poor vasculature is the limiting factor and have demonstrated improved graft survival by co-transplanting angiogenic devices or materials (Dufrane et al., 2010, Lacy et al., 1991, Golocheikine et al., 2010), or using pre-vascularised devices (Tatarkiewicz et al., 1999, Juang et al., 1996, Pileggi et al., 2006).

The failure of microencapsulation to improve islet graft outcomes at the subcutaneous site suggests that protection from mechanical stress and provision of a stable matrix which is likely to contribute to graft success at the intraperitoneal site, is not of primary importance in the maintenance of graft function at the subcutaneous site. This is consistent with the different physical environments at the respective implantation sites, with intraperitoneal graft islets more likely to be free floating in the intraperitoneal fluid whereas subcutaneous islets are likely to be held stationary by direct contact between the skin and muscle. In accordance with

this, graft success has been improved at the subcutaneous site by encapsulating islets in macrocapsules or other devices but the improvement has generally been attributed to the biomaterial stimulating angiogenesis (Lacy et al., 1991, Tatarkiewicz et al., 1999, Juang et al., 1996) rather than by providing mechanical protection or a stable matrix. Indeed it has been demonstrated that transplantation of non-encapsulated islets to a pre-vascularised subcutaneous site without the use of any biomaterial device is sufficient to reverse the diabetic state (Kawakami et al., 2000, Kawakami et al., 2001). This indicates that neovascularisation is the predominant factor in the success of islet transplantation at the subcutaneous site, and confirms our observations that provision of mechanical protection or a stable matrix may not be as important at the subcutaneous at they are at the intraperitoneal site.

It cannot be ruled out that using a larger number of islets may have yielded better transplant outcomes at the subcutaneous site. However using a relatively large dose suitable for the intraperitoneal site we were unable to detect any impact of microencapsulation on graft efficacy. Additionally we have anecdotal evidence that implantation of 1750 microencapsulated islets also failed to induce normoglycaemia. Even if a greater number of islets were to be effective it would be difficult to justify using more islets than required for microencapsulated islet grafts implanted in the intraperitoneal cavity. One of the reasons for exploring the subcutaneous site is because the number of microencapsulated islets required at the intraperitoneal site is over double the number required for non-encapsulated grafts at sites such as under the kidney capsule. Given the donor shortage it is critical that alternate sites for islet transplantation have similar or better efficiency to current clinical protocols. However it should be possible to justify the use of a greater number of islets for immunoisolated grafts as immunosuppressant-free islet transplantation is highly desirable.

Although intraperitoneal implantation of microencapsulated islets was successful in reversing hyperglycaemia, the site is suboptimal and detrimental to islet viability. Islet ATP content of microencapsulated islets transplanted intraperitoneally was reduced by 55% from controls maintained in culture within 7 days of implantation, and 48% of the islets contained 50-100% dead cells after 28 days. However, microencapsulated islets transplanted subcutaneously showed an even more pronounced loss of viability and function. At 7 days GSIS was reduced by 68%, insulin content was reduced by 59%, and ATP content was reduced by 82% from controls maintained in culture. In addition 78% of the islets had 50-100% dead cells at 28 days. These observations suggest that relative graft success is likely to be related to the respective ability of the site to support the viability and function of the microencapsulated islets.

The low viscosity, high-G barium-alginate microcapsules used in the current study were selected due to their resistance to mechanical stress, inert properties and also on the basis of their success at the intraperitoneal site. Using these microcapsules we were unable to utilise the subcutaneous site for successful islet transplantation. A study by Veriter et al. (2010) reported that high-M calcium-alginate microencapsulated pig islets transplanted into diabetic rats were able to reverse the diabetic state when transplanted subcutaneously but not intraperitoneally. The authors did not offer suggestions of why the intraperitoneal grafts failed, however it was reported in a previous study that compared to the subcutaneous site a higher proportion of broken capsules was observed in similar high-M calcium-alginate microencapsulated islets transplanted in the peritoneal cavity (Dufrane et al., 2006). The strength of high-M calcium-alginate microcapsules may not be enough to tolerate the intraperitoneal site leading to capsule breakage. Microcapsules with the highest mechanical strength are made with alginate with >70% G content and cross-linked with Ba^{2+} (Martinsen et al., 1989).

The relative success at the subcutaneous site between the current study and the study by Veriter et al. (2010) indicates that the success of microencapsulated islets transplanted to the subcutaneous site may be dependent upon the selection of the alginate and composition of the microcapsules. Indeed implanting high-M calcium-alginate discs was reported to be more biocompatible, less permeable, and induced a greater level of neovascularisation and oxygen tension than high-G calcium-alginate discs at the subcutaneous site (Veriter et al., 2010). The authors infer the most likely cause of graft success with high-M calcium-alginate microcapsules at the subcutaneous site is enhanced induction of neovascularisation. However, a direct comparison of the graft efficacy of high-G calcium-alginate microencapsulated islets and high-M calcium-alginate microencapsulated islets transplanted subcutaneously was not reported.

Another factor impacting graft outcome at the subcutaneous site may be the source of the islet tissue. The study by Veriter et al. (2009) used pig islets xenotransplanted into rats whereas the current study used syngeneic islets transplanted into mice. It is possible that xenogeneic tissue which elicits a stronger immune reaction in the host may also provoke a stronger angiogenic response. Interestingly there is some crossover of signalling pathways between the inflammatory response and angiogenesis (Mor et al., 2004). Pancreatic insulitis has been reported to increase blood flow in islets (Carlsson et al., 1998) and inflammatory tissue is prone to induce new vessel formation in conditions such as bacterial infection (Kilcullen et al., 1998), rheumatoid arthritis (Esposito et al., 2004), and cancer (Chen et al.,

2005). However the relative capacity of syngeneic and xenogeneic islets to induce angiogenesis has not been reported.

In summary, the subcutaneous site has a number of features that make it attractive as an implantation site for encapsulated islets to treat diabetic hyperglycaemia. Our study suggests that using low viscosity, high-G barium-alginate microcapsules in an unmodified subcutaneous site is unsuitable to maintain encapsulated islet function and viability. Consequently the subcutaneous site is not a suitable alternative to the intraperitoneal site using these specific microcapsules.

4 Co-Encapsulation of Islets with an L Cell Line Improves Insulin Secretion *In Vitro*

4.1 Introduction

GLP-1 increases insulin secretion (Mojsov et al., 1987), insulin synthesis (Alarcon et al., 2006), β cell proliferation (Buteau et al., 2003) and neogenesis (Xu et al., 2006), and protects β cells against apoptosis (Li et al., 2003). Additionally GLP-1 may have anti-inflammatory and immunosuppressive functions (Cechin et al., 2011). Consequently GLP-1 therapy can be used to improve islet transplantation outcomes. Systemic delivery of GLP-1R agonists causes side effects (Kolterman et al., 2003) and cannot be administered clinically at doses high enough to mediate β cell proliferation. Consistent stimulation of β cell proliferation *in vivo* would greatly enhance graft sustainability. Due to the higher doses required for full utilisation of GLP-1, delivery localised close to the islets may be preferable. High doses delivered locally to the islets may avoid GLP-1 inactivation in the circulation and also provide GLP-1-mediated benefits which require high concentrations such as stimulation of β cell proliferation. With localised delivery this may be achievable without accumulating systemic GLP-1 levels at sufficiently high concentrations to cause side effects. Islet localised delivery of GLP-1 can be achieved by ectopic expression, however due to safety concerns and limited expression longevity this approach is unlikely to be developed in the clinic. GLP-1 protein can also be co-encapsulated with islets however as GLP-1 will readily diffuse out of conventional biomaterials it must be altered in order to immobilise it for sustained release. Modification of proteins can result in unforeseen functional variation which may compromise the therapeutic efficacy or safety of GLP-1. Additionally, the longevity of GLP-1 action with protein delivery is ultimately limited by the amount of protein incorporated to begin with. It has been shown that GLP-1R agonist treatment is beneficial to islet function in culture (King et al., 2005) and when initiated immediately post-transplantation. It has also been shown that discontinuation of treatment can reverse the beneficial effect on grafts (Merani et al., 2008). Consequently a continuous supply of GLP-1R agonist throughout culture and the post-transplantation period may offer the most benefit.

As an alternative to systemic delivery, localised ectopic expression or localised protein delivery of GLP-1, a cell-based delivery system could utilise GLP-1 secreting L cells co-encapsulated with islets. Cellular delivery of GLP-1 would provide unmodified GLP-1 continuously within the device for the lifetime of the L cells. Additionally L cells in transplanted devices would be

responsive to physiological cues such as blood glucose levels, therefore providing GLP-1 under physiologically appropriate circumstances, and hence reducing the risk of overstimulation. Protein incorporation into encapsulation materials can alter the properties of the material and may result in altered encapsulation device performance *in vivo*. Conventional islet encapsulation approaches such as alginate microencapsulation are designed for cell encapsulation. Therefore incorporation of L cells is unlikely to compromise the function of such immunoisolation devices. Co-encapsulation of L cells with islets is an attractive concept. The potential benefits of co-encapsulation of islets and L cells in alginate microcapsules for islet transplantation have been investigated using an L cell line as an experimental model.

4.2 Materials and Methods

4.2.1 Animals

Male ICR mice aged 6-8 weeks were used to isolate islets for *in vitro* experiments. Male C57BL/6J mice aged 6-8 weeks were used as islet donors and STZ diabetic male C57BL/6J mice weighing 25g (8-10 weeks) were used as recipients.

4.2.2 L Cell Culture and Seeding

GLUTag and STC-1 cells were cultured in fully supplemented low glucose (5.6 mM) DMEM. L cell co-encapsulated islets were cultured in fully supplemented RPMI islet culture medium (11.1 mM). Matrigel (BD) was used as an attachment factor for seeding L cells in experiments to prevent the loss of cells due to washing during experiments. Matrigel was defrosted for 16 h on ice at 4°C and then diluted 1/100 in serum-free RPMI. 200 µl of diluted matrigel was added to wells of a 24-well plate (Nunc) and the plate was incubated at 37°C for >30 min to solidify the matrigel coating. Prior to seeding the L cells, the residual diluted matrigel was removed by aspiration, and the L cells were strained and counted. The desired number of cells was then added to the matrigel coated wells of a 24-well plate. The volume was made up to 1 mL per well in low glucose RPMI and the plate was returned to the incubator to allow the cells to attach for >16 h before starting co-culture experiments.

4.2.3 L Cell Secretion

The medium of the seeded L cells was removed and the cells were washed with 1 mL glucose-free 138 buffer (138 mM NaCl, 4.5 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_26H_2O$, 10 mM HEPES, 1 $mg.mL^{-1}$ BSA, Sigma). The test substrates were added in 250 µl 138 buffer and the plate was returned to the incubator for 2 h. Following secretion the

plate was put on ice, 750 µl of ice cold glucose-free 138 buffer was added to each well and the samples were transferred from the wells into 1.5 mL tubes on ice. Samples were centrifuged at 300g for 5 min at 4°C and the supernatant was collected, snap frozen on dry ice, and stored at -80°C. GLP-1 secretion was measured using an ELISA.

4.2.4 L Cell Conditioned Buffer and Co-Culture

The medium of the seeded L cells was removed, the cells were washed with 1 mL glucose-free 138 buffer. 200 µl of 11 mM glucose 138 buffer was added to each well, and the plate was returned to the incubator for 2 h. For L cell conditioned buffer experiments the buffer was transferred to wells containing 10 microencapsulated islets in 50 µl of 11 mM glucose 138 buffer and the plate was returned to the incubator for 1 h. For co-culture experiments after L cell incubation 10 microencapsulated islets were added to each well in 50 µl of 11 mM glucose 138 buffer and the plate was returned to the incubator for 1 h. Following islet incubation the plate was put on ice, 750 µl of ice cold glucose-free 138 buffer was added to each well and the samples were transferred from the wells into 1.5 mL tubes on ice. Samples were centrifuged at 300g for 5 min at 4°C and the supernatant was collected and stored at -20°C. Insulin secretion was measured using a RIA.

4.2.5 L cell and Islet Co-Encapsulation

L cells were encapsulated with islets with $8x10^5$ or $4x10^6$ cells per mL of alginate to yield a density of 1 islet and approximately 50-100 or 400-500 L cells per capsule respectively as estimated by DNA measurements. Following culture overnight islet containing microcapsules were either picked and cultured or picked and transplanted. Capsules were assessed with the GSIS assay and insulin secretion and content were measured with a RIA. Some microcapsules were stained with live/dead (FDA/PI) staining and visualised using a confocal microscope.

4.2.6 Transplantation

Islets isolated from several animals were pooled and half were used for islet alone encapsulation and half for islet and L cell co-encapsulation. A minimal mass of 250 capsules in serum-free RPMI medium were delivered into the peritoneal cavity using an endotoxin-free, sterile pipette. Mice were considered cured when the average of 4 consecutive blood glucose measurements was ≤11.1 mM and if the average of the final 4 consecutive measurements was ≤11.1 mM.

4.2.7 Post-Transplantation Studies

The mice were killed by cervical dislocation at the study end-point and the capsules were recovered. Insulin secretion and insulin content samples were measured from groups of 10 encapsulated islets explanted from each mouse or picked from each culture dish. Some microcapsules were stained with FDA then viewed under a fluorescence microscope.

4.3 Results

4.3.1 Co-Encapsulation and Secretion of L Cells

4.3.1.1 Microencapsulation of L Cells

The compatibility of L cells in an alginate microencapsulated environment was assessed by measuring the DNA content of L cells in microcapsules over time. Microencapsulation of $4x10^6$ cells per mL of alginate resulted in approximately 400-500 cells per capsule. Both STC-1 and GLUTag cells survived the microencapsulation process and grew within capsules, as demonstrated by increasing DNA content which reached a plateau at day 7 and 3 for STC-1 and GLUTag encapsulated cells respectively (Figure 7). Accordingly, encapsulated cells on day 8 had larger individual points of FDA fluorescent staining compared to day 1 confirming cell growth. Co-encapsulated islets and L cells had strong FDA staining in both islets and cells. Microencapsulated L cells cultured long term (>3 weeks) were observed with cells growing on the surface of capsules and even causing capsule breakage at 6-8 weeks.

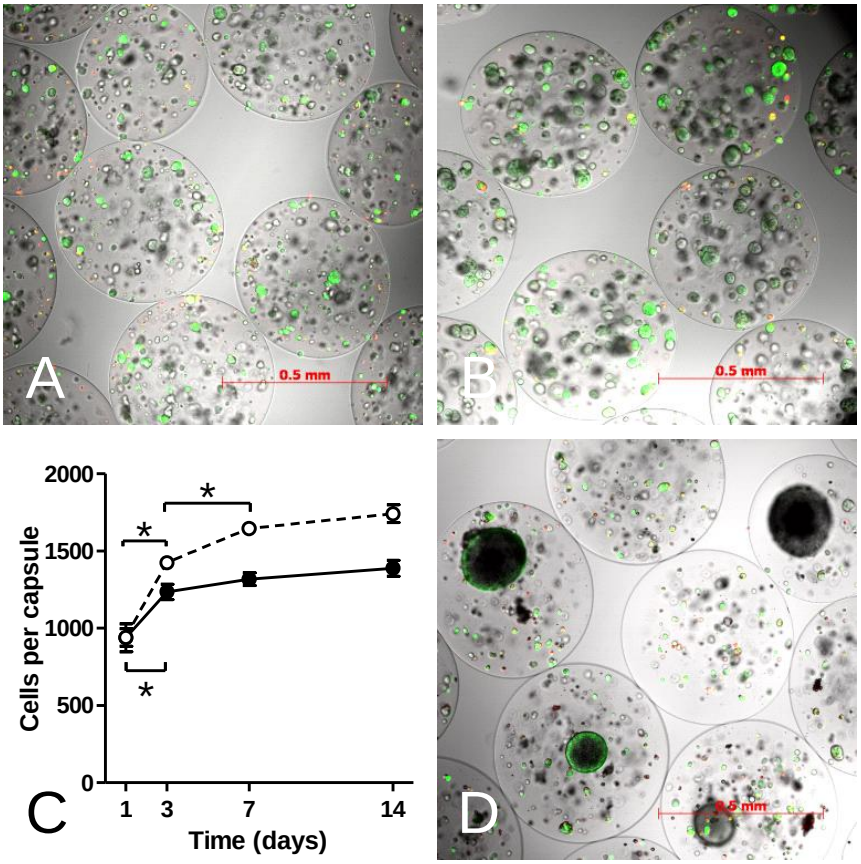


Figure 7. Microencapsulated L cell growth and islet co-encapsulation.
Representative confocal microscope images of microencapsulated STC-1 cells with live/dead staining post-encapsulation on: **A.** day 1 **B.** day 8. **C.** Growth curve of STC-1 (white circles, dotted line) and GLUTag (black circles, solid line) microencapsulated with $4x10^6$ cells per mL of alginate. *P<0.05 vs. previous data point (Two way repeated measures analysis of variance, n=3(1)). STC-1 and GLUTag growth curves were significantly different (P<0.001). **D.** Representative confocal microscope image of co-encapsulated mouse islets with $4x10^6$ GLUTag cells per mL of alginate with live/dead staining on day 1.

4.3.1.2 Glucagon-Like Peptide-1 Secretion from L Cells

The L cell lines were tested for GLP-1 secretion in the presence of glucose, FBS and diprotin A (DPA). Glucose was used to stimulate insulin secretion and DPA was used to inhibit any potential DPP-IV activity in FBS. Both L cell lines secreted basal GLP-1 which could be increased by stimulation with glucose (Figure 8). GLUTag cells had a higher level of glucose-stimulated GLP-1 secretion than STC-1 cells. Supplementation with FBS did not reduce detectable GLP-1 in GLUTag cells but did in STC-1 cells. The reduction observed in the STC-1 cells could be recovered with DPA treatment. Treatment with DPA alone did not alter detectable GLP-1 from glucose stimulated levels in either cell line.

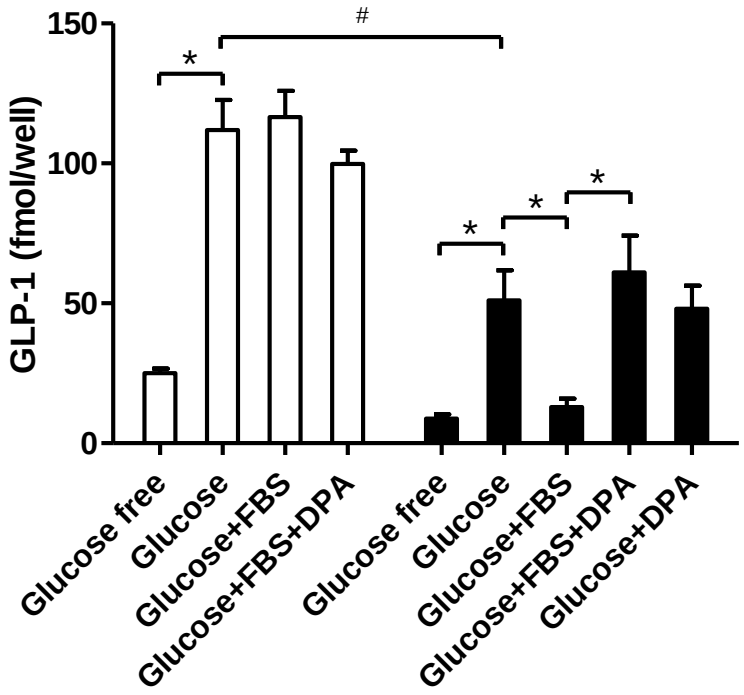


Figure 8. Glucagon-like peptide-1 secretion from L cells.
GLP-1 secretion of $1x10^6$ GLUTag (white) or STC-1 (black) cells per well in 138 buffer with bovine serum albumin in the absence of glucose or supplemented where stated with glucose (11 mM), 10% foetal bovine serum or 100 µM diprotin A. **P*<0.05 (One way analysis of variance on ranks, *n*=6-17(1-3), $^{\#}P$=0.001 (Mann-Whitney Rank Sum Test, *n*=14-17(3))

4.3.2 Co-Culture and Co-Encapsulation of L cells

4.3.2.1 Conditioned Buffer and Co-Culture of L Cells

Microencapsulated islets were co-cultured with L cells seeded in the wells of culture plates to assess the impact of L cell co-culture on islet insulin secretion. $2x10^5$ GLUTag or STC-1 cells per well conditioned buffer or co-culture with $2x10^5$ or $1x10^6$ GLUTag or STC-1 cells per well did not impact insulin secretion in microencapsulated islets (Figure 9). After L cell incubation the glucose in the buffer was measured with a blood glucose meter. The starting concentration was 11 mM glucose and the final concentration was 10.9±0.08 mM (*n*=7).

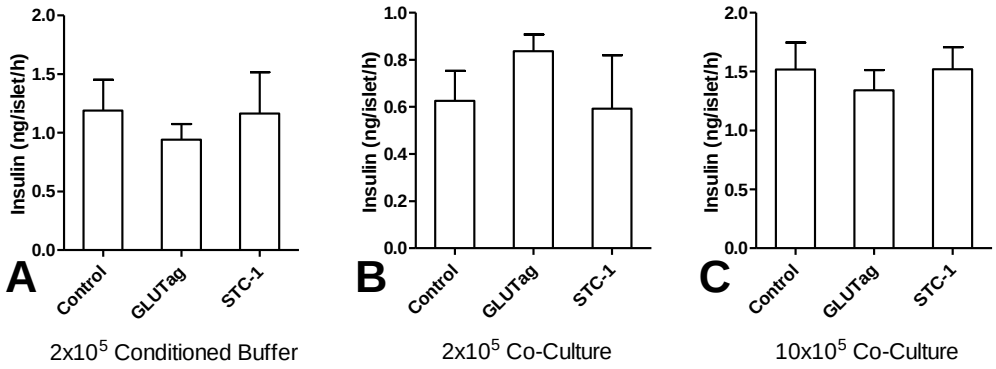


Figure 9. Microencapsulated islet insulin secretion with L cell conditioned buffer and L cell co-culture.
Microencapsulated islets were exposed to: **A.** Conditioned buffer from empty wells (Control) or wells with $2x10^5$ GLUTag or STC-1 cells. *P*=0.774 (One way analysis of variance, *n*=6(1)) **B.** Co-culture with empty wells (Control) or wells with $2x10^5$ GLUTag or STC-1 cells. *P*=0.523 (One way analysis of variance, *n*=6(1)) **C.** Co-culture with empty wells (Control) or wells with $1x10^6$ GLUTag or STC-1 cells. *P*=0.771 (One way analysis of variance, *n*=12(2)).

4.3.2.2 Inhibition of L cell Derived Glucagon-Like Peptide-1 with Exendin-9

To investigate a potential mechanism for the lack of stimulatory effect of GLP-1 from GLUTag cells on the insulin secretion of islets, we used GLP-1R agonist exendin-4 and GLP-1R antagonist exendin-9. Microencapsulated islets respond to exendin-4 by increasing insulin secretion (Figure 10). Exendin-9 in combination with exendin-4 resulted in reduced stimulation compared to exendin-4 alone. Exendin-9 alone treatment resulted in reduced insulin secretion compared to glucose alone treatment. Insulin secretion was not increased compared to exendin-9 alone treated by co-culture with GLUTag cells.

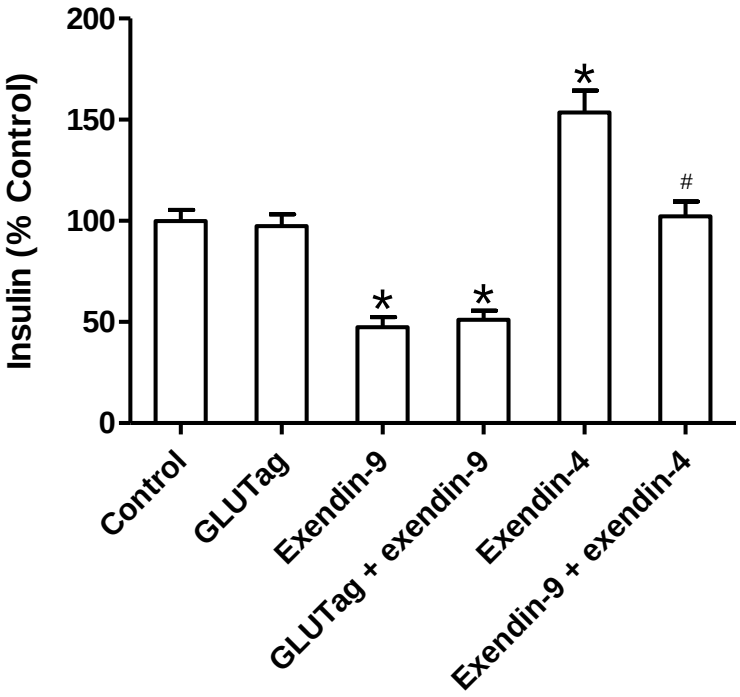


Figure 10. Insulin secretion of microencapsulated islets in response to GLUTag cells, exendin-4 and exendin-9.
All treatments were in 138 buffer supplemented with 11 mM glucose and where indicated $1x10^6$ GLUTag cells per well, 10 nM exendin-4, and 100 nM exendin-9. **P*<0.05 vs. Control, $^{\#}P$<0.05 vs. Exendin-4 (One way analysis of variance, *n*=11-22(3-4))

4.3.2.3 Co-Encapsulation of Islets with GLUTag Cells

Islets were co-encapsulated with GLUTag cells to determine the impact on insulin secretion and insulin content. 3 days after encapsulation, glucose stimulated insulin secretion was increased from controls in islets that were co-encapsulated with $0.8x10^6$ GLUTag cells per mL of alginate (Figure 11). Islets co-encapsulated with $4x10^6$ GLUTag cells per mL of alginate had higher basal insulin secretion at day 3 and lower glucose stimulated insulin secretion at day 14 than controls. Glucose stimulated insulin secretion was increased at day 14 compared to day 3 in both islets encapsulated alone (*P*<0.001, ANOVA on ranks, *n*=19) and islets co-encapsulated with $0.8x10^6$ GLUTag cells (*P*<0.001, ANOVA, *n*=15). At day 3, insulin content was lower in islets co-encapsulated with $4x10^6$ GLUTag cells per mL of alginate compared with islets microencapsulated alone. This difference was not evident by 14 days after microencapsulation.

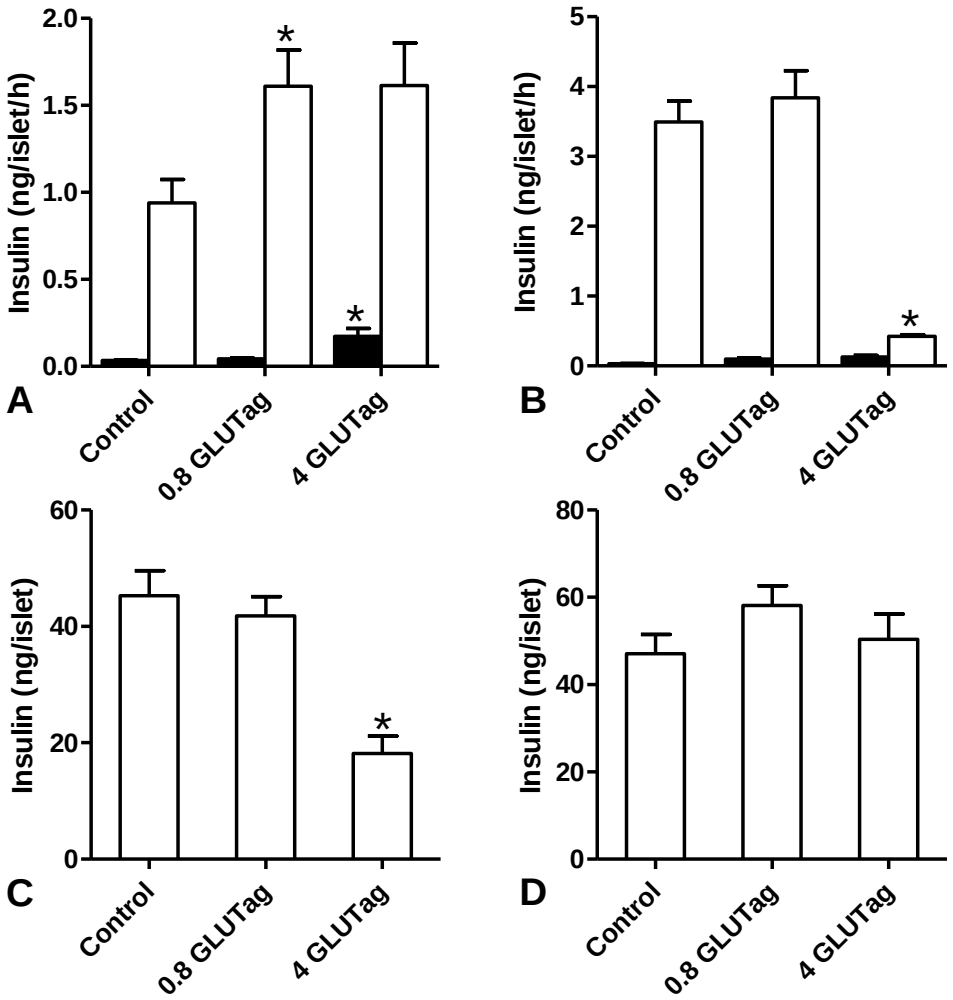


Figure 11. Insulin secretion and insulin content of co-encapsulated islets and GLUTag cells. Islets were microencapsulated alone (Control, *n*=19(4)) or co-encapsulated with $8x10^5$ (0.8 GLUTag, *n*=15(3)), or $4x10^6$ (4 GLUTag, *n*=9(2)) GLUTag cells per mL of alginate. Microencapsulated islets were exposed to 2 mM (black) and 20 mM (white) glucose at: **A.** 3 days **B.** 14 days post-encapsulation. Insulin content was measured at: **C.** day 3 and **D.** day 14 post-encapsulation. **P*<0.05 vs. Control (One way analysis of variance on ranks, *n*=9-19(2-4)).

4.3.3 Transplantation of Islets Co-Encapsulated With GLUTag Cells

4.3.3.1 Pilot Study of Co-Encapsulated Islets and GLUTag Cells Transplanted into Diabetic Mice

The impact of incorporation of GLUTag cells into microcapsules with islets for transplantation into diabetic mice was assessed in a pilot study (n=2). A minimal mass of 250 islets were encapsulated alone or with GLUTag cells into the peritoneal cavity of diabetic mice (Figure 12). Both recipients of GLUTag co-encapsulated islet grafts and one recipient with islets alone cured at week 2 and maintained normoglycaemia for 8 weeks. The stimulated insulin secretion from recovered capsules at 8 weeks was higher in GLUTag co-encapsulated islets than islet alone encapsulated islets. Islets but not GLUTag cells stained positively for FDA in recovered capsules from the GLUTag co-encapsulated group at 8 weeks.

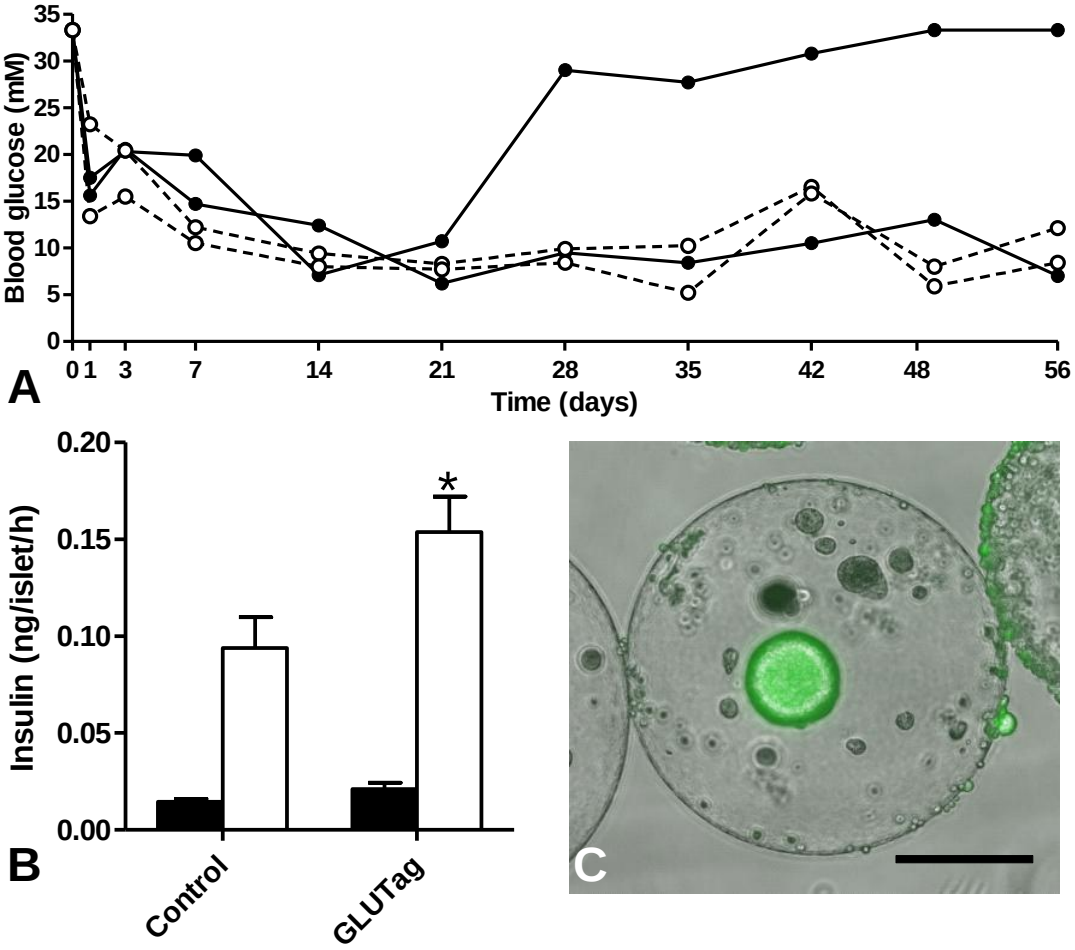


Figure 12. Pilot study of co-encapsulated islets and GLUTag cells transplanted into diabetic mice.

Diabetic mice were implanted with syngeneic intraperitoneal grafts of 250 encapsulated islets alone or 250 islets co-encapsulated with $8x10^5$ GLUTag cells per mL of alginate. **A.** Blood glucose measurements of individual mice with microencapsulated islet alone grafts (black circles, solid line) or GLUTag co-encapsulated islet grafts (white circles, dotted line). **B.** Insulin secretion of recovered microcapsules from microencapsulated islet alone grafts (Control) or GLUTag co-encapsulated islet grafts (GLUTag) exposed to 2 mM (black) and 20 mM (white) glucose. **P*=0.030 (One way analysis of variance, *n*=12(2)). **C.** Fluorescein diacetate stained microscope images of GLUTag co-encapsulated islet recovered at 8 weeks (200 μm scale bar).

4.3.3.2 Expanded Study of Co-Encapsulated Islets and GLUTag Cells Transplanted into Diabetic Mice

The pilot study of GLUTag cell co-encapsulated islet transplantation into diabetic mice was expanded. Overall, recipients in both the islet encapsulated alone and the GLUTag co-encapsulated islet groups did not cure and resulted in graft failure by 4 weeks (Figure 13). The average blood glucose of the recipients was no different overall between groups but the average blood glucose was lower in the GLUTag co-encapsulated islet recipient group at 2 time points. There was a non-significant trend for more rapid graft failure in the islet encapsulated alone group.

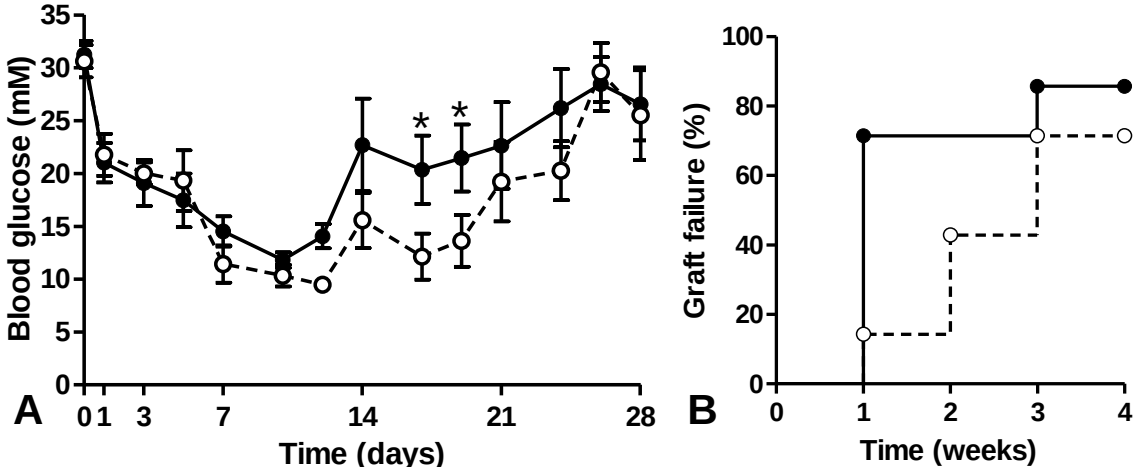


Figure 13. Graft outcome of diabetic mice transplanted with islets co-encapsulated with GLUTag cells.
Diabetic mice were implanted with syngeneic intraperitoneal grafts of 250 encapsulated islets alone (black circles, solid line, *n*=7) or 250 islets co-encapsulated with $8x10^5$ GLUTag cells per mL of alginate (white circles, dotted line, *n*=7). **A.** Average blood glucose measurements.**P*<0.05 vs. islet alone group (Two way repeated measures analysis of variance, multiple comparisons versus control group (Holm-Sidak method)). **B.** Percentage of cured mice. *P*>0.05 (Kaplan-Meier survival analysis (Log-rank).

4.4 Discussion

GLP-1 has multiple beneficial effects on β cells including stimulating glucose-dependent insulin secretion, proliferation, islet neogenesis and protecting β cells against apoptosis. It was hypothesised that incorporating GLP-1 secreting L cells with islets into microcapsules would provide the islet with a constant, physiologically regulated source of GLP-1, thereby improving islet survival, function and graft outcome in models of transplantation for T1DM. It was established that the L cell lines STC-1 and GLUTag were tolerant to the microencapsulation process and proliferated in microcapsules. Long term survival of L-cells and therefore secretion of GLP-1 in microcapsules is desirable as local delivery of GLP-1 could potentially provide an advantage in culture and post-transplantation for the lifetime of the graft. It was also observed that after 1 or 2 weeks the cell number of encapsulated L cells reached a plateau, suggesting the capacity of the capsule had been reached. However, eventually cells were observed on the outside of capsules and broken capsules were observed suggesting the L cells were able to overcome the capacity if the capsules. In a transplantation setting this would be disadvantageous as protruding cells would cause an inflammatory reaction and broken capsules would negate the immunoprotective capacity of the microcapsules. Additionally, aggressive growth of L cells co-encapsulated with islets would use oxygen and nutrient resources, resulting in islet starvation. The aggressive growth observed in microcapsules after several weeks is likely to be because the cell lines used in this study are transformed transgenic

mouse tumour cell lines which are also observed to grow rapidly in conventional adherent culture. L cell lines would never be used in a clinical setting however they do provide a convenient model for L cells in the current study. Encapsulated GLUTag cells reached a growth plateau at a lower density than STC-1 cells which suggests they may have less aggressive growth and therefore may be more suitable for proof of principle co-encapsulation and transplantation studies.

The secretion of GLP-1 from the L cell lines could be stimulated by glucose, with a higher level secreted from GLUTag cells than STC-1 cells. Interestingly the cell lines responded differently to FBS, with GLUTag cell GLP-1 secretion remaining unchanged and STC-1 cell GLP-1 secretion being reduced. FBS was included as it is used under standard culture conditions and is necessary for longer term studies. However FBS can contain DPP-IV which inactivates GLP-1, therefore the impact of FBS in the GLP-1 secretion studies was of interest. In the STC-1 line, FBS decreased glucose stimulated GLP-1 secretion, indicating that DPP-IV may have been present in the FBS. Accordingly, addition of the DPP-IV inhibitor DPA was able to reverse the effect. The same effect was not seen in the GLUTag cell line, with FBS not affecting GLP-1 secretion. It should be noted that this experiment was not carried out simultaneously with the STC-1 FBS experiment. Thus it is currently not possible to conclude whether the differences seen between the two cells lines are due to their respective responses to the contents of FBS, or whether the DPP-IV content of the FBS differed sufficiently between the two experiments to yield these disparate results.

The secreted factors from L cells were transferred onto microencapsulated islets in conditioned buffer experiments and microencapsulated islets were co-cultured with L cells. The conditions were similar to the conditions for the GLP-1 secretion studies which allowed the theoretical amount of GLP-1 being produced to be estimated. Stimulation of insulin secretion from microencapsulated islets in the L cell conditions was not detected in any of the experiments. The highest number of cells used for co-culture was $1x10^6$ GLUTag cells which resulted in a highly confluent well surface. This number of cells should produce GLP-1 in the range of 400-500 pM, and it has been shown that as little as 100 pM GLP-1 in 10 mM glucose media can stimulate approximately a 40% increase in insulin secretion in islets (Goke et al., 1993). Therefore it is surprising that co-culture of islets with GLUTag cells did not stimulate insulin secretion. As microencapsulated islets were used, the ability of GLP-1 to diffuse through the cross-linked alginate should be considered. GLP-1 has a molecular weight of 3.2 kDa and exendin-4 has a molecular weight of 4.3 kDa. We have shown that exendin-4 is able to stimulate the insulin secretion of microencapsulated islets, therefore it is likely that GLP-1

should readily diffuse through alginate. Resource limitation may be a factor preventing stimulation of insulin secretion in the experiments. It is possible that although the secreted GLP-1 does have a positive effect on islet insulin secretion the L cells have used the oxygen and nutrients in the buffer which acts to limit insulin secretion. The glucose concentration did not change due to L cell culture, therefore ruling out glucose as a limiting factor. Additionally, the lower cell density co-culture condition did not yield better results. Another possibility is that the L cells also secrete a factor which has negative implications on insulin secretion. Peptide-YY is secreted from L cells including STC-1 cells (Geraedts et al., 2009, Hand et al., 2012) and has been shown to inhibit the insulinotropic action of GIP and also directly inhibit insulin secretion in islets (Bertrand et al., 1992). STC-1 cells are reported to secrete approximately 1.6 fmol PYY per $1x10^6$ cells (Hand et al., 2012) which would equate to around 150 pM in our conditions. It has been shown that 100 pM PYY can inhibit insulin secretion in the perfused rat pancreas (Bertrand et al., 1992), therefore PYY may be an inhibitory factor on insulin secretion in our experiments. Another possibility is that the islets are secreting a factor which inhibits GLP-1 secretion from the L cells. There is some evidence that somatostatin inhibits GLP-1 secretion from L cells (Brubaker, 1991). This scenario seems unlikely as the conditioned buffer experiments would not be affected and in the co-culture experiments at the point the encapsulated islets are transferred the L cells have already produced stimulatory levels of GLP-1.

To investigate the hypothesis that GLUTag cell derived GLP-1 was stimulating insulin secretion but another factor or factors were cancelling out the effect, studies were carried out with the GLP-1 antagonist exendin-9. Exendin-9 alone inhibited insulin secretion but the effect could be cancelled out with exendin-4. It has been reported that GLP-1 is locally produced in α cells in adult islets which is likely to cause basal GLP-1R stimulation (Masur et al., 2005). Exendin-9 therefore can inhibit basal insulin secretion but exendin-4 counteracts the effect. A similar effect was not observed with L cell produced GLP-1 which suggests either that it does not stimulate the GLP-1R or the concentration is too low to counteract the effect of exendin-9. If GLUTag-derived GLP-1 does not stimulate the GLP-1R then it is possible the gene is mutated which is can occur in a long-lived transformed cell lines. In opposition to this theory the ELISA could detect active GLP-1 which suggests at least the active site is not mutated. Another possibility is that GLP-1 does stimulate the GLP-1R but another factor secreted from the L cells prevents downstream signalling.

Despite the poor response of islets to L cells in acute studies it was investigated if co-encapsulation could be beneficial. Co-encapsulation locates the L cells in closer proximity to

the islet which may result in a higher islet localised GLP-1 concentration. It also allows for convenient long term co-culture in comparison to adherent cell culture where cells must be sub-cultured every few days and the islets are at risk of direct overgrowth by the L cells. It was found that with islets co-encapsulated with $0.8x10^6$ GLUTag cells per mL of alginate had increased insulin secretion at day 3 compared to islets encapsulated alone. It is possible that the closer proximity of the L cells to the islets and the longer time period compared to the co-culture experiments resulted in the difference in outcome. Another possibility is that the microencapsulated environment stimulates the L cells to produce more GLP-1. There was also a trend increase in GSIS with islets co-encapsulated with $4x10^6$ GLUTag cells per mL of alginate at 3 days. However basal insulin secretion was also increased and insulin content was decreased suggesting aberrant insulin secretion. By day 14 the insulin secretion of $0.8x10^6$ GLUTag cells per mL of alginate capsules was not different to controls. Both control and $0.8x10^6$ GLUTag cells per mL of alginate co-encapsulated islets had increased insulin secretion compared to day 3 without a decline in insulin content. There are reports demonstrating approximately a 25% increase in the GSIS of microencapsulated islets after a period of culture (King et al., 1999). However a >300% increase was observed in control microencapsulated islets in the current study, but it is not clear by what mechanism. At 14 days the GSIS of $4x10^6$ cells per mL of alginate co-encapsulated islets were poor. This is likely to be related to the expansion of GLUTag cells in the capsules using resources to the detriment of the islets.

The enhancement of insulin secretion mediated by the incorporation of GLUTag cells with islets into microcapsules suggested that the approach may improve graft efficacy in a diabetic transplantation model. A pilot study was established to determine the strategy for a larger study using 250 microencapsulated islets as a minimal mass model. It was found that GLUTag co-encapsulated islets showed a trend for improved graft efficacy for up to 8 weeks and enhanced GSIS in recovered capsules compared to islets encapsulated alone. Interestingly the L cells in explanted capsules were not alive after 8 weeks *in vivo* which suggests the benefit of the L cells could be during culture and the early transplantation period. The data from the pilot study suggested the model was appropriately stringent and a benefit could be observed by co-encapsulating with L cells. As a result the pilot was expanded, however, the same trends were not repeated in the blood glucose measurements and there was no overall difference between GLUTag co-encapsulated group and the islet alone group. The time to graft failure showed a trend for faster failure in the islet alone group but was not significantly different. The *in vitro* insulin secretion, blood glucose data of the pilot study and differences in blood glucose at 2 time points in the full study suggest that GLUTag cells could provide some benefit when co-

encapsulated with islets. In that case the minimal mass model may have been too stringent and the data from pilot study was not representative of the model.

Co-encapsulation of L cells with islets improved islet insulin secretion but the mechanism is unclear. Using a stringent minimal mass model L cells were unable to improve microencapsulated graft outcome. If it were apparent that L cells did improve microencapsulated islet transplantation there would be major hurdles limiting the development of such an approach for clinical islet transplantation. Isolation of purified primary L cells is not currently possible and there are few descriptions of primary human intestinal epithelial cell isolation (Beaulieu and Menard, 2012). L cells are a rare cell type in primary human intestinal epithelial cell populations and therefore some form of cell sorting would have to be optimised to get a good purity of L cells. Exploiting the phenotype of naturally occurring cells presents a far more complicated system then conventional drug delivery due to the number of different factors which are secreted from a single cell type and the dynamic secretion responses to environmental cues. L cells were used in the current study as a vehicle for localised delivery of a single molecule of interest, GLP-1. Conceptually, localising L cells with islets should provide GLP-1 mediated benefits to islet survival and function. However attempting to demonstrate the concept has resulted in some unexpected results which probably represents the complexity of the interaction between the assorted L cell secretions and islet responses.

5 Co-Encapsulation of Islets with Mesenchymal Stem Cells Improves Graft Outcome in Diabetic Mice

5.1 Introduction

Recently there have been several studies reporting the benefit of co-transplanting MSCs with non-encapsulated islets to improve graft efficacy in animal models (Figliuzzi et al., 2009, Ito et al., 2010, Sordi et al., 2010b, Rackham et al., 2011, Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Berman et al., 2010). MSCs are pluripotent cells located in the perivascular niche of the majority of vascularised organs (da Silva Meirelles et al., 2006) which can differentiate into mesenchymal cell types. Native MSCs promote tissue repair by simultaneously secreting trophic factors and inhibiting inflammatory and immune responses (Xu et al., 2008). Accordingly the beneficial effects of MSCs in islet co-transplantation have been attributed to improving graft revascularization (Figliuzzi et al., 2009, Ito et al., 2010, Sordi et al., 2010b, Rackham et al., 2011) and suppressing immune or inflammatory responses (Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Berman et al., 2010). Recently it has been suggested that MSCs can also improve graft outcome by maintaining islet organization and morphology (Rackham et al., 2011).

Immunoisolation of the islets by microencapsulation in alginate offers the prospect of restoring physiological glycaemic control without the need for immunosuppression. Microencapsulation prevents the passage of cells in or out of the capsule which protects the islet from immune rejection but also prevents any direct cellular contact with the host or other islets in the graft. Microencapsulation completely prevents islet revascularisation and therefore can function as a tool to study its role in graft success (Bohman et al., 2006). Additionally, microencapsulation prevents islets from aggregating and therefore can also be used to study the role of islet morphology preservation on graft success. Microencapsulation therefore offers an isolated-graft model of islet transplantation which excludes some of the factors impacting non-encapsulated islet transplantation. Microencapsulated islet grafts also have the advantage of yielding greater insight into the mechanisms of graft success as it is possible to retrieve individual encapsulated islets for functional and morphological assessment.

Microencapsulated islet transplantation is suboptimal with at least double the number of islets necessary to cure diabetic animals compared to non-encapsulated islet grafts (Siebers et al.,

1993). In the current study we investigated whether co-encapsulation with MSCs could improve microencapsulated islet graft efficiency. We also used microencapsulation as an isolated-graft model of islet transplantation with the aim of determining whether the effects of MSCs are sustained in the absence of MSC-mediated enhancement of revascularization and preservation of islet morphology.

5.2 Methods

5.2.1 Animals

Male ICR aged 6-8 weeks were used to isolate islets for *in vitro* experiments. Male C57BL/6J mice aged 6-8 weeks were used as islet donors and STZ diabetic male C57BL/6J mice weighing 25g (8-10 weeks) were used as recipients.

5.2.2 Mesenchymal Stem Cells

I am grateful to Pedro Chagastelles (Universida de Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil) and Chloe Rackham (King's College London) for isolating and characterising the MSCs. A brief description of the isolation and characterisation methods are detailed below.

5.2.2.1 Mesenchymal Stem Cell Isolation

Kidney-derived MSCs were isolated from C57BL/6J mice, immunophenotyped for MSC markers and validated for pluripotency as previously described (Rackham et al., 2011). Briefly, the kidneys were cut into small pieces and digested for 30-45 min at 37°C with 1 $mg.mL^{-1}$ collagenase type 1 in calcium and magnesium free Hank's balanced salt solution (HBSS, PAA Laboratories, Pasching, Austria) with 10 mM HEPES. Following trituration the cells were centrifuged for 10 min at 400 g, resuspended in DMEM (10% FBS, 100 $units.mL^{-1}$ penicillin, 100 $ng.mL^{-1}$ streptomycin) and cultured at 37 °C, 5% CO_2. Non-adherent cells were removed during 4-6 passages and once an aesthetically uniform population of cells emerged the cells were characterised.

5.2.2.2 Mesenchymal Stem Cell Immunophenotyping

The MSCs were assessed for the presence or absence of specific cell surface markers which indicate a mesenchymal stem cell phenotype. MSCs were washed in PBS and then incubated for 30 min with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for CD11b, CD31, CD44, CD45, CD73, CD90.2 and stem cell antigen-1 (BD Pharmingen, San Diego, CA, USA). Cells were analysed for antibody binding in a fluorescence activated cell sorter (BD Pharmingen). Cells were negative for CD11b, CD31 and CD45 which are markers for

macrophages, endothelial cells, and haematopoietic cells respectively, and positive for CD44, stem cell antigen-1, CD73, CD90.2 which are characteristic of MSCs.

5.2.2.3 Mesenchymal Stem Cell Differentiation

To confirm a pluripotent phenotype, MSCs were cultured in an induction medium of DMEM containing 20% FBS, 2.5 $\mu g.mL^{-1}$ insulin, 100 µM indomethacin, 5 µM rosiglitazone and 10 nM dexamethasone to induce adipocyte formation. MSCs were cultured in an induction medium of DMEM containing 10% FBS, 10 mM β-glycerophosphate, 5 $\mu g.mL^{-1}$ ascorbic acid and 10 nM dexamethasone to induce osteocyte formation. The cells were able to differentiate *in vitro* into both adipocyte- and osteoblast-like cells as determined by Oil Red O and Alizarin Red S staining respectively.

5.2.2.4 Mesenchymal Stem Cell Culture

MSCs were cultured in 5.6 mM glucose DMEM supplemented with 10% FBS, 100 $units.mL^{-1}$ penicillin, 100 $ng.mL^{-1}$ streptomycin, and 20 mM L-glutamine. Cells were subcultured every 3-4 days and MSCs up to passage 12 were used for experiments.

5.2.2.5 Islet and Mesenchymal Stem Cell Co-encapsulation

MSCs were encapsulated with $8x10^5$ or $4x10^6$ cells per mL of alginate to yield a density of approximately 50-100 or 400-500 MSCs per capsule respectively as estimated by DNA content measurements, and 1 islet per capsule.

5.2.3 *In vitro* Assessment of Islets Co-Encapsulated with Mesenchymal Stem Cells

Insulin secretion and insulin content samples were from groups of 10 encapsulated islets picked from each culture dish. Insulin was measured by RIA.

5.2.4 Transplantation of islets Co-Encapsulated with Mesenchymal Stem Cells

Islets isolated from several animals were pooled and half were used for islet alone encapsulation and half for islet and MSC co-encapsulation. A minimal mass of 350 capsules in serum-free RPMI medium were delivered into the peritoneal cavity using an endotoxin-free, sterile pipette. Mice were considered cured when the average of 4 consecutive blood glucose measurements was ≤11.1 mM and the average of the final 4 consecutive measurements was ≤11.1 mM.

5.2.5 Post-Transplantation Studies

The mice were killed by cervical dislocation 6 weeks after transplantation. Individual capsules and capsules in small aggregates were counted and the numbers of capsules in large aggregates were estimated. Insulin secretion and insulin content samples were from groups of 10 encapsulated islets explanted from each mouse or picked from each culture dish. In addition, capsules were histologically examined as outlined below.

5.2.5.1 Microcapsule and Islet Histology

60-180 capsules recovered from each mouse were fixed in 10% formaldehyde, rinsed in graded concentrations of ethanol, embedded in paraffin, and cut into 5 µm sections. Microcapsule sections were stained with haematoxylin and eosin. Microcapsules (13-51 per graft) were scored for overgrowth on an increasing scale of 1-4 where 1 represents little or no overgrowth and 4 represents heavy overgrowth. Islet sections from recovered capsules (13-51 per graft) were assessed for islet section area using Image J software (Rasband, National Institute of Health).

5.2.5.2 Mesenchymal Stem Cell Histology

Haematoxylin and eosin stained microcapsule sections from recovered capsules (13-51 per graft) or cultured capsules from day 1-3 (9-13 per encapsulation) were assessed for MSC survival by counting the number of cells with a nucleus and normal morphology against the number of missing or anucleated cells. 10-30 capsules recovered from each mouse were stained with FDA. The number of FDA positive MSCs was estimated from images of microcapsules focused on a single plane.

5.2.5.3 Immunostaining

Microcapsule sections from recovered capsules (1-6 per graft) were stained with insulin or glucagon to count β and α cell number per islet section respectively and detect MSC phenotype. Insulin staining was carried out with rabbit polyclonal anti-insulin primary (1:1000) (Gene Tex, Irvine, CA, USA) with goat anti-rabbit immunoglobulin G (IgG) secondary (Alpha Diagnostic International, San Antonio, TX, USA). Glucagon staining was carried out with mouse monoclonal anti-glucagon primary (1:1000) (Sigma) with a biotinylated universal link secondary (Dako, Glostrup, Denmark). Staining was developed with Dako Liquid DAB + substrate chromogen system (Dako).

5.3 Results

5.3.1 *In vitro* Function of Islets Co-Encapsulated with Mesenchymal Stem Cells

MSCs were co-encapsulated with islets at a density of $8x10^5$ or $4x10^6$ cells per mL of alginate to determine the impact on islet insulin secretion and insulin content. Co-encapsulation of islets with MSCs increased stimulated insulin secretion compared with encapsulated islets alone at both MSC densities (Figure 14). The insulin secretion of islets co-encapsulated with $4x10^6$ MSCs per mL was higher than islets co-encapsulated with $8x10^5$ MSCs per mL. Co-encapsulation of islets with MSCs at a density of $4x10^6$ MSCs per mL of alginate increased insulin content compared with islets encapsulated alone at day 3.

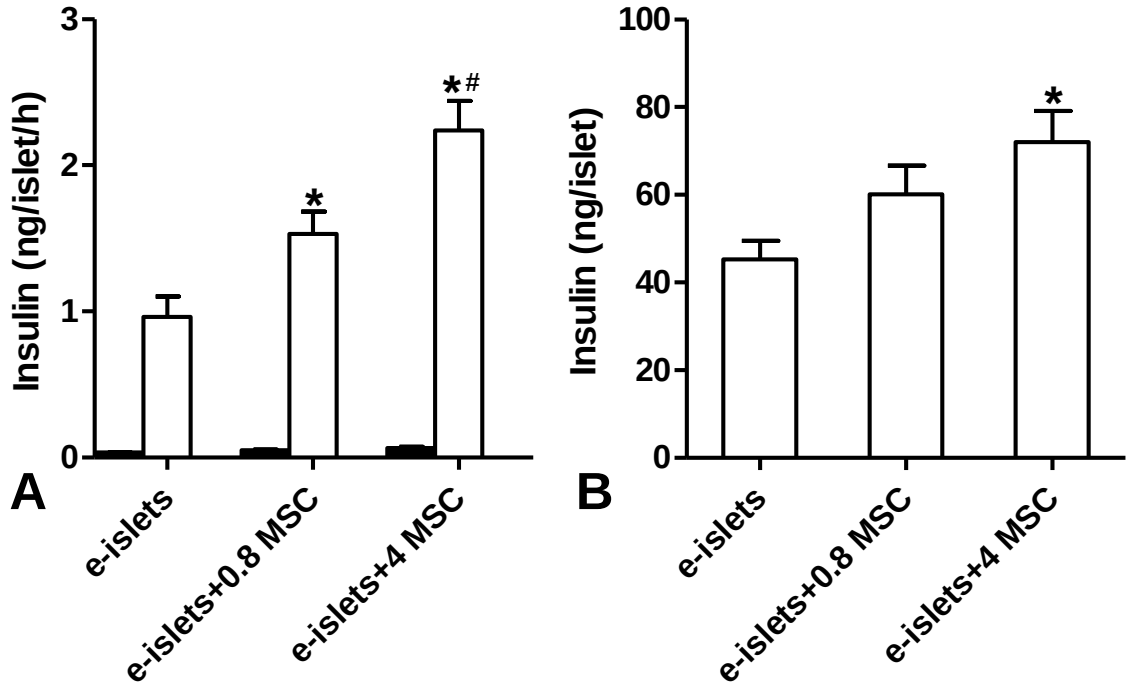


Figure 14. Insulin secretion and content of islets microencapsulated alone or co-encapsulated with mesenchymal stem cells.
Encapsulated islets alone (e-islets) were compared to co-encapsulated islets with $8x10^5$ mesenchymal stem cells per mL of alginate (e-islets+0.8 MSC) and $4x10^6$ mesenchymal stem cells per mL of alginate (e-islets+4 MSC). **A.** Insulin secretion of encapsulated islets at day 3 post-encapsulation in 2 mM (black) and 20 mM (white) glucose solutions. **P*<0.05 vs. e-islets, #*P*<0.05 vs. e-islets+0.8 MSC (One way analysis of variance with Holm-Sidak post hoc test *n*=17-18(3)). **B.** Insulin content of encapsulated islets at day 3. **P*<0.05 vs. e-islets, (Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's method), *n*=18-19(3)).

5.3.2 *In vivo* Function of Islets Co-Encapsulated with Mesenchymal Stem Cells

To determine the efficacy of islets co-encapsulated with MSCs in a transplantation setting, $4x10^6$ MSCs per mL of alginate co-encapsulated islets were transplanted into syngeneic STZ diabetic mice. Co-encapsulation of islets with MSCs improved the graft outcome compared to encapsulated islets alone. The average blood glucose level for the co-encapsulated group was

significantly lower on day 21, 35, and 42 (Figure 15). At 6 weeks 71% of the co-encapsulated group were cured compared with 16% of the islet alone group.

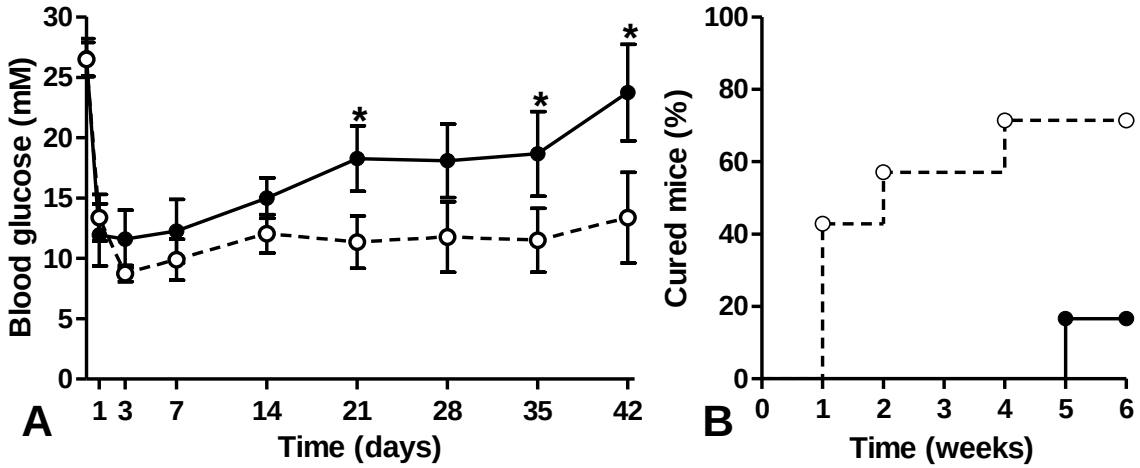


Figure 15. Graft outcome of diabetic mice transplanted with islets co-encapsulated with mesenchymal stem cells.
Mice were transplanted with 350 encapsulated islets alone (black circles, solid line, *n*=6) or 350 islets co-encapsulated with $4x10^6$ mesenchymal stem cells per mL of alginate (white circles, dotted line, *n*=7). **A.** Average blood glucose measurements.**P*<0.05 vs. islet alone group (Two way repeated measures analysis of variance, multiple comparisons versus control group (Holm-Sidak method)). **B.** Percentage of cured mice. *P*<0.05 (Kaplan-Meier Survival Analysis (Log-Rank)).

5.3.3 *Ex Vivo* Islet Function and Histology

Encapsulated islets were recovered from mice at 6 weeks after transplantation for functional and histological assessment of the islets. Stimulated insulin secretion and insulin content were higher in recovered islets that had been co-encapsulated with MSCs compared with islets encapsulated alone (Figure 16). Islets stained positively for insulin and glucagon with glucagon positive cells located on the islet periphery in both groups. The islet area and number of β cells per islet section were higher in the islets co-encapsulated with MSCs compared to islets encapsulated alone. The number of α cells was not different between the groups.

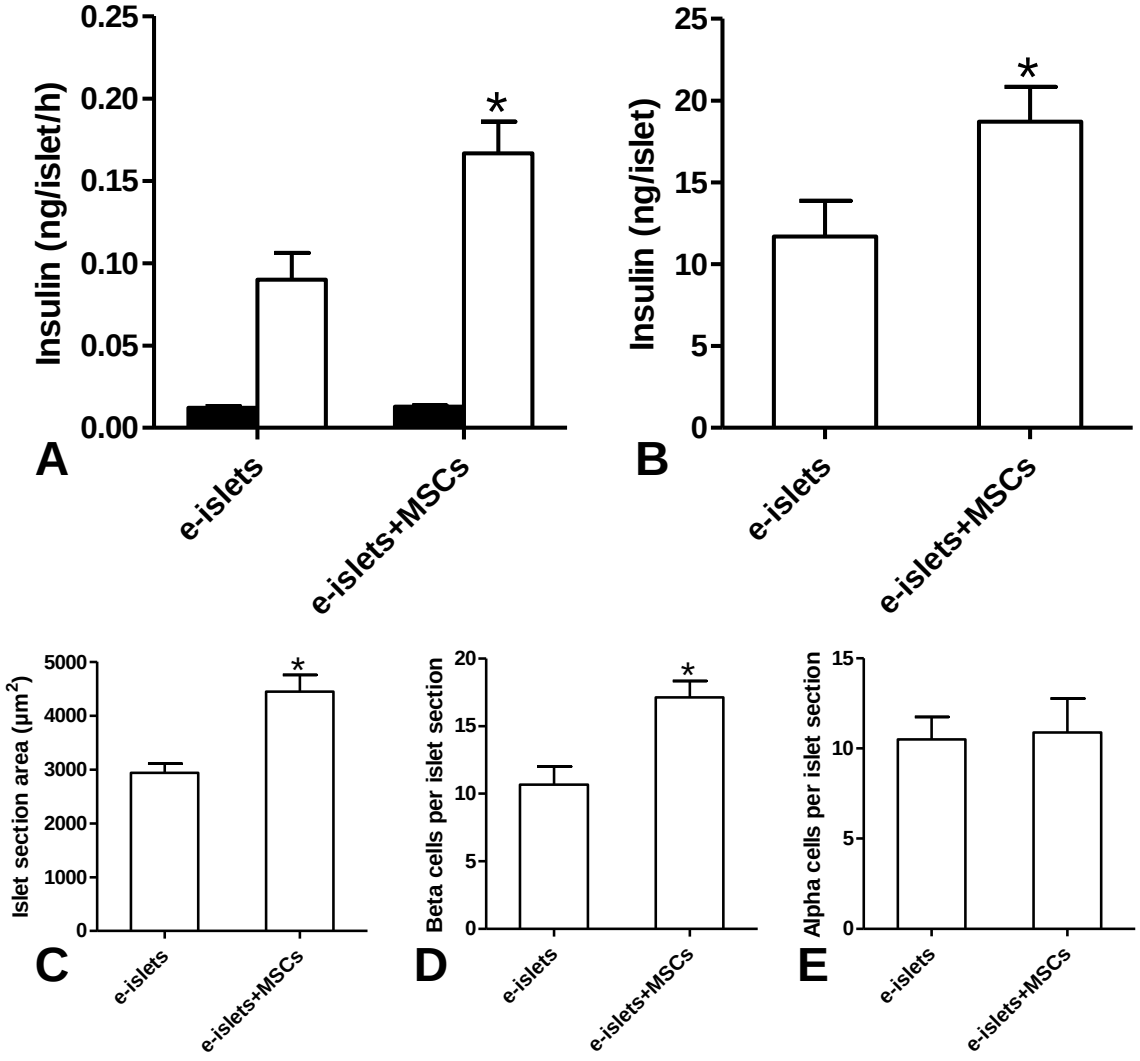


Figure 16. Insulin secretion, insulin content, and islet area of recovered islets co-encapsulated with mesenchymal stem cells.
Capsules were recovered at 6 weeks and control encapsulated islets alone (e-islets) were compared to co-encapsulated islets with $4x10^6$ MSCs per mL (e-islets+MSCs). **A.** Insulin secretion of capsules exposed to 2 mM (black) and 20 mM (white) glucose. **P*<0.05 vs. e-islets (Mann-Whitney Rank Sum Test, *n*=6-7). **B.** Insulin content of recovered capsules. **P*<0.05 vs. e-islets (Mann-Whitney Rank Sum Test, *n*=6-7). **C.** The islet area. **P*<0.001 vs. e-islets (Mann-Whitney Rank Sum Test, *n*=5-7). **D.** The number of β cells per islet section. **P*=0.004 vs. e-islets (t-test, *n*=8-12(3-5)). **E.** The number of α cells per islet section (*n*=8-10(3-5)).

5.3.4 Capsule Recovery and Overgrowth

The number of capsules recovered and the amount of pericapsular overgrowth indicate the capsule stability and biocompatibility respectively. The number of capsules recovered from the mice was not different between groups (Figure 17). Recovered capsule sections were scored for overgrowth and no differences between the overgrowth scores for each group.

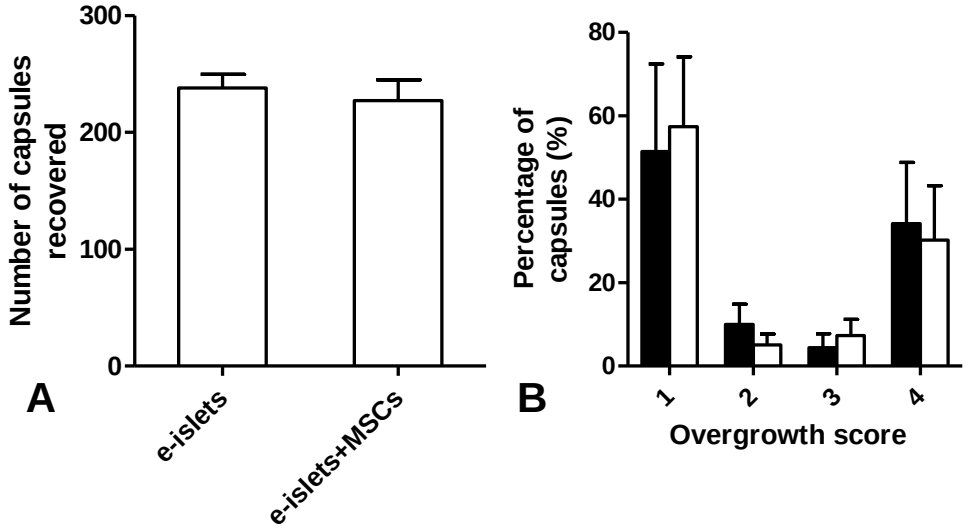


Figure 17. Capsule recovery and overgrowth score of recovered islets co-encapsulated with mesenchymal stem cells.
Capsules were recovered at 6 weeks and control encapsulated islets alone (e-islets) were compared to co-encapsulated islets with 4x10^6 mesenchymal stem cells per mL (e-islets+MSCs). **A.** Number of capsules recovered. *P*=0.634 (One way analysis of variance, *n*=6-7) **B.** Overgrowth score of recovered capsule sections. Control encapsulated islets alone (black) were compared to co-encapsulated islets with 4x10^6 mesenchymal stem cells per mL (white). *P*>0.05 (Two way analysis of variance *n*=5-7)).

5.3.5 Mesenchymal Stem Cell Histology

Encapsulated islets were recovered from mice at 6 weeks after transplantation for functional and histological assessment of the islets. A smaller percentage of MSCs in capsule sections and fewer FDA stained MSCs per capsule were found in capsules recovered from mice compared to capsules cultured for 1-3 days (Figure 18). MSCs did not stain positively for insulin or glucagon.

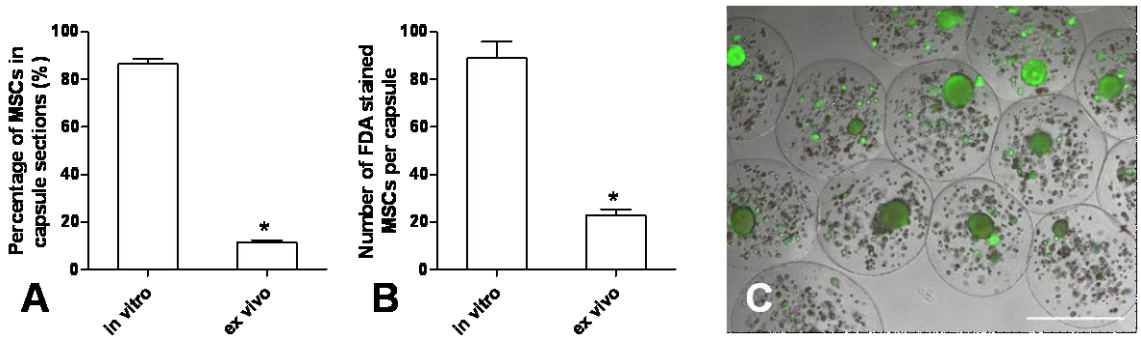


Figure 18. Survival of mesenchymal stem cells.
Co-encapsulated islets with 4x10^6 mesenchymal stem cells per mL either cultured for 1-3 days (*in vitro*) or recovered at 6 weeks from transplanted mice (*ex vivo*) were assessed. **A.** The percentage of mesenchymal stem cells in capsule sections, **P*<0.001 vs. *in vitro* (Mann-Whitney Rank Sum Test, *n*=3-7)). **B.** The number of fluorescein diacetate stained mesenchymal stem cells counted per capsule image **P*<0.001 vs. *in vitro* (Mann-Whitney rank sum test, *n*=13-24(3-7)). **C.** Representative image of islet and mesenchymal stem cell co-capsules recovered from mice at 6 weeks. Merged bright field and fluorescein diacetate cell stained merged image (500 µm scale bar).

5.4 Discussion

Co-encapsulation of islets with MSCs improved the GSIS and insulin content of islets *in vitro*. Several groups have also reported benefits from co-culturing non-encapsulated islets with MSCs including increasing insulin secretion (Park et al., 2010b, Jung et al., 2011, Park et al., 2009), insulin content (Jung et al., 2011), viability (Park et al., 2010b, Jung et al., 2011, Karaoz et al., 2010), and reducing apoptosis (Park et al., 2010b, Karaoz et al., 2010). Microencapsulation of MSCs results in the random distribution of cells throughout the capsule. Consequently a small proportion may be located at the islet periphery by chance although the vast majority of MSCs would not be in direct contact with the islet. It has been demonstrated that direct co-culture with MSCs is not necessary to improve islet viability and function either *in vitro* (Jung et al., 2011, Karaoz et al., 2010), or *in vivo* (Park et al., 2010a). In agreement, the mechanism of MSC-mediated enhancement of islet viability and function in our model is likely to be mostly through stimulation by soluble factors. Using a higher density of MSCs resulted in higher GSIS and a trend for higher insulin content at day 3 indicating a dose-response interaction. It has been shown that co-transplantation of MSCs pre-conditioned to produce more secretions were able to improve graft efficacy more than MSCs without pre-conditioning (Cavallari et al., 2012). It is likely that increasing the concentration of secreted MSC factors will increase the positive effects on islets, although it may be possible to over stimulate the islets. Additionally, where MSCs share the same environment as the islets, increasing the number of MSCs may be detrimental to islet viability and function due to the MSCs competing against the islets for limited resources such as oxygen and nutrients.

We validated the incorporation of MSCs co-encapsulated with islets *in vitro* and proceeded to translate the system into a transplantation setting in diabetic mice. Transplantation of a minimal mass of 350 islets alone into STZ diabetic mice resulted in a gradual decline in graft efficacy. The model was based on using 50% of the number of islets necessary for robust graft efficacy, and resulted in a similar pattern of graft failure reported in a related study (Bohman et al., 2006). The islet and MSC co-encapsulation group had significantly lower average blood glucose as early as 3 weeks post-transplantation and a greater proportion of the mice were cured by six weeks. The *ex vivo* assessment between the groups revealed a similar pattern to the *in vitro* data, with higher GSIS, higher insulin content, greater islet area, and a greater number of β cells in the islet and MSC co-encapsulation group. This finding demonstrates that the beneficial effects of co-encapsulating islets with MSCs observed *in vitro* is sustained *in vivo* for at least 6 weeks. MSCs appear to enhance the survival of islet cells as demonstrated by the larger islet area in islet and MSC co-encapsulated graft sections. Interestingly the number of β

cells is greater in the islet and MSC co-encapsulation group islets than the islet alone group islets, but the number of α cells is the same. It is possible that an islet cell type selective advantage is mediated by MSCs. However a more obvious explanation may be that there is proportionally more cell death in the β cell rich islet core of transplanted islets (MacGregor et al., 2006). Preservation of β cell survival is causally linked to the increase in islet insulin secretion and content observed in islet and MSC co-encapsulated graft islets. It is unclear if enhanced survival is the only cause of the greater amount of insulin produced and secreted or if the MSCs are enhancing the insulin production and secretion capability of the β cells.

The beneficial effect of MSCs in a number of transplantation models has been attributed to several mechanisms. It has been suggested that the transplantation of MSCs without islets can reduce the blood glucose concentration of STZ diabetic rodents (Ezquer et al., 2008, Dong et al., 2008, Lee et al., 2006d, Xu et al., 2012). The explanation for this finding was attributed to a small proportion of infused MSCs migrating to the pancreas and predominantly encouraging β cell regeneration, although there is some evidence of a small number of MSCs transdifferentiating into IPCs. In the current study microencapsulation resulted in the random distribution of MSCs throughout the capsule. By chance a few MSCs will be located at the periphery of the capsule. We have observed MSCs located at the periphery of the capsule escaping using time-lapse microscopy (data not shown). However, the vast majority of microencapsulated MSCs are trapped within the cross-linked alginate and have been observed to only move within the defined space created during the cross-linking of the alginate. In total approximately 25,000 to 32,000 MSCs were transplanted in a single graft and it is likely that a very negligible proportion of those would be located at the periphery of the capsules and therefore at risk of escape. MSC therapy for STZ diabetic rodents has been efficacious with as few as 500,000 cells (Ezquer et al., 2008), however other studies have reported using a few million (Dong et al., 2008, Lee et al., 2006d) or even up to 12 million cells (Xu et al., 2012). Given the large number of MSCs that have been reported for MSC therapy and the negligible chance of MSCs escaping from microcapsules, it is highly likely that the improvement in glycaemic control observed in the current study was not due to MSCs migrating to the pancreas and inducing endogenous β cell regeneration.

The improved performance of islets co-transplanted with MSCs has also been attributed to the immunosuppressive effects mediated by cytokine (Solari et al., 2009, Longoni et al., 2010, Berman et al., 2010) or metalloproteinase (Ding et al., 2009) secretion. The attenuation of the alloimmune response by MSCs is not relevant to the current study as both the islets and MSCs were syngeneic. In addition to regulating the alloimmune response MSCs have also been

implicated in suppressing the inflammatory response (Oh et al., 2008, Jung et al., 2011). The interaction between the inflammatory response and MSCs is relevant to the current study and may be reflected by the capsule recovery data at six weeks. The number of capsules recovered was equal for both groups which suggests that co-encapsulation with MSCs does not have a significant effect on capsule stability and biocompatibility. Poor biocompatibility results in overgrowth which is detrimental to islet survival and function and also may increase the chance of capsules becoming adhered in the tissues in the peritoneal cavity. There was also no difference in overgrowth between the groups which suggests that microencapsulated MSCs do not have a major impact on the inflammatory process which causes overgrowth. Consequently the suppressive effects of MSCs on inflammation are unlikely to be a major influence in our model.

Microencapsulation effectively impedes the formation of blood vessels connecting the islet to the general circulation. Co-transplantation with MSCs improved graft success and *ex vivo* islet function without revascularisation. This finding suggests that the effect MSCs exert on revascularisation is not essential to improving graft outcome by co-transplantation. Most islet and MSC co-transplantation studies have used the subcapsular kidney site which has an oxygen tension of 12-16 mmHg, which is hypoxic relative to the 40 mmHg oxygen tension of native islets (Carlsson et al., 2000). The intraperitoneal site has a higher oxygen tension of 40 mmHg (Renvall and Niinikoski, 1975) although due to the absence of revascularisation and poor diffusion through the alginate, hypoxia is an often cited cause of graft failure of microencapsulated islets at this site (Vaithilingam and Tuch, 2011, De Vos et al., 1999). It has been demonstrated that the oxygen tension of islets in small 180 µm diameter capsules is reduced by 55-73% compared to the oxygen tension outside of the capsule (Chen et al., 2012). In addition, mathematical modelling studies have suggested the oxygen tension in the core of islets in 500 µm alginate microcapsules in a 40 mmHg environment may be as low as <2 mmHg (Avgoustiniatos and Colton, 1997, Johnson et al., 2009). It is likely that the oxygen tension of microencapsulated islets transplanted intraperitoneally is at least as hypoxic as non-encapsulated subcapsular kidney grafts. Therefore there is an equal need for alleviation of hypoxia by revascularisation at both sites. We have demonstrated that revascularisation is not essential to gaining an advantage by co-transplanting with MSCs in hypoxic conditions. Therefore this indicates that the advantage we have observed in the absence of revascularisation is also likely to play a role in co-transplantation at the subcapsular kidney site.

Microencapsulation effectively preserves the morphology and organisation of the islets (Bohman and King, 2008). Several previous studies have indicated that under the kidney capsule, non-encapsulated islet alone grafts form into an amorphous mass of islet cells post transplantation (Davalli et al., 1996, Rackham et al., 2011, Biarnes et al., 2002). A previous study in our lab found that co-transplantation with MSCs prevented islet aggregation and preserved morphology more similar to that of native islets (Rackham et al., 2011). Aggregation of islets is likely to limit the diffusion of oxygen and nutrients. Indeed it has been observed that small islets are more efficacious than large islets for transplantation (Su et al., 2010, Lehmann et al., 2007). Graft tissue divided into small subunits is likely to have better availability of oxygen and nutrients than graft tissue in one aggregated mass. Microencapsulation keeps the islets in the graft fully isolated from each other and therefore preserves islet morphology. In the current study, islet alone transplantation had diminished graft success and *ex vivo* islet function compared to islet and MSC co-encapsulated grafts despite the preservation of islet morphology in both groups. This finding suggests that the effect MSCs exert to preserve islet morphology is also not essential to improving graft outcome by co-transplantation.

The outcome of microencapsulated islet transplantation in animal models is suboptimal (De Vos et al., 1999) with at least double the number islets necessary for graft success in animal models compared to non-encapsulated grafts (Siebers et al., 1993). Indeed in the current study we observed approximately a 10 fold reduction in insulin secretion and MSC viability which is likely to be related to the poor diffusion of oxygen and nutrients to microencapsulated islets implanted in the intraperitoneal environment. Our findings suggest that the efficiency can be greatly improved using a simple co-encapsulation strategy. MSCs are considered safe for infusion into humans and have been used in clinical trials. Consequently co-encapsulation of islets with MSCs may be a route worth perusing in higher animals. However our studies were carried out with syngeneic transplantations and the next logical step would be to carry out allogeneic transplantations. Microcapsules are designed for immunoprotection of their cellular contents so it might be predicted that an allogeneic transplantation would yield similar results to syngeneic transplantations. Indirect activation of T cells by allogeneic islet antigens from microencapsulated islets can cause an inflammatory environment with activation of macrophages and cytokine production. It is possible that the immunomodulatory cytokines secreted from MSCs may inhibit T cell responses to allogeneic islet antigens, attenuating the inflammatory reaction, and thereby contributing the enhanced graft outcome. It is possible that MSCs could attenuate the pericapsular overgrowth response which can be detrimental to graft outcome.

The improvement in insulin secretion at 6 weeks *ex vivo* suggests that the advantage of MSCs is not limited to the immediate post-isolation period but can be sustained *in vivo*. Co-encapsulation results in the continuous exposure of the islets to the MSCs which may confer an advantage for the lifetime of the graft. Indeed a proportion of recovered encapsulated MSCs were found to be viable after 6 weeks *in vivo*. The positive effect is triggered by the initial exposure to the MSCs but it unclear if the continued exposure is necessary to sustain the effect, although our data show it is not detrimental. It has been shown that islets cultured in MSC conditioned medium have enhanced graft functionality, suggesting co-transplantation is not necessary to utilise MSCs for islet transplantation (Park et al., 2010a). Although MSCs are being used in clinical trials the long term safety of MSC therapy has not been established. Therefore an MSC and islet co-culture approach without direct implantation of MSCs may be favoured for clinical islet transplantation.

Co-encapsulation of islets with MSCs robustly improved islet function *in vitro* and graft function *in vivo*, confirming that MSCs can improve the efficacy of microencapsulated islet transplantation. Using an isolated-graft model we were able to eliminate the impact of MSC-mediated enhancement of revascularisation, preservation of islet morphology and putative effects of the MSCs on the endogenous pancreas. From this we are able to confirm that the improvement in islet insulin secretion and insulin content observed *in vitro* is sustained *in vivo* and can significantly improve islet graft outcome.

6 Nano-Scale Encapsulation Improves Allogeneic Islet Transplantation in Mice

6.1 Introduction

The implantation site of immunoisolated islet grafts is typically limited by the relatively large size of immunoisolation devices. Microcapsules are the most commonly used immunoisolation device for islet transplantation which increase the graft volume by around 40 fold. Consequently microencapsulated grafts are only suitable for large sites such as the intraperitoneal cavity. As a result the development of islet immunoisolation strategies which minimise overall graft volume are desirable. Islet conformal coatings conform to the shape of the islet and thereby limit unnecessary increase in volume. If such coatings can be developed to provide immunoisolation they may be suitable for preferred sites such as the intraportal site in allogeneic islet transplantation.

A nano-scale layer-by-layer conformal coating approach has been developed in our lab (Zhi et al., 2010). The encapsulation was formed by sequential adsorption of chitosan then alginate to form electrostatically complexed bilayers. The desired number of bilayers was added followed by a final bilayer composed of chitosan and PC modified chondroitin-4-sulfate. The properties of alginate have been fully described in this thesis (1.2.4). The most significant property of alginate with regard to the nano-scale encapsulation is the negative charge of the polymer which allows electrostatic interaction with the positively charged polymers such as chitosan. The properties and relevant applications of the other materials used in the coating are described below.

6.1.1 Nano-Scale Encapsulation Materials

6.1.1.1 Chitosan

Chitosan is formed by deacetylation of chitin which is a structural element of the exoskeleton of crustaceans and cell walls of fungi. The amino groups in chitosan are positively charged in acidic solution conferring water solubility and an affinity for negatively charged surfaces such as cell membranes. Chitosan is also biocompatible and non-toxic which makes it amenable to use in implantable applications such as wound healing, drug and gene delivery, and tissue engineering. Chitosan has been used as an immunoisolation material for islet transplantation (Yang et al., 2010b). An injectable solution of chitosan and glycerol 2-phosphate disodium salt

hydrate which solidifies at >37°C was used in the study. 500 rat islets suspended in the solution were injected under the kidney capsule of diabetic mice resulting in maintenance of normoglycaemia for 28 days. Control islets rejected within 7 days which suggests that chitosan per se is able to function as an immunobarrier in a non-discordant xenograft islet transplantation model.

6.1.1.2 Chondroitin Sulfate

Chondroitin sulfate is a glycosaminoglycan present in the ECM which is widely distributed and abundant in mammalian tissues. It is a structural component of cartilage and is often derived from cartilage. It is also used to treat cartilage related pathologies such as osteoarthritis. It has been established that chondroitin sulfate has immunomodulatory and anti-inflammatory effects (du Souich et al., 2009) and there is some evidence to suggest it has anti-oxidant properties (Campo et al., 2006). Chondroitin sulfate has been used as a biomaterial in islet transplantation studies. A microencapsulation approach using agarose and PSS based capsules with a chondroitin sulfate coating has been described (Takagi et al., 1994). 1000 encapsulated hamster islets were intraperitoneally implanted in diabetic mice resulting in maintenance of normoglycaemia for 77 days. Non-encapsulated grafts were rejected in 7 days and agarose microcapsules without PSS were rejected in 32 days. No experiment was performed to decipher the impact of the chondroitin sulfate coating. The authors did offer the suggestion that as chondroitin sulfate is degraded in the body it may have limited functional longevity. One step microcapsules composed of an alginate and chondroitin sulfate mixture have been reported (Campos-Lisboa et al., 2008). 3,500 microencapsulated human islets were implanted intraperitoneally into diabetic mice resulting in maintenance of normoglycaemia for 60 days. Non-encapsulated islet grafts failed within 3 days. An alginate alone control microcapsule group was not included so the impact of chondroitin sulfate cannot be determined from this study.

6.1.1.3 Phosphorylcholine

Phosphorylcholine (PC) is the hydrophilic, uncharged head group found on some phospholipids which can be bound to polymers to mimic a biomembrane surface which is resistant to non-specific adsorption. Protein adsorption can result in coagulation, complement activation, and lymphocyte activation. Consequently PC modification has been applied to implantable devices to provide haemocompatibility (Iwasaki and Ishihara, 2005). Glucose sensors for an artificial pancreas have been coated with PC-modified polymer to prevent protein absorption to improve the functional longevity of the device (Nishida et al., 1995). PC coating of an islet

chamber has been described which prevented protein adsorption *in vivo* and permitted diffusion of insulin (Yang et al., 2004).

6.1.2 Polysaccharide Multilayer Nanoencapsulation of Insulin-Producing β Cells Grown as Pseudoislets for Potential Cellular Delivery of Insulin

The nano-scale encapsulation approach developed in our laboratory was tested *in vitro* using self-assembling islet-like aggregates (pseudoislets) generated from MIN6 β cells (Zhi et al., 2010). Coating or encapsulation of cells requires the use of non-toxic materials which can be applied under physiological conditions. The encapsulation strategy effectively coated the islets as demonstrated by electron microscopy and fluorescence microscopy. No immediate reduction in cell viability was detected upon encapsulation and the encapsulated cells remained viable for over 5 weeks. An islet coating should ideally prevent the infiltration or adsorption of cytotoxic antibodies but permit the diffusion of insulin. Coating with an outer bilayer of chitosan-alginate partially prevented antibody adsorption compared to non-coated pseudoislets. However with an outer bilayer of chitosan-chondroitin-4-sulfate the adsorption of antibody was further reduced. When stimulated with glucose or glucose and protein kinase C (PKC) activator phorbol myristate acetate (PMA), pseudoislets coated with 3 bilayers where found to be responsive to the stimuli, but insulin secretion was found to be 20-30% reduced compare to non-coated controls. The authors suggested that the nano-scale encapsulation may partially inhibit the diffusion of insulin.

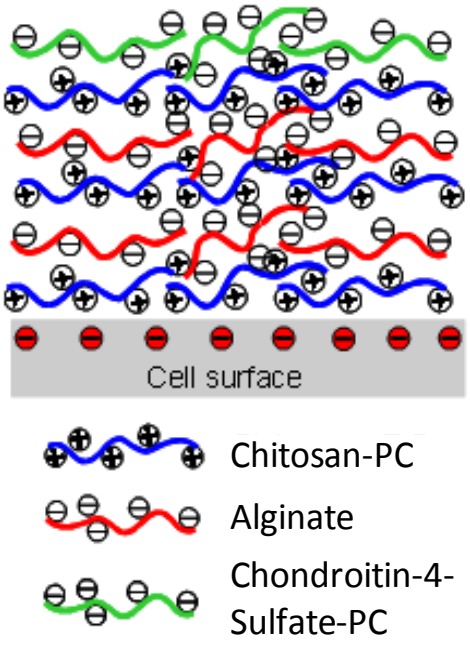


Figure 19. Schematic of nano-scale encapsulation with 3 bilayers.

6.1.3 Islet Nano-scale Encapsulation for Islet Transplantation

The current study involved a group of experiments testing a similar nano-scale encapsulation approach with the inclusion of PC modification of chitosan (Figure 19). Chondroitin-4-sulfate-PC has been shown to effectively reduce protein adsorption. Chitosan is amenable to PC modification and may provide further resistance to protein adsorption. The sulfate group of each chondroitin-4-sulfate disaccharide unit is able to form a strong complex with positively charge chitosan to provide a durable outer layer.

A series of *in vitro* studies were carried out by Dr Zhi which are detailed in the publication in the appendix (P173). To summarise, fluorophore tagged chitosan or PLL was incorporated into the nano-scale encapsulation to confirm islet coverage. Fluorescence could be detected for over 10 days and increasing the number of layers resulted in an increase in fluorescence. Transmission electron microscopy also confirmed coverage and a coating thickness of around 80 nm with 4 bilayers. Using live/dead staining no difference was observed between coated islets at 1 week or 4 weeks, suggesting long term compatibility of the nano-scale encapsulation. There was no difference in apoptosis levels between control and coated islets at 48 h. The incorporation of alginate protected islets from apoptosis when exposed to a cocktail of cytokines. The incorporation of chondroitin-4-sulfate in the coating protected islets from apoptosis when exposed to blood serum containing complement proteins and antibodies. The nano-scale encapsulation also prevented the adsorption of a fluorophore tagged MHC class II antibody.

These studies show that the nano-scale encapsulation effectively coats and is biocompatible with islets. The nano-scale encapsulation also offers some protection against infiltration of cytokines, complement, and antibodies. Following the encouraging results from the *in vitro* studies the following experiments including transplantation studies were carried out as detailed below.

6.2 Methods

6.2.1 Animals

Islets isolated from male Bagg albino C inbred mice (BALB/c) aged 6-8 weeks were used for perifusion studies. Male C57BL/6J mice aged 6-8 weeks were used as islet donors for syngeneic transplantations and male BALB/c mice aged 6-8 weeks were used as islet donors for allogeneic transplantations. STZ diabetic male C57BL/6J mice weighing 25g (8-10 weeks) were used as recipients for all transplantations.

6.2.2 Nano-Scale Islet Encapsulation

Islets were nanoencapsulated by the sequential adsorption of chitosan-PC then alginate to form electrostatically complexed bilayers. This was repeated until the desired number of bilayers was reached. A final bilayer composed of chitosan-PC and chondroitin-4-sulfate-PC was then added.

6.2.3 Perifusion

Islets or nano-scale encapsulated islets were cultured for >16 h before perifusion then picked in groups of 40 per chamber. Islets were perifused with Gey & Gey buffer with 2 mM glucose from 0-10 min, 20 mM glucose from 10-30 min, and 2 mM glucose from 30-50 min.

6.2.4 Non-Encapsulated and Nano-Scale Encapsulated Islet Transplantation

Non-encapsulated and nano-scale encapsulated islets were both transplanted under the kidney capsule of diabetic mice. Mice were considered cured if blood glucose concentrations were ≤11.1 mM on at least 2 consecutive measurements and did not revert to hyperglycaemia for the remainder of the study. Reversion to hyperglycaemia was defined as the point at which 2 consecutive measurements and all following measurements were >11.1 mM. Graft rejection was defined as 2 consecutive measurements >20 mM.

6.2.5 Glucose Tolerance Test

Glucose tolerance tests were carried out in cured animals at 14 days. Weight-matched, non-diabetic, non-transplanted male C57BL/6J mice were used as controls.

6.2.6 Nephrectomy

Transplanted mice were killed when graft rejection was confirmed and the graft-bearing kidney removed for histological analysis. Mice which did not revert to hyperglycaemia by day 28 had the graft-bearing kidney nephrectomised on or after day 28. Nephrectomised mice with 2 consecutive blood glucose readings >20 mM were considered diabetic and were killed.

6.2.7 Histology

Nephrectomised kidneys were fixed in neutral-buffered formalin and the graft-bearing area of the kidney was paraffin-embedded and sectioned. Immunostaining was carried out by Dr Z. Zhi by insulin staining of β cells to show the persistence and morphology of recovered grafts. The lymphocyte infiltration of grafts was also assessed by CD3 staining for T cells, and CD68 staining for macrophages. The results of the histology are detailed in the results section and the images can been found the publication in the appendix of this thesis (P173).

6.3 Results

6.3.1 Functional Assessment of Nano-Scale Encapsulated Islets *In Vitro*

The dynamic insulin secretion of non-encapsulated and nano-scale encapsulated islets were assessed by perifusion. There were differences between the insulin secretion profile of non-encapsulated islets and islets nano-scale encapsulated with 2 bilayers and 4 bilayers (Figure 20A and 20B, respectively). There were also differences between the insulin secretion profile of islets nano-scale encapsulated with 2 bilayers and 4 bilayers (Figure 20C). The pattern and reversibility of the stimulated glucose response was similar in all groups.

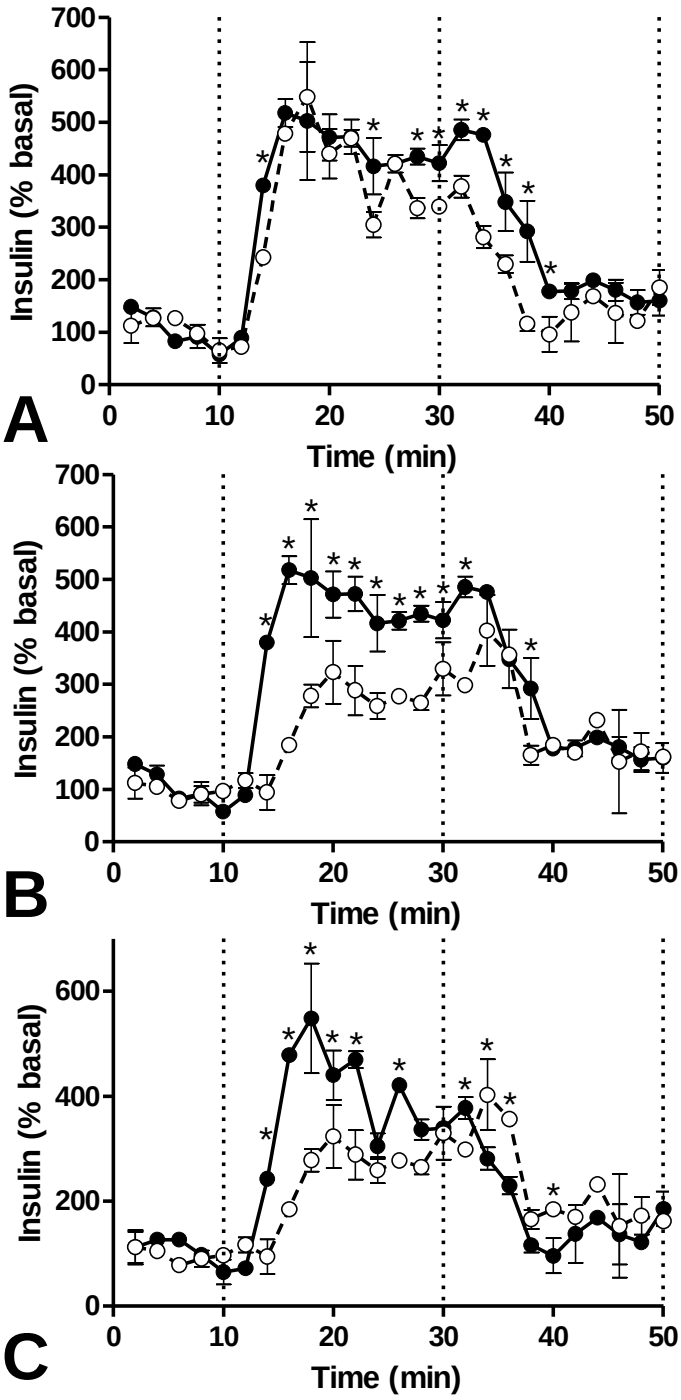


Figure 20. Dynamic insulin secretion of perifused nano-scale encapsulated islets.
Islets were exposed to 2 mM glucose from 0-10 min, 20 mM glucose from 10-30 min, and 2 mM glucose from 30-50 min. These data are from a single experiment with three conditions but are displayed with separate plots for each combination of pairs of conditions (**P*<0.05, Two way analysis of variance, *n*=4(1)). **A.** Non-encapsulated islets (black circles, solid line) and 2 bilayer nano-scale encapsulated islets (white circles, dotted line). **B.** Non-encapsulated islets (black circles, solid line) and 4 bilayer nano-scale encapsulated islets (white circles, dotted line). **C.** 2 bilayer nano-scale encapsulated islets (black circles, solid line) and 4 bilayer nano-scale encapsulated islets (white circles, dotted line).

6.3.2 Functional Assessment of Nanoencapsulated Islets *In Vivo*

6.3.2.1 Nano-Scale Encapsulation with 2 Bilayers

To determine the curative potential of nano-scale encapsulated islets in the absence of allogeneic immune challenge, syngeneic transplantations were performed. 300 non-encapsulated islets or nano-scale encapsulated islets with 2 bilayers were transplanted under the kidney capsule of diabetic mice. In both groups the mice were cured by day 14 and there was no difference overall between the groups (Two way repeated measures ANOVA, P=0.585, n=3-4) (Figure 21A). Removal of the graft bearing kidney resulted in reversion to hyperglycaemia in all mice. Insulin staining of recovered graft sections of non-encapsulated and nano-scale encapsulated grafts had similar morphology. To determine the ability of the nano-scale encapsulation to protect against allogeneic immune challenge, allogeneic transplantations were performed. In preliminary experiments, 300 non-encapsulated islets or nano-scale encapsulated islets with 2 bilayers were transplanted under the kidney capsule of diabetic mice. In both groups the mice were initially cured by day 4, however the non-encapsulated group reverted to hyperglycaemia by day 10 and the non-encapsulated group reverted to hyperglycaemia by day 16 (Figure 21B).

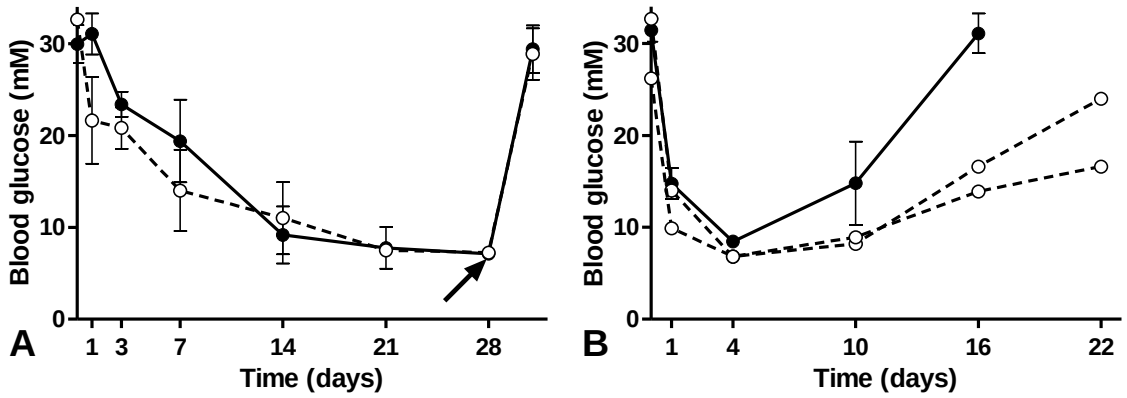


Figure 21. Blood glucose of mice transplanted with syngeneic or allogeneic islets nano-scale encapsulated with 2 bilayers.
A. Blood glucose of diabetic mice with syngeneic transplantation of 300 non-encapsulated islets (black circles, solid line, n=3) or nano-scale encapsulated islets with 2 bilayers (white circles, dotted line, n=4). Recipients were nephrectomised at 28 days as indicated by the arrow. **B.** Blood glucose of diabetic mice with allogeneic transplantation of 300 non-encapsulated islets (black circles, solid line, n=5) or nano-scale encapsulated islets with 2 bilayers (individual plots for each mouse, white circles, dotted lines, n=2).

6.3.2.2 Nano-Scale Encapsulation with 4 Bilayers

Due to the failure of islets nano-scale encapsulated with 2 bilayers to fully protect against rejection it was decided to increase the number of bilayers in the nano-scale encapsulation. Non-encapsulated islets or nano-scale encapsulated islets with 4 bilayers were transplanted

under the kidney capsule of diabetic mice. In the non-encapsulated group the mice were initially cured by day 6 however they rejected by day 13 (Figure 22). The nano-scale encapsulated group were cured by day 10, 2 mice rejected on day 16 and 24, but the rest of the mice were cured up to day 28. There was a difference overall between the groups up to the last measurement for the control group (day 0-17) (Mann-Whitney Rank Sum Test $P<0.001$, $n=7\text{-}9$). By day 28, 71% of the nano-scale encapsulated graft recipients were cured compared to 0% of the non-encapsulated graft recipients (Figure 22). The proportion of cured mice was greater in the nano-scale encapsulation group compared to the non-encapsulated group. There was no difference in the glucose tolerance of cured nano-scale encapsulated islet graft recipients and non-diabetic mice (Figure 22). Insulin staining of graft sections was present in nano-scale encapsulated islet grafts but almost absent in non-encapsulated islet grafts. The presence of T cells identified by CD3 staining and macrophages indentified by CD68 staining were detected in non-encapsulated islet grafts but not nano-scale encapsulated islet grafts.

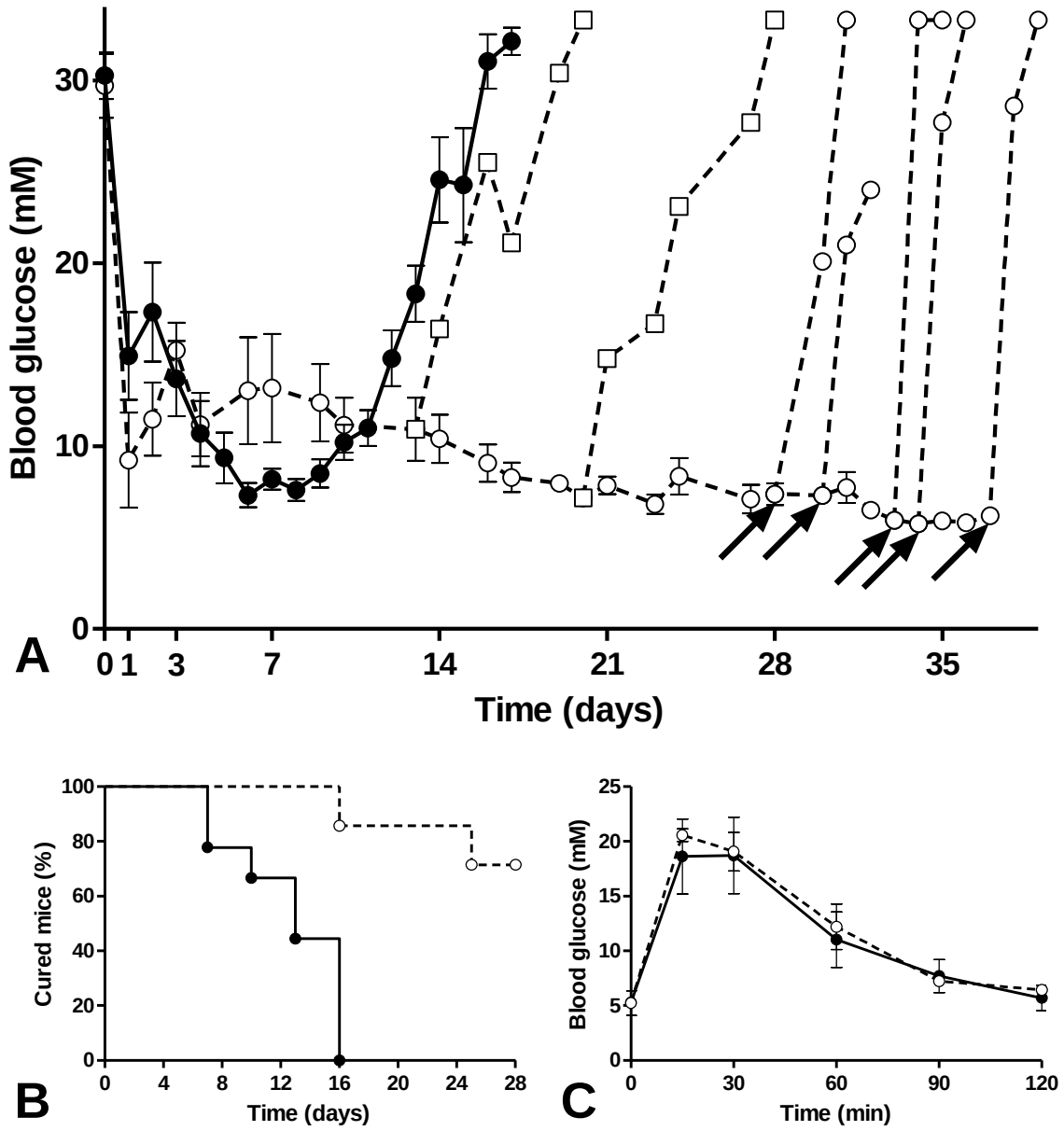


Figure 22. Transplantation of allogeneic islets nano-scale encapsulated with 4 bilayers.
A. Blood glucose of diabetic mice with allogeneic transplantations of 300 non-encapsulated islets (black circles, solid line, *n*=9) or nano-scale encapsulated islets with 4 bilayers (white circles, dotted line, *n*=7). Rejected nano-scale encapsulated graft recipients (white squares, dotted line, *n*=2) and nephrectomised nano-scale encapsulated recipients (white circles, dotted line, *n*=5) are shown as individual plots from the point of rejection or nephrectomy. Mice which were cured at 28 days were nephrectomised on or after day 28 as indicated by the arrows. **B.** Survival curve of diabetic mice transplanted with non-encapsulated islets (black circles, solid line) or nano-scale encapsulated islets (white circles, dotted line). *P*<0.001 (Kaplan-Meier Survival Analysis (Log-Rank), *n*=7-9). **C.** Glucose tolerance test of non-diabetic, non-transplanted mice (black circles, solid line) and 4 bilayer nano-scale encapsulated islet transplanted diabetic mice (white circles, dotted line) at day 14. *P*>0.05 (One way analysis of variance, *n*=4).

6.4 Discussion

We have described the nano-scale encapsulation of islets with a novel layer-by-layer polyion complex conformal coating to improve islet transplantation outcome in allogeneic diabetic mouse recipients. The polymers used are charged on each monosaccharide unit which provides hundreds of binding sites on each polysaccharide molecule. Negatively charged alginate or chondroitin-4-sulfate polymers therefore interact electrostatically with positively charged chitosan, and chitosan interacts with the negative charge on the cell membrane. We have demonstrated that using the nano-scale encapsulation the layer-by-layer technique effectively covers islets. A uniform fluorescence on the islet was observed when a fluorophore was incorporated into the nano-scale encapsulation and remained stable for at least 10 days *in vitro*. The modification of chitosan with PC increased the solubility of the polysaccharide so that it became soluble at physiological pH as opposed to the acidic conditions required for unmodified chitosan. Consequently the nano-scale encapsulation could be applied under mild physiological conditions, and was found not affect cell viability over a period of 4 weeks.

The recruitment of activated cytokine producing macrophages to the site of immunoisolated islets has a detrimental effect on islet viability and function (de Vos et al., 2003). We have demonstrated the ability of the alginate in the nano-scale encapsulation to partially inhibit cytokine induced apoptosis which may confer an advantage in a transplantation setting. Upon transplantation rapid infiltration of complement components can occur leading to lysis of islet cells (Tjernberg et al., 2008). We have demonstrated the ability of chondroitin-4-sulfate-PC to partially inhibit complement induced apoptosis which may be of particular advantage for transplantations in the intraportal site. MHC class II molecules present self antigens which trigger the host immune response in allogeneic transplantations. Using an antibody exclusion assay we were able to demonstrate that an anti-MHC class II was prevented from binding when the islet was coated. Therefore the nano-scale encapsulation may prevent an adaptive immune response in allogeneic transplantation models.

Nano-scale encapsulation of islets with 2 bilayers inhibited insulin secretion compared to non-encapsulated islets. A greater inhibition of insulin secretion could be observed with nano-scale encapsulated islets with 4 bilayers. The significant differences observed in islets with a greater number of layers were mostly during the 20 mM stimulated glucose period of the perifusion. This suggests the encapsulation is permissive to diffusion of low concentrations of insulin but imposes a diffusion limitation at higher secretion rates. Increasing the number of bilayers from

2 to 4 increases the diffusion limitation therefore it is probable that further increasing the number of layers would further lower the diffusion threshold. A similar finding was reported in the previous study in our lab coating pseudoislets in 3 bilayers (Zhi et al., 2010). In both studies no evidence was found to suggest that the nano-scale encapsulation has any impact on cell viability, which suggests that β cell death is not a contributing factor to the reduction in insulin secretion observed.

Syngeneic transplantations were carried out to assess the impact of the nano-scale encapsulation on islet graft function in the absence of an allogeneic immune response. Islets coated with 2 bilayers had a similar impact to non-encapsulated islets on the recipients' blood glucose kinetics, effectively reversing hyperglycaemia and maintaining normoglycaemia for up to 28 days. Insulin staining of graft sections were similar between non-encapsulated and nano-scale encapsulated islet grafts suggesting the coating does not affect graft structure. No deleterious effects were observed in the recipients and no major inflammatory reaction was observed by eye in graft sections, which suggests that the nano-scale encapsulation is likely to be non-toxic and biocompatible. Removal of non-encapsulated or nano-scale encapsulated islet grafts resulted in reversion to the diabetic state thereby confirming the curative ability of the grafts.

Allogeneic transplantations were carried out to assess the ability of the nano-scale encapsulation to protect the islets from the allogeneic immune response. Transplantation of islets coated with 2 bilayers resulted in a short delay in the onset of rejection whereas transplantation of islets coated in 4 bilayers resulted in graft success in 5/7 of recipients for 28 days. This indicates that the thickness of the nano-scale encapsulation is critical to graft success. It is likely that increasing the number of bilayers reduces the permeability and provides a more substantial immunobarrier to protect the islets from an immune mediated attack, or from triggering an immune attack. Removal of nano-scale encapsulated islet grafts resulted in reversion to the diabetic state thereby confirming the curative ability of the grafts.

To date the most successful islet conformal coating tested in immunocompetent allogeneic diabetic animal models without the use of any immunosuppression has been to coat the islets sequentially with 3 layers of reactive PEG (Lee et al., 2007). Using a single layer of reactive PEG 7/7 grafts rejected whereas using a triple layer of reactive PEG 4/7 graft rejected. The layer-by-layer coating allows sequential layers of reactive PEG deposits to form a more substantial and effective immunobarrier. The findings from the study are similar to the current study which indicates that an immunobarrier of great enough thickness and impermeability is required to

prevent allorejection. Our nano-scale encapsulation was unable to protect all of the grafts from rejection which suggests that the addition of more bilayers may be necessary for full protection. However, as there is some limitation of insulin diffusion out of the coating, increasing the number of bilayers may ultimately be detrimental to graft function. The best solution may be a balance between improving immunoprotection at the cost of decreasing insulin diffusion. It has been demonstrated that microencapsulated islets recovered at 6 weeks from grafts implanted intraperitoneally can have as much as a 10 fold reduction in insulin secretion compared to pre-implantation levels and still maintain normoglycaemia in recipients (Figure 15). It is possible that maintaining islet viability by immunoprotection may be preferable to preserving maximal insulin diffusion.

Recovered graft sections from cured mice with encapsulated islet grafts at 28 days revealed grafts with normal morphology and no infiltration of T cells or macrophages. Conversely graft sections from rejected grafts had little insulin staining and infiltration of T cells and macrophages indicating allorejection. Recruitment of lymphocytes around immunoisolated islet grafts has been reported. Microencapsulated islet grafts transplanted intraperitoneally are detrimentally affected by recruitment and overgrowth of macrophages and fibroblasts on the capsule surface (de Vos et al., 2003). Although it has been shown that there is less overgrowth under the kidney capsule compared to the intraperitoneal site (Bakeine et al., 2007). However, triple coated reactive PEG conformal coated islets transplanted under the kidney capsule do recruit lymphocytes (Lee et al., 2007). It is surprising that no recruited lymphocytes can be detected at all in the encapsulated graft sections. Chondroitin-4-sulfate can reduce the formation of pro-inflammatory cytokines (du Souich et al., 2009) and PC modification confers resistance to protein adsorption. It is possible that the properties of these biomaterials may play a role in inhibiting the recruitment of lymphocytes to the immediate graft area. The nano-scale encapsulation may also prevent the leakage of antigens out of the encapsulation thereby reducing immune recruitment and activation.

The nano-scale encapsulation is held together by electrostatic forces and is weak in comparison to other immunoisolation methods such as cross-linked alginate hydrogel microcapsules. Consequently it is predicted that the nano-scale encapsulation would provide short term protection from the inflammatory and immune responses in the early post-transplantation period. The negligible increase in graft volume of the nano-scale encapsulation confers suitability for intraportal transplantation. Intraportal allogeneic transplantation of reactive PEG coated islets has been reported (Wee et al., 2008) although 4 times as many islets were used and graft survival was around 4-13 times shorter than an equivalent studies (Lee et

al., 2006c, Yun Lee et al., 2007) using the subcapsular kidney site. If these studies are representative, the allogeneic intraportal site may present a significant challenge for conformally coated islet grafts. It is therefore likely that using the current nano-scale encapsulation design, intraportal transplantation may be need to be augmented with low levels of immunosuppressants or tolerance induction mechanisms for long term graft success. Alternatively, it may be necessary to alter the design of the nano-scale encapsulation so that it is tailored for the intraportal site. For example the immobilisation of heparin or thrombomodulin onto the outer surface may be necessary to combat the IBMIR.

We have demonstrated that a conformal coating composed of a layer-by-layer polyion complex can improve graft outcome in an allogeneic mouse model of T1DM. The incorporation of polymers with functional properties and the ability to alter the number of layers make the nano-scale encapsulation a unique and flexible approach. Long term and intraportal transplantation studies will determine if the nano-scale encapsulation is clinically relevant.

7 General Discussion

Clinical islet transplantation is currently an experimental therapeutic strategy for T1DM patients. The necessity for immunosuppression limits islet transplantation as a suitable therapeutic option to a small subset of patients with recurrent severe hypoglycaemic episodes, or to those who are already using immunosuppressants for kidney grafts. The practical reality for islet alone graft recipients is swapping hypoglycaemic episodes with the risks associated with immunosuppression. Islet transplantation without immunosuppression would reduce the risks predominantly to the risks associated with the surgical procedure alone. As a consequence islet transplantation could become a realistic option for the majority of T1DM patients. This is the goal of immunoisolation approaches such as microencapsulation. Recent clinical microencapsulated islet transplantation studies report reduction in insulin requirements and eradication of hypoglycaemic episodes for up to 3 years (Basta et al., 2011). Compared to the breakthrough study using the Edmonton protocol (Shapiro et al., 2000) of insulin independence in 7/7 cases, the graft outcomes are modest. However, as current islet transplantation recipients are selected on the basis of severity of hypoglycaemic episodes, reduction in hypoglycaemic episodes should be the primary therapeutic goal. If microencapsulated grafts are able to treat hypoglycaemic episodes with minimal risk due to the absence of immunosuppression then this approach may be preferable to current clinical islet transplantation protocols. It is early in the development of clinical microencapsulated islet transplantation however the future looks promising.

The studies in this thesis were designed to investigate novel strategies to improve immunoisolated islet transplantation. It has been demonstrated that islet viability and functionality are greatly diminished by microencapsulated transplantation into the peritoneal cavity after several weeks. Transplantation into the peritoneal cavity avoids the IBMIR, and therefore the acute loss of islets upon transplantation, as reported with intraportal transplantations. Indeed after 7 days the GSIS and insulin content of microencapsulated islets are not significantly reduced compared to cultured islets, although ATP content levels are reduced. At 6 weeks there is a 4 fold reduction in insulin content and at 6 or 8 weeks there is a 10 fold reduction in GSIS. From these results it is apparent that a gradual reduction in islet viability and function occurs. However microencapsulated islets can cure diabetic rodents for several months despite the reduced viability and functionality of the islets (Bohman and King, 2008). This suggests that the reduction in viability and functionality of islets reaches a plateau and the remaining β cells are able to maintain a sufficient degree of functionality to sustain blood glucose control. As a consequence microencapsulated islets grafts require at least

double the number of islets compared to non-encapsulated islet transplantations under the kidney capsule. There is therefore a need to investigate new approaches for microencapsulated islet transplantation in order to improve the maintenance of graft viability and efficacy.

A major factor influencing islet viability and functionality of microencapsulated grafts is the site of transplantation. The intraperitoneal site is conventionally used for microencapsulated islet grafts predominantly due to the large capacity of the site. Implanted microcapsules are randomly dispersed throughout the peritoneal cavity which may locate them next to a vascularised tissue. However the distance to the nearest capillary is likely to be large enough that the islets predominantly rely on passive diffusion through the intraperitoneal fluid for acquisition of oxygen, nutrients and glucose, and the dissemination of insulin. This results in insufficient oxygen and nutrients getting to the islets and a lag in insulin entering the circulation. Consequently the subcutaneous site was investigated as an alternative to the intraperitoneal site. It was discovered that microencapsulated islet GSIS, insulin content, and ATP content were all lower after a week transplanted in the subcutaneous site compared to the intraperitoneal site. Accordingly, equivalent grafts were ineffective at the subcutaneous site but efficacious at the intraperitoneal site. Successful transplantation of islets to the subcutaneous site has been reported by inducing angiogenesis at the site (Pileggi et al., 2006) (Dufrane et al., 2010, Lacy et al., 1991, Golocheikine et al., 2010). Indeed it has been reported that microcapsules composed of high-M alginate were efficacious at the subcutaneous site (Veriter et al., 2010). Taken together this suggests that the passive diffusion at the subcutaneous site is worse than at the intraperitoneal site but can be improved by induction of angiogenesis by materials of specific composition. The high-G alginate capsule composition used for the study in this thesis was not appropriate for the specific demands of the site. With this work we were able to highlight the comparatively poor environment the unmodified subcutaneous site provides compared to the intraperitoneal site, and also the importance of optimising the composition of alginate microcapsules for the specific task.

It was established that the conventional site in the intraperitoneal cavity is preferable for microencapsulated islet transplantation. We decided to investigate the use of helper cells to enhance intraperitoneal microencapsulated islet transplantation. Helper cells have several advantages to drug based enhancement of islet transplantation including the ability to localise helper cells with the islets, physiological responsiveness, adaptation to microenvironments, and the ability to secrete factors continuously for the lifetime of the graft. GLP-1R agonists have been shown to increase GSIS (Mojsov et al., 1987), insulin synthesis (Alarcon et al., 2006),

β cell proliferation (Buteau et al., 2003) and neogenesis (Xu et al., 2006), and protect β cells against apoptosis (Li et al., 2003). The L cells of the intestine are the major endogenous source of GLP-1. A study was undertaken to demonstrate the impact of co-encapsulating L cells with islets to improve microencapsulated islet transplantation. Transformed L cell lines were used due to their convenience for this proof of principle study. It was found that the L cells secreted GLP-1 in a glucose responsive manner and but did not stimulate GSIS in islets when co-cultured. However, when L cells were co-encapsulated with islets it was found that GSIS was increased in islets, although it was not determined if the increase was GLP-1-mediated. In a pilot study using a minimal mass of 250 microencapsulated islets, there was a trend improvement in graft outcome in L cell co-encapsulated islet grafts. However when the study was extended, the incorporation of L cells did not improve graft outcome. It was hypothesised that either L cells are not beneficial for microencapsulated islet grafts or the minimal mass model was too stringent to detect a positive effect. Overall, it is possible that L cells can offer an advantage in an islet transplantation setting, however the results from the study were inconclusive.

The use of mesenchymal stem cells as helper cells in non-encapsulated islet transplantation has been shown to benefit islet viability, function, and revascularisation, and to protect islets from alloimmune rejection by immunomodulation (Figliuzzi et al., 2009, Ito et al., 2010, Sordi et al., 2010b, Rackham et al., 2011, Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Berman et al., 2010). Co-encapsulation of islets with MSCs was hypothesised to improve graft outcome and also demonstrate the importance of MSC-mediated enhancement of revascularisation. MSC co-encapsulated islets had improved GSIS and insulin content, and improved graft outcome in a minimal mass transplantation model of 350 islets per graft. Recovered co-encapsulated islets had greater GSIS, insulin content, and islet area than recovered encapsulated islets alone. MSCs had a positive effect on islet function in pre-transplantation culture which is sustained after transplantation. The MSC co-encapsulation study also demonstrated that MSC-mediated enhancement of revascularisation and preservation of morphology is not necessary to improve graft outcome in islet and MSC co-transplantation. Studies have reported enhanced islet revascularisation and preservation of morphology by co-transplanting with MSCs, however we have demonstrated that MSC-mediated enhancement of GSIS and insulin content alone enhances graft outcome.

The minimal mass model of 350 islets used for the MSC study was of appropriate stringency as the impact of the MSCs on graft outcome could be detected. If the model is not stringent enough, too many of the controls would cure so any beneficial effects of the treatment would

not be detected. Conversely if the model were too stringent, the benefit of the treatment would have to be large in order to be detected. From the results of the L cell study it was suggested that the minimal mass model of 250 islets was too stringent. Comparing the control groups from all of the microencapsulated studies it was found that graft masses of 250, 350, and 700 islets resulted in average blood glucose measurements and graft failure proportions (Figure 23) which were significantly different from each other over 28 days. 350 islet grafts were only different to 700 islet grafts at individual time-points after 21 days. However 250 islet grafts were different from 700 islet grafts in the first 3 days post-transplantation and then from 14 days onward. This data indicates that the 250 islet minimal mass model is highly stringent and it may therefore be difficult to detect differences in treatments using this model. The 350 microencapsulated islet graft seems to act as an appropriate minimal mass model, with average blood concentrations at day 28 between 10 mM and 20 mM.

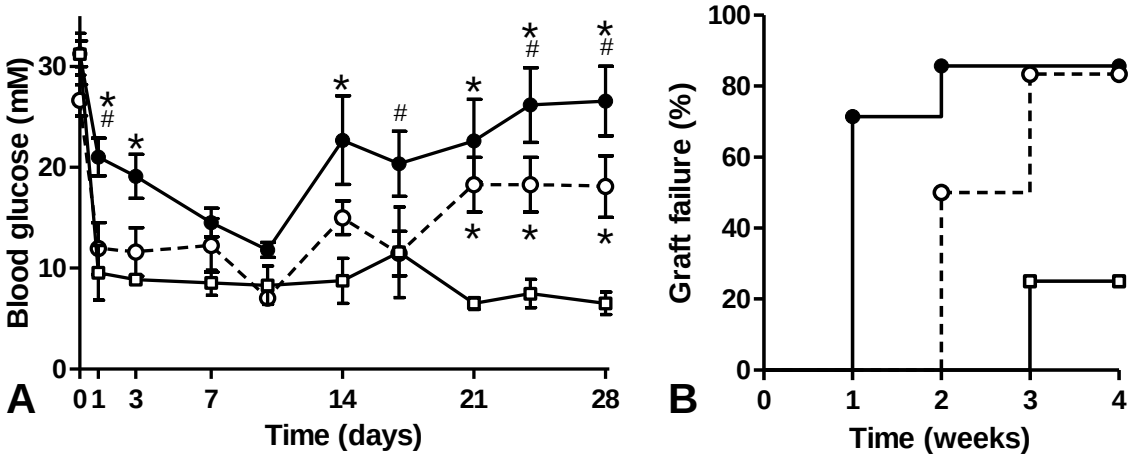


Figure 23. The impact of graft mass on microencapsulated graft outcome.
Microencapsulated islets were transplanted intraperitoneally in graft masses of 250 (black circles, solid line, *n*=7), 350 (white circles, dotted line, *n*=6) and 700 (white squares, solid line, *n*=5) syngeneic islets in separate studies. **A.** Blood glucose measurements. **P*<0.05 vs. 700 islet grafts, $^{\#}$*P*<0.05 vs. 350 islet grafts (Two way repeated measures analysis of variance, multiple comparisons versus control group (Holm-Sidak method)) **B.** Percentage of failed grafts. *P*<0.05 (Kaplan-Meier survival analysis (Log-rank).

The MSC study demonstrated that intraperitoneal microencapsulated islet transplantation can be improved. However the reduction in islet GSIS and insulin content decreased from pre-transplantation levels proportionally in both control islet alone and MSC co-encapsulated islets post-transplantation. It is therefore clear that although MSCs improve graft outcome they cannot completely protect islets from the course of functional decay. Consequently we sought to investigate the efficacy of a novel conformal coating immunoisolation approach. The concept of conformal coating is to create a minimal islet immunobarrier with maximal diffusion and minimal impact on islet volume. Conformally coated islet grafts may therefore better conserve islet function and constitute a graft size which is acceptable for preferable

sites such as under the kidney capsule or the intraportal site. It was demonstrated that the layer-by-layer nano-scale encapsulated islets secreted insulin, although the coating did inhibit secretion to some degree which was dependent on the number of layers. However nano-scale encapsulated islets maintained curative potential as demonstrated by blood glucose normalisation in syngeneic transplantations. Subsequently it was found that nano-scale encapsulation with 4 bilayers but not 2 bilayers was able to protect islets from allorejection for 1 month in 5/7 recipients. This result represents a significant advance in the field of conformal coating for immunoisolation of islets grafts. The study demonstrated that the layer-by-layer method allows a balance between diffusion limitation and immunoprotection to be optimised. Immunosuppressants have been used in many islet conformal coating studies (Lee et al., 2006b, Lee et al., 2006c, Lee et al., 2006a, Yun Lee et al., 2007, Wee et al., 2008, Jeong et al., 2011), however no immunosuppressant was used in our study. Interestingly the only other study to successfully transplant conformally coated islets in a fully immunocompetent allogeneic setting also used a layer-by-layer approach (Lee et al., 2007). Consequently the layer-by-layer approach is an attractive option for the development of conformal coating strategies.

In summary, the studies presented in this thesis have made some contribution to the advancement of immunoisolated islet transplantation. The use of MSCs to improve immunoisolated islet transplantation and a novel immunoisolation approach have been validated. These approaches show promise for development into the clinic. The use of MSCs for immunoisolated islet grafts has the potential to reduce the number of islets necessary for successful transplantation and may improve outcomes in higher animal studies. The use of nano-scale encapsulation has the potential to equalise the number of islets used in non-immunoisolated and immunoisolated grafts. With improved graft efficacy a stronger case can be made for the development of immunoisolation approaches for islet transplantation into the clinic.

7.1 Future Work

The subcutaneous site was unable to support the viability of implanted microencapsulated islets. As safety is a priority in the development of novel therapeutic approaches the subcutaneous site is worth perusing for immunoisolated islet transplantation despite the challenges it presents. Strategies which may improve the outcome at the site are based around maximising the availability of oxygen and nutrients to the islets. This could be achieved by

minimising the immunoisolation device by using nano-scale encapsulation for example. It is likely that this alone would be insufficient and would need to be coupled with angiogenic modification of the site. It is possible that the angiogenic properties of MSCs could be applied in subcutaneous islets grafts by co-transplantation or co-encapsulation of MSCs and islets.

The positive effect of co-localising islets with L cells was not as reliable or pronounced in comparison to the effects observed using MSCs. As a result it is difficult to justify the continuation of the L cell work. However one important distinction between the two bodies of work is the derivation of the cells. It is possible that primary cells are better suited for application as helper cells and it would satisfy curiosity to try using primary L cells.

As MSCs are already used therapeutically the development of co-encapsulation of islets with MSCs for clinical application is feasible. The MSC study demonstrated that MSCs can enhance microencapsulated islet graft function in a syngeneic transplantation model. As MSCs are immunomodulatory it is hypothesised that in addition to the enhancement of islet functionality MSCs mediated in a syngeneic setting, they may also attenuate immune responses such as overgrowth in an allogeneic setting. The immunomodulatory properties of MSCs may also be applicable to pig islet xenotransplantation. There is interest in pig islets as an alternate source of islets to fill the donor shortage gap. However due to the high immunological discordance, immunosuppression at efficacious doses cannot be safely used and therefore immunoisolation approaches are necessary. Therefore it would be of interest to investigate if MSCs are also able to improve microencapsulated pig islet xenotransplantation.

By co-encapsulating islets with MSCs, the MSCs were located with the islet throughout the islet and MSC co-encapsulation transplantation study. The MSCs enhanced islet function in culture, however it was not determined if MSCs continue to have an impact on islet function post-transplantation. Studies simulating the conditions *in vivo* such as using cytokines or hypoxic conditions could give an indication of whether it is beneficial to have MSCs co-encapsulated with the islets post-transplantation. Alternatively co-culture of MSCs with microencapsulated islets alone prior to transplantation may be sufficient, which would avoid transplanting the MSCs and therefore avoid increasing the risk of transplantation.

The conformal coating study had an endpoint of 28 days as the initial objective was to determine if the nano-scale encapsulation could protect islets from early post-transplantation rejection. It would be interesting to determine if grafts stabilised at 28 days could continue to maintain blood glucose for an unlimited period. Transplantations for the study were under the

kidney capsule of mice as the model allows for ease of implantation and the possibility of retrieving the graft and confirming graft efficacy. However, there is interest in developing conformal coatings which can immunoprotect islets in the conventional clinical intraportal site. It is possible that the nano-scale encapsulation could provide an advantage at the site. However poor results from other studies suggest that the rapid inflammatory and immune responses in this lymphocyte rich site may present challenges not present to the same degree under the kidney capsule (Wilson et al., 2011, Wee et al., 2008). It may be necessary to modify the nano-scale encapsulation by functionalisation. For example anticoagulant molecules could be incorporated onto the outer layer of the coating to combat the IBMIR. Overall the nano-scale encapsulation approach which was validated in an allogeneic transplantation model in this thesis represents a novel and potentially versatile approach for immunoisolation of islets.

The use of MSCs and nano-scale encapsulation have both proved to enhance islet graft outcome, it is possible that these two modalities may also provide a synergistic effect if combined.

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Publications and Abstracts from this Thesis

Publications

- **Kerby A**, Jones P, King A (2012) Co-transplantation of islets with mesenchymal stem cells in microcapsules demonstrates graft outcome can be improved in an isolated-graft model of islet transplantation in mice. (Accepted at "Cytotherapy")

- **Kerby A**, Bohman S, Westberg H, Jones P, King A (2012) Immunoisolation of Islets in High Guluronic Acid Barium-Alginate Microcapsules Does Not Improve Graft Outcome at the Subcutaneous Site. Artif Organs. 2012 Feb 28. doi: 10.1111/j.1525-1594.2011.01411.x.

- Zhi ZL, **Kerby A**, King AJ, Jones PM, Pickup JC (2012) Nano-scale encapsulation enhances allograft survival and function of islets transplanted in a mouse model of diabetes. Diabetologia. 2012 Apr;55(4):1081-90.

Abstracts

- **Kerby A**, Rackham CL, Chagastelles PC, King A (2012) Co-Encapsulation of Islets with Mesenchymal Stem Cells Improves Islet Function. Molecular Therapy 20 Suppl 1:S163. Poster presentation at ASGCT conference 2012.

- **Kerby A**, Zhi Z, Jones PM, Bowe JE and King AJ (2011) A novel method of islet nanoencapsulation reverses hyperglycaemia in diabetic mice. Rev. Diabetic Studies 8(1) MO-141. Oral presentation at IPITA conference 2011, Prague.

- **Kerby A**, Zhi Z, Jones PM, Bowe JE and King AJ (2011) A novel method of islet nanoencapsulation reverses hyperglycaemia in diabetic mice. Diabetic Medicine 28 Suppl 1:P21. Poster presentation at Diabetes UK conference 2011, London.

- **Kerby A**, Checketts S, Bohman S, Jones P, King A (2010) The subcutaneous site is inferior to the intraperitoneal site for microencapsulated islet transplantation. Diabetic Medicine 27 Suppl 1:P10. Poster presentation at Diabetes UK conference 2010, Liverpool.

Appendix

Zhi ZL, **Kerby A**, King AJ, Jones PM, Pickup JC (2012) Nano-scale encapsulation enhances allograft survival and function of islets transplanted in a mouse model of diabetes. Diabetologia. 2012 Apr;55(4):1081-90.

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ARTICLE

Nano-scale encapsulation enhances allograft survival and function of islets transplanted in a mouse model of diabetes

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Abstract
Aims/hypothesis The success of islet transplantation as a treatment for type 1 diabetes is currently hampered by post-transplantation loss of functional islets through adverse immune and non-immune reactions. We aimed to test whether early islet loss can be limited and transplant survival improved by the application of conformal nano-coating layers to islets.
Methods Our novel coating protocol used alternate layers of phosphorylcholine-derived polysaccharides (chitosan or chondroitin-4-sulphate) and alginate as coating materials, with the binding based on electrostatic complexation. The in vitro function of encapsulated mouse islets was studied by analysing islet secretory function and cell viability. The in vivo function was evaluated using syngeneic and allogeneic transplantation in the streptozotocin-induced mouse model of diabetes.
Results Nano-scale encapsulated islets retained appropriate islet secretory function in vitro and were less susceptible to complement- and cytokine-induced apoptosis than non-encapsulated control islets. In in vivo experiments using a syngeneic mouse transplantation model, no deleterious responses to the coatings were observed in host animals, and the encapsulated islet grafts were effective in reversing hyperglycaemia. Allo-transplantation of the nano-coated islets resulted in preserved islet function post-implantation in five of seven mice throughout the 1 month monitoring period.
Conclusions/interpretation Nano-scale encapsulation offers localised immune protection for implanted islets, and may be able to limit early allograft loss and extend survival of transplanted islets. This versatile coating scheme has the potential to be integrated with tolerance induction mechanisms, thereby achieving long-term success in islet transplantation.

Keywords Allogeneic transplantation · Graft survival · Immunoisolation · Islet transplantation · Nano-coating · Nano-scale encapsulation · Type 1 diabetes

Abbreviations

HBSS	Hanks' buffered salt solution
PC	Phosphorylcholine
PEG	Polyethylene-glycol
TEM	Transmission electron microscopy

Introduction

Islet transplantation is arguably one of the most important conceptual advances in the treatment of type 1 diabetes and has the potential to cure the disease [1]. A major barrier to successful transplantation is early post-transplantation depletion of functional islets in response to activated complement and coagulation systems, and to a chronic inflammatory and immunogenic environment [2–5]. This has obvious detrimental effects on the outcome of individual grafts and further exacerbates the scarcity of donor tissue. Moreover, allogeneic transplantation requires pharmacological suppression of the host immune system to circumvent graft rejection, while current immunosuppressive regimens are likely to contribute

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to the early loss of engrafted islets [6]. A strategy that conceals implanted islets from the host immune system may help to minimise or even prevent the post-transplantation loss of islet mass and function by immune rejection. Thus physical isolation of islets from the host environment by encapsulation, while maintaining islet responsiveness to metabolic changes, could be an effective means of improving islet survival and function after transplantation.

Islet encapsulation strategies to date have mainly focused on macrocapsules (encapsulation of the whole islet graft) and microcapsules (encapsulation of individual islets) [7]. Previous studies in animal models [8, 9] and in human participants [10, 11] have demonstrated that physical isolation of islets from the host immune system by, for example, alginate microencapsulation is effective in preventing beta cell loss and in maintaining long-term secretory function in transplanted allogeneic and xenogeneic islets without systemic immunosuppression [12–16]. Although conferring the immunological advantages predicted from graft/host isolation, microcapsules have some significant drawbacks. Thus the relatively large volume of a typical alginate capsule (diameter ~500–800 μm) compared with a typical islet (~150 μm) results in a greatly increased diffusion length, which can lead to impaired diffusion of oxygen and nutrients to the islet, with consequent hypoxic cell death or malfunction. A more immediate consequence of the size of microcapsules is the choice of anatomical location for the graft material, with most experimental studies focussing on intraperitoneal or subcutaneous compartments due to their large capacity [17]. In contrast, intraportal infusion is the current site of choice for clinical programmes [1], with the transplanted islets lodging in the hepatic microcirculation. An encapsulation technology designed for application to current clinical transplantation of human islets must therefore be compatible with intraportal delivery to the hepatic capillary bed, excluding microcapsules or macrocapsules on the basis of their size.

Conformal nano-coating avoids these problems by generating a biocompatible nanometre-scale isolating layer close to the cell surface, thus reducing barriers to diffusion, while ensuring that the encapsulated islets can be implanted into any site suitable for non-encapsulated islets [7]. The challenge with conformal nano-coating is to nano-engineer an efficient and lasting immune-protective layer that covers islets completely or nearly completely, and is biocompatible with the recipient.

Several methods of conformal coating have been developed recently, including covalent surface attachment of polyethylene glycol (PEG), known as 'PEGylation' [18], and layer-by-layer encapsulation [19–28]. Nano-layers with different combinations of components, including streptavidin and biotin–PEG derivatives [19, 24], complement receptor 1 and heparin [25], and PEG–lipid and poly(vinyl alcohol) [26, 27], have been previously researched as islet surface coatings. Most of these attempts at islet encapsulation have focused on development of the method, with relatively little emphasis on in vivo evaluation of the technology, and particularly on the maintenance of functional beta cells in an allogeneic environment. Teramura and Iwata demonstrated that coating of islet surfaces with PEG-lipid and urokinase could effectively protect against blood-mediated inflammatory reactions in a syngeneic mouse model of transplantation [23]. However, a recent study using conformal coating to encapsulate islets via layer-by-layer deposition of poly(L-lysine)-*g*-PEG-(biotin) and streptavidin failed to show any improvement in the survival and function of the implanted allo-islets [19]. Effective immunoprotection using nano-coating thus remains a challenge in islet transplantation.

Layer-by-layer nanofilm deposition has been studied extensively in connection with the coating of biodevices such as implants in biomedical applications [29, 30]. In the current study, we aimed to adapt this technology to produce a conformal nano-coating for islets, which covers individual islets in nano-layers that possess anti-coagulation and anti-inflammatory properties [31, 32], thus achieving effective encapsulation without significantly increasing the size of the islet. Charged linear polysaccharides carrying phosphorylcholine (PC) modification were used as coating materials (Fig. 1), with the binding being based on electrostatic interactions. As demonstrated previously [33], layer-by-layer deposition of alginate–chitosan nano-layers provides a mild process (near physiological conditions) that does not involve covalent cross-linking, it is therefore able to leave the cell surfaces and cell interior undisturbed. In this study, we investigated the localised immune protective effects of biopolymer nano-coating of islets and tested whether nano-scale encapsulation improves early outcomes from syngeneic and allogeneic islet transplantation in the commonly used streptozotocin-induced mouse model of type 1 diabetes.

Methods

A more detailed description of research design and methods, including regents and materials, is available in the electronic supplementary material (ESM Methods).

Experimental animals For syngeneic transplantation, male C57BL/6 mice aged 8 weeks and weighing 20–25 g (Charles River, Margate, UK) were used as donors and recipients of grafts. For allogeneic transplantation, male C57BL/6 mice were used as islet graft recipients and Balb/c mice as tissue donors. Recipient mice were made diabetic by a single i.p. streptozotocin injection (180 mg/kg; Sigma-Aldrich, Poole, UK) 5–6 days prior to transplantation and those with a non-fasting blood glucose concentration of

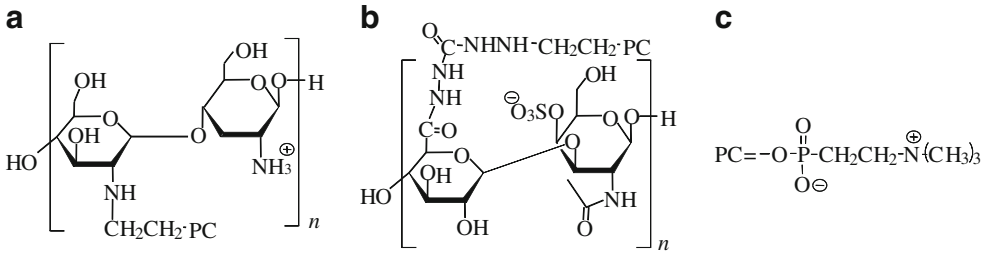

Fig. 1 Chemical structure of the PC moiety-functionalised polysaccharides used in the nano-scale encapsulation. (**a**) PC-modified (40% of the amine groups) chitosan (protonated); (**b**) PC-modified (100% of the carboxylic group) chondroitin-4-sulphate (deprotonated); and (**c**) PC

≥20 mmol/l were used as recipients. All animal procedures were approved by our institution's Ethics Committee and carried out under licence, in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Islet isolation Islets were isolated from mice by injecting collagenase (type XI, 1 mg/ml; Sigma-Aldrich) into the pancreas via the common bile duct, followed by digestion for 10 min at 37°C. The islets were purified by centrifugation (3,500 rpm, 25 min; Universal 320R, Hettich Zentrifugen, Tuttingen, Germany) in a density gradient (Histopaque-1077; Sigma-Aldrich). Before being used for encapsulation, the purified islets were incubated for 16 h in RPMI-1640 medium (Sigma-Aldrich) that was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Nano-coating of islets The working solutions of the polysaccharides, including PC-chitosan, alginate and PC-modified chondroitin-4-sulphate (1 mg/ml; Fig. 1), were prepared by dissolving the solids in Hanks' buffered salt solution (HBSS; PAA Laboratories, Pasching, Austria), which was pH-adjusted to 6.9–7.0 and supplemented by 2 mmol/l $CaCl_2$. Positively charged chitosan-PC and negatively charged alginate (unmodified) were alternately deposited into a multilayer film on individual islets in suspension in a 1.5 ml Eppendorf tube. Cationic chitosan-PC (400 μl) was added first to a suspension of the islets (pre-washed with HBSS) to form a seeding layer. After 5 min deposition time, two intermediate washings with the buffer were made using gravity sedimentation for 1 to 2 min to remove any excess, unadsorbed chitosan-PC. Subsequently, anionic alginate (400 μl) was adsorbed in the same manner. This process was repeated *n* times per batch of islets according to the following layering scheme: islets/(chitosan-PC–alginate layers)$_n$, where *n* represents the number of bilayers. Finally, a PC-modified chondroitin-4-sulphate (400 μl) layer was added as the outermost layer in the same manner as for alginate. Some loss of islets was incurred, totalling <10% for a typical eight-layer coating process. All encapsulation solutions were filtered through a sterile 0.2 μm membrane filter cartridge. All coating, washing and sample solutions were kept on ice during the coating process.

Dynamic insulin secretion from the encapsulated islets Encapsulated islets were incubated for 16 h (37°C) before perifusion. The rate and patterns of in vitro insulin secretion from encapsulated and control islets were assessed using a temperature-controlled (37°C) multi-channel perifusion system, as described previously [34]. Briefly, 40 islets were loaded on to nylon filters in Swinnex filter holders (Millipore, Cork, Ireland) and perifused with a bicarbonate-buffered physiological salt solution (Gey & Gey buffer, made in house) supplemented by 2 mmol/l $CaCl_2$, 0.5 mg/ml bovine serum albumin and a concentration of glucose as indicated below. Fractions were collected every 2 min during (1) a 10 min perifusion period with buffer containing 2 mmol/l glucose, (2) a 20 min perifusion with 20 mmol/l glucose and (3) an additional 20 min perifusion with 2 mmol/l glucose. Insulin content was assessed by radioimmunoassay [34].

Transplantation of encapsulated islets in diabetic mice Mice were anaesthetised by inhalation of isoflurane and transplanted with 300 islets under the kidney capsule, according to a procedure reported previously [35]. Briefly, a lumbar incision was made, the kidney exposed and an incision made in the capsule. Encapsulated and control islets that had been centrifuged into pellets in PE50 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ, USA) were placed underneath the kidney capsule using a Hamilton syringe (Fisher, Two Rivers, WI, USA). All islets were transplanted with a delay of no more than 2 h after encapsulation.

In vivo function of islet graft The body weight and blood glucose concentrations of recipient mice were monitored every 1–2 days. Reversal of hyperglycaemia was defined as non-fasting blood glucose concentrations ≤11.1 mmol/l on at least two consecutive readings. In cured animals, we assessed the in vivo function of the transplanted islets by an intraperitoneal glucose tolerance test at 1 month after transplantation. Weight-matched, non-diabetic, non-transplanted male C57BL/6 mice were used as controls. Fasting blood

glucose concentrations were measured prior to an i.p. injection of 2 g/kg of glucose dissolved in saline solution and then after 15, 30, 60, 90 and 120 min. The islet graft-bearing kidneys were removed 1 or 2 days later to assess whether graft removal would result in a reversion to hyperglycaemia. Other mice in which the graft was rejected in less than 28 days were killed and the graft-bearing kidney removed for histological analysis.

Immunohistochemistry Detailed descriptions of histological and immunohistological analysis of the graft-bearing kidneys is available in the ESM Methods.

Statistical analysis Independent *t* tests were used to test for significant difference between individual groups of the cytokine assay results and glucose tolerance data. Values of $p < 0.05$ were considered significant.

Results

Deposition of coating layers on islets We used a nano-scale encapsulation approach that uses spontaneous deposition of alternate layers of charged linear polysaccharides (PC-chitosan/alginate) as coating materials, with a final layer of PC-modified chondroitin-4-sulphate, as illustrated in Fig. 2. The nano-coated C57BL/6 mouse islets showed better physical strength than the uncoated ones, as evidenced by an increase of 20–30% in packed tissue volume for the same amounts of islets when loaded in PE50 polyethylene transplantation tubing.

The deposition of eight layers was assessed by fluorescence microscopy following the incorporation of two layers of PC-chitosan tagged with FITC in the fifth and seventh layers. As shown in Fig. 3a, the addition of the FITC-tagged layer to the islets resulted in strong fluorescence localised on the surface of the islets, which is consistent with their extracellular architecture and indicates uniform coverage.

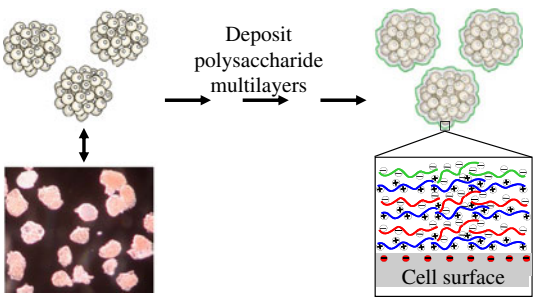

Fig. 2 Schematic representation of the nano-scale encapsulation of pancreatic islets, with layer-by-layer deposition of charged polysaccharide multilayers on individual islet surfaces. Blue lines with + symbol, chitosan-PC; red lines with − symbol, alginate; green lines with − symbol, chondroitin-4-sulphate-PC

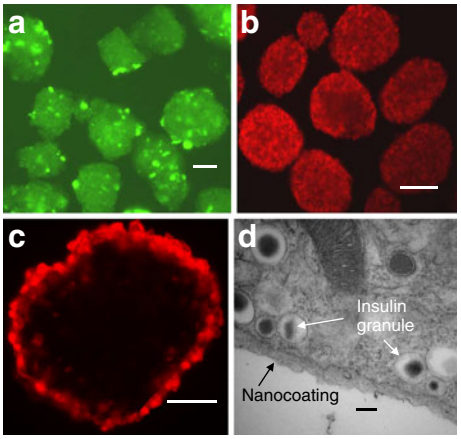

Fig. 3 Confirmation of the nano-scale encapsulation of mouse islets. **a** Deposition of eight layers with incorporation of two layers of PC-chitosan tagged with FITC into the fifth and seventh layers. Scale bar 50 μm. **b** Fluorescence labelling of islets by incorporation of Alexa Fluor 647-tagged poly-L-lysine into the seventh layer as a fluorescent marker. Scale bar 100 μm. **c** Cross-section of a coated islet with an incorporated layer of Alexa Fluor 647-tagged poly-L-lysine (formalin-fixed and paraffin-embedded), showing that the nano-layer appears to completely cover the islet surface. Scale bar 50 μm. **d** TEM micrograph of the cross-section of an islet encapsulated by an eight-layer coating (consisting of PC-chitosan, alginate and condroitin-4-sulphate as described in Methods, non-labelled). Islets were imaged shortly after completion of coating. Scale bar 100 nm

A few bright spots found on the surfaces of islets may have been due to the higher surface charges that can occur on cell or extracellular surfaces. The above image (Fig. 3a) was taken shortly after encapsulation; however, the coating was found to be stable in culture throughout 10 days of monitoring (ESM Fig. 1). Due to the instability of FITC under paraffin-embedding conditions, we also generated fluorescence-labelled islets by incorporating poly-L-lysine tagged with Alexa Fluor 647 in the seventh layer as a fluorescent marker (see ESM Methods) (Fig. 3b). The cross-section of the labelled islet showed that the exterior islet surfaces were completely covered and that the coating was localised extracellularly (Fig. 3c). Note that the thickness of the fluorescent layer ring could have been affected by the surface roughness of the islet and the position of the section taken from the islet.

The deposition of the non-labelled polysaccharide multilayers on the individual islets was further confirmed by high-resolution transmission electron microscopy (TEM) imaging of the encapsulated islets (Fig. 3d; ESM Methods). The ultrastructural image showed an intact coating consisting of nano-layers of approximately 80 nm thickness that covered the outer surface of cells on the islet periphery. The nano-layer was not found in the extracellular space between the islet cells. Insulin-secreting vesicles (dense-core granules) were seen by TEM to be aligned along the plasma membrane ready for exocytosis, suggesting that beta cells were healthy and unaffected by the coating.

In vitro cytotoxicity exerted by the coating materials After maintenance in culture for up to 4 weeks, the survival of encapsulated mouse islet cells within eight layers was assessed using a two-colour fluorescence live–dead cell assay with the cell-permeable esterase substrate, fluorescein diacetate, and the cell-impermeant nucleic acid stain, propidium iodide (ESM Methods). Most of the cells in the islet were viable after 1 and 4 weeks of culturing, indicating that islets tolerated the multi-step nano-coating process with no or little detectable loss in cell viability (Fig. 4a, b).

In addition, no elevated level of apoptosis in the nano-coated islets was detected after 48 h in culture compared with non-encapsulated islets, as shown in Fig. 4c (see ESM Methods). The rate of islet cell apoptosis (determined as caspase-3/7 activity) in control islets was increased by approximately 620% following exposure to combined cytokines (IL-1β 1.7 ng/ml, TNF-α 1.7 ng/ml and IFN-γ 3 ng/ml), but this was significantly less in the nano-coated islets. The negative coating layer with alginate was more potent in inhibiting cytokine-induced cell damage than chondroitin-4-sulphate-PC. Nano-coated islets were also protected against apoptosis mediated by complement (50% rabbit serum; Fig. 4d). Chondroitin-4-sulphate-PC, an anti-coagulatory molecule [31, 32], was found to provide a better protective effect than alginate. Based on the findings above, the coating scheme adopted for our in vivo experiments used chitosan-PC and alginate in repeated layers, with the last layer being chondroitin-4-sulphate-PC.

Assessment of the in vitro function of nano-scale encapsulated islets Dynamic insulin secretion of the encapsulated islets was studied and compared with control using a perifusion system that simulates physiological conditions. As shown in Fig. 5, the four-layer encapsulated mouse islets retained glucose-induced insulin secretory responses. Increasing the glucose concentration from 2 to 20 mmol/l induced a rapid, biphasic and reversible stimulation of insulin secretion from islets encapsulated with four layers, with identical or similar response patterns from control islets. Increasing the nano-film thickness to eight layers caused a small reduction in the kinetics and overall amount of insulin released, but the pattern and reversibility of the glucose-induced response were maintained.

In vivo assessment of nano-scale encapsulated islet function: syngeneic transplantation Figure 6a shows the data from islet transplantation under the kidney capsule from C57BL/6 donor mice (300 islets/graft) to diabetic recipients of the same strain. As expected, implantation of non-encapsulated islets as a control reversed hyperglycaemia and restored normoglycaemia for the duration of the study (4 weeks). Nano-scale encapsulated islets with four layers were equally effective in reversing hyperglycaemia, with a

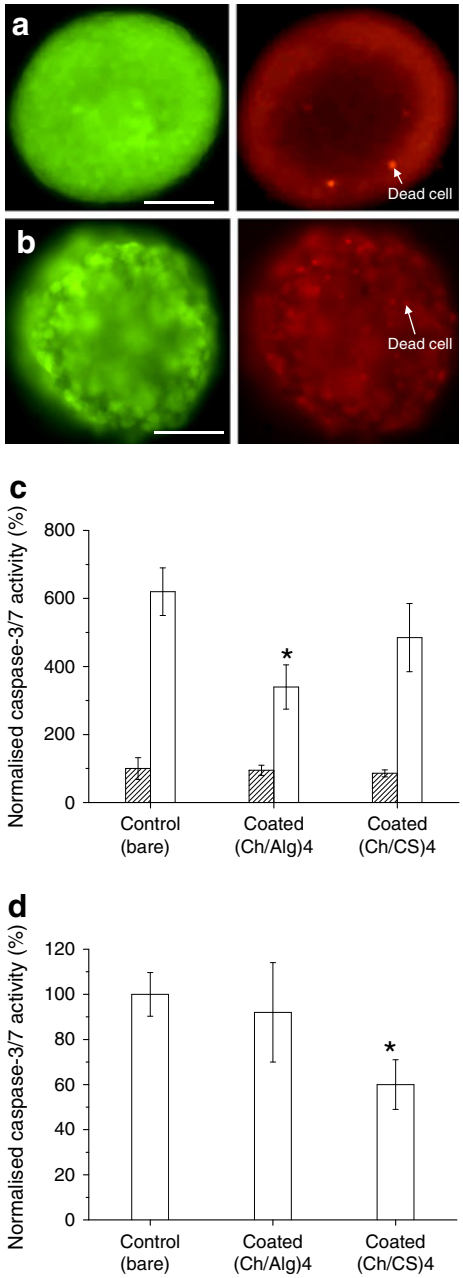

Fig. 4 Live–dead cell staining in coated islets. Nano-coated islets within eight layers of PC-chitosan and alginate were stained with fluorescein diacetate (green) and propidium iodide (red), and visualised (**a**) after 1 week and (**b**) at 4 weeks post-encapsulation of maintenance in culture. Green shows viable and red (dots) non-viable cells/islets. Similar data on uncoated islets can be found in our previous paper [28]. **c** Activity of caspase-3 and -7 in the eight-layer-encapsulated and control (non-encapsulated) islets, with (white bars) and without (hatched bars) exposure to cytokine combination (IL-1β, TNF-α and IFN-γ). Data were normalised against the value of control non-coated islets without cytokines. Ch, PC-chitosan; Alg, alginate; CS, chondroitin-4-sulphate. **d** Activity of caspase-3 and -7 in islets as above (**c**) after 24 h in culture with 50% rabbit serum. Data were normalised against the value of control non-coated islets exposed to the serum. Values (**c, d**) are mean±SD; $n=3$; *$p<0.05$ for PC-chitosan/Alg or PC-chitosan/condroitin-4-sulphate-coated islets vs control islets

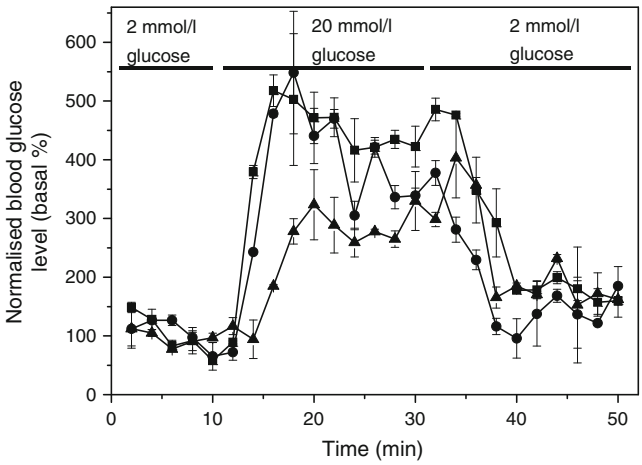

Fig. 5 Dynamic insulin production in vitro in control (non-encapsulated) and encapsulated (PC-chitosan, alginate, condroitin-4-sulphate) Balb/C mouse islets in response to changes in extracellular glucose. Insulin secretion in response to the glucose challenge as a function of time was studied in a temperature-controlled cell perifusion system. Non-encapsulated islets (black squares) and encapsulated islets with four (black circles) and eight layers (black triangles) are shown. Periods of glucose stimulation and glucose concentration were as indicated. Values are mean±SD; n=4

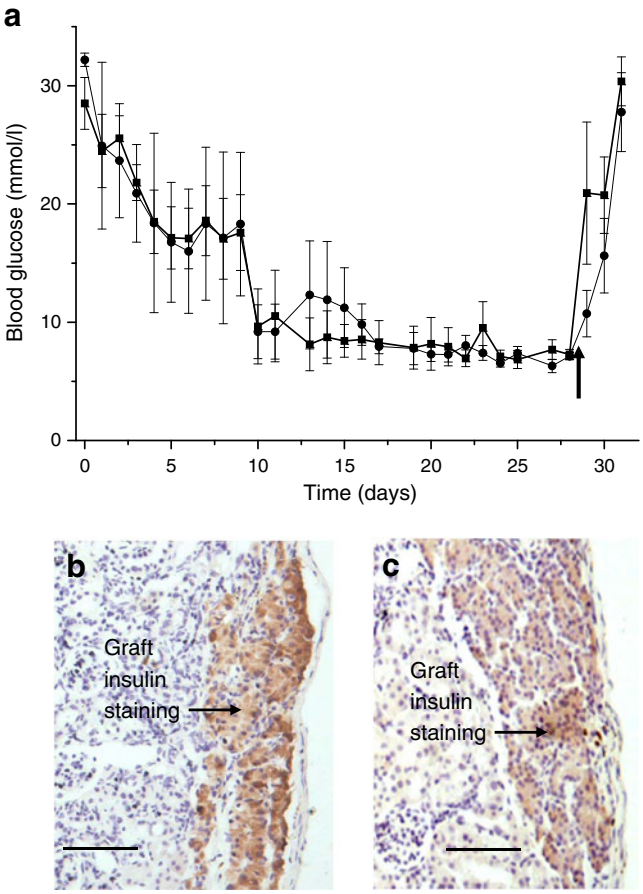

Fig. 6 Syngeneic transplantation of the encapsulated islets. **a** STZ-induced hyperglycaemia was reversed by implantation of 300 islets of either control (black circles) islets or islets encapsulated with four layers (PC-chitosan, alginate, PC-chitosan, PC-condroitin-4-sulphate) (black squares). Graft removal (upright arrow) caused reversion to hyperglycaemia. Values are mean±SEM; n=6. **b** Histological analysis of recovered representative graft sections (5 μm) of encapsulated and (**c**) non-encapsulated (control) islets using insulin immune-staining (brown). Scale bars 50 μm

time course similar to that of control islet grafts, in agreement with the functionality displayed in in vitro analyses. All mice reverted to the hyperglycaemic state on removal of the graft-bearing kidney (Fig. 6a). Subsequent histological assessment of graft material retrieved 28 days after implantation showed the presence of insulin-positive islet cell mass in the graft (Fig. 6b, c). There were no morphological differences between control and encapsulated islet grafts, with normal patterns of immunostaining for insulin, and no detectable major T cell (CD3 as the biomarker) and host macrophage (CD68 as the biomarker) infiltration at the graft sites (ESM Fig. 2).

In vivo assessment of nano-scale encapsulated islet function: allogeneic transplantation To assess whether encapsulation offers effective protection against immune rejection, we also performed studies using an allogeneic islet transplantation model, in which 300 Balb/c islets (H-2d) were transplanted below the kidney capsule of streptozotocin-induced diabetic C57BL/6 mice (H-2b). Transplanted islets reversed hyperglycaemia within 2–4 days. Animals implanted with control islets reverted to hyperglycaemia (blood glucose >11.1 mmol/l) within 10 to 14 days (median survival time 12 days) (Fig. 7a), consistent with graft rejection by the host immune system. Subsequent histological analysis of the non-encapsulated islet graft material revealed complete loss of the islet mass with little or no immunoreactive insulin being detectable (Fig. 7d). T lymphocytic and macrophage recruitment at the subcapsular implantation site was observed (ESM Fig. 3). Islets nano-encapsulated with four layers also reverted to hyperglycaemia, albeit a few days later (median survival time 15 days), suggesting that this level of encapsulation was not sufficient to prevent immune rejection (ESM Fig. 4).

Protection from immune assault was more successful in a further study, in which we implanted islets coated with eight layers in the allogeneic model. In this study, the graft material was retrieved after about 1 month for histological analysis. Nano-scale encapsulated islets maintained normoglycaemia for 28–37 days (Fig. 7b) in five of seven mice. Reversion to hyperglycaemia for mice receiving encapsulated islet transplants was caused by graft-bearing kidney removal. Two mice receiving the encapsulated islets reverted to hyperglycaemia in less than 28 days due to rejection.

To determine whether transplanted islets secrete insulin and maintain glycaemic control in response to increases in blood glucose levels, we carried out intraperitoneal glucose tolerance tests at 4 weeks post-transplantation in mice that

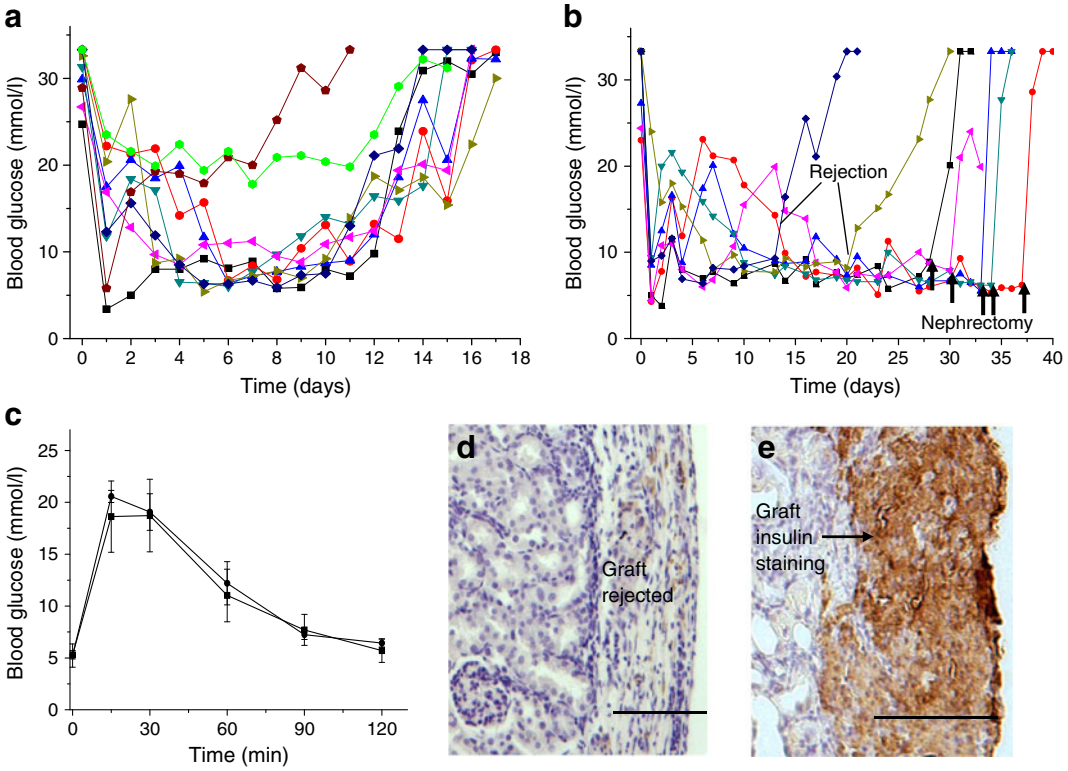

Fig. 7 Allogeneic transplantation of non-encapsulated (**a**) and eight-layer-encapsulated ((PC-chitosan and alginate)$_3$ + PC-chitosan + PC-condroitin-4-sulphate) (**b**) Balb/c islets reversed STZ-induced hyperglycaemia in diabetic C57BL/6 mice. Blood glucose concentrations of the individual animals (colour + symbol) are shown. Upright arrows (**b**), day of nephrectomy. **c** Intraperitoneal glucose tolerance tests were performed 4 weeks post-transplantation on mice with encapsulated islets (black circles) and on non-diabetic, non-transplanted mice as controls (black squares). Values (**a**–**c**) are mean±SEM; n=5; (**c**) $p>0.05$ for each data pair. **d** Histological analysis (insulin immune-staining, brown) of recovered representative graft-bearing kidney capsules in control non-encapsulated islets and (**e**) eight-layer-encapsulated islets. Scale bars 50 μm

had remained normoglycaemic. Figure 7c shows that the change in glucose level was similar to that of non-diabetic mice. Histological analysis of the recovered grafts showed significant amounts of insulin-positive tissue (Fig. 7e) and no evidence of infiltration of T cells into the islets, although a few macrophages were detected (ESM Fig. 3).

Discussion

We describe here a new protocol for nano-scale encapsulation of pancreatic islet cells and for the first time demonstrate in an animal model improved post-transplant survival of such coated islet cells in comparison with uncoated islets.

Alternate layers of PC-grafted polysaccharides (chitosan and chondroitin-4-sulphate) were used with alginate as the coating materials, with the binding being based on electrostatic complexation. Multilayer film formation is possible because of charge reversal on the film surface after each adsorption step. The polysaccharides we used contain at least one charge in each monosaccharide unit, which serves as the binding site, offering hundreds of binding sites for each polysaccharide molecule, thus providing a strong affinity to the charged cellular surfaces. The rationale for using these polymers in the layer architecture was based on our in vitro studies, which identified potential complement- and inflammation-inhibitory activities of these polysaccharides (Fig. 3c, d). Any success of islet encapsulation in extending graft survival in this study is likely to be dependent to a large part on the ability of the nano-coating to control the local transplant microenvironment and restrict immune cell infiltration, essentially blocking a variety of protein–protein interactions involved in complement (molecular mass 500 kDa), macrophage and T cell activation. In this regard, we introduced the protein-repelling zwitterionic PC modification (Fig. 1) in the coating constructs to minimise interactions between islets and the environment. The PC moiety, which is a component of plasma cell membranes, confers hydrophilicity, haemocompatibility and resistance to non-specific protein absorption, thus inhibiting the development of fibrosis, supporting endothelial cell growth [36] and also carrying anticoagulatory properties [37, 38], all of which should enhance islet survival in vivo and encourage host integration. In addition, the 40% amine group content of the PC modification for

chitosan increased the solubility of the polysaccharide up to about 2 mg/ml under physiological pH conditions (pH ~ 7.0).

Using the layer-by-layer deposition technique, a defined multilayer coating containing PC modification could be immobilised on the islet cell surfaces (ESM Fig. 5). The thickness (80 nm) of a typical eight-layer coating, as measured by TEM, is consistent with that previously reported with similar polymer sizes (200 nm with 20 layers) [39]. The addition of a polymer, chondroitin-4-sulphate, that carried a strongly ionic sulphate group into the complexation pair confers anti-coagulatory properties to the coating. It also contributes greatly to the stability of the layered nanofilm [40], making it a very durable coating even under harsh physiological conditions.

The nano-scale encapsulated mouse islets were found to preserve appropriate islet secretory function and survival in vitro. This indicates that the multilayers of the polysaccharides were deposited non-covalently on to the cell surfaces without perturbing cellular physiology or compromising cell survival. Pro-inflammatory cytokines, including IFN-γ, TNF-α and IL-1β, are major products of activated effective T cells and macrophages, and are known to be damaging to pancreatic islets via apoptosis induction [41]. In this study, we found that the nano-coating also rendered the islets less susceptible to cytokine- and complement-induced apoptosis (Fig. 4c, d). In addition, we found that the PC-displayed nano-coating effectively inhibited specific adsorption of large molecules of the immune systems (IgG, 150 kDa) on to the islet cell surfaces (ESM Fig. 6), indicating the effectiveness of the non-fouling PC-modification of the coating materials.

We used the syngeneic transplantation model to assess the in vivo secretory functionality of nano-scale encapsulated mouse islets and their ability to reduce hyperglycaemia and maintain normoglycaemia thereafter. These studies were used to avoid any influences of graft-versus-host immune rejection on islet function, while focusing on how the coating layers could impact on the immediate inflammatory reaction. Our in vivo tests using the syngeneic mouse transplantation model showed no deleterious responses from host animals to the coating materials, suggesting the materials used for encapsulation are non-toxic. Allo-transplantation studies in present work have shown that tailored encapsulating layers could optimise islet function post-implantation, allowing a degree of protection against inflammation and immune rejection in the majority of the studies. In the current study, nano-scale encapsulated islets were responsive to a hyperglycaemic environment, secreted appropriate amounts of insulin to restore normoglycaemia and survived for an extended period in vivo.

The failure of encapsulation to prevent rejection in two of the seven animals studied is of note and may indicate incomplete coating before transplantation or degradation at some time after transplantation. Future studies will need to test whether the robustness or completeness of the coating can be improved.

Since the purpose of our study was to prevent or reduce early-stage islet loss, we considered that a 4–5 week post-transplant observation period was appropriate. The rejection in controls normally occurs at 10–14 days (Fig. 7a), so the chosen study period showed that graft survival was twice as long. We have to look at grafts when the animals are normoglycaemic to determine whether there are any signs of graft destruction or whether the graft has a normal histological appearance. A long-term study of the function and graft survival with this multilayer coating scheme has not yet been performed. Nevertheless, unlike the arguably more expected durability of alginate microcapsules [15, 42], the nano-layers used in this study, while being non-biodegradable, are not expected or intended to last for many months under in vivo conditions. Rather, we propose that nano-scale encapsulation is an alternative technology that may promote engraftment by allowing intraportal administration and by limiting early islet loss caused by exposure to an inflammatory environment. In fact, our in vitro cytokine exposure study (Fig. 3c, d) confirmed that the encapsulation layers were protective against the cytotoxic effects of cytokines and complement proteins, as assessed by a reduction of apoptosis. Thus by the integration of graft tolerance induction mechanisms, the encapsulated environment may promote the development of regulatory T cells, which inhibit unintended host immune system activation, thereby achieving permanent survival of the transplanted islets [43, 44].

Studies are now needed to test whether modifications of the nano-layers will enhance engraftment and prevent rejection of islets in the longer term. This may involve: (1) incorporating bioactive molecules such as complement and coagulation inhibitors into the layers to prevent islet loss caused by the instant blood-mediated inflammatory reaction; (2) incorporating anti-inflammatory agents to reduce localised inflammation and fibrosis; or (3) incorporating natural and/or artificial extracellular matrices to provide optimal cell functioning after engraftment. Since there is little or no increase in size and volume of the islets after encapsulation, we expect this protocol will be potentially suitable for hepatic implantation via intraportal infusion. Such an option now needs to undergo experimental testing.

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Contribution statement ZlZ contributed to the conception and design of the study, performed the experiments and wrote the manuscript. AK contributed to the interpretation of in vivo data. AJFK contributed to the conception and design of in vivo experiments. PMJ contributed to the conception and design of the study, and reviewed the manuscript. JCP contributed to the conception and design and reviewed/edited the manuscript. All authors have read and given critical input during preparation of the manuscript and all have approved the final version.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Electronic supplementary material

Detailed Research Design and Methods

Reagents and materials. Tissue culture reagents, including RPMI-1640 medium, penicillin, streptomycin, fetal bovine serum, chitosan (low molecular weight, 50−190 kDa, 75% deacetylation) and alginic acid sodium salt from crown algae, viscosity of 2% solution at 25 °C, 250 cps, molecular weight, 75−100 kDa, poly-L-lysine (40-60 kDa) and chondroitin-4-sulphate (20 kDa) were from Sigma-Aldrich (Poole, UK). Rabbit polyclonal antibody to insulin and rabbit polyclonal antibody to CD68 were purchased from Gene Tex (Irvine, CA, USA). Rabbit anti-human T cell CD3 (cross-reacted to mouse T cell CD3) were provided by Sigma. Goat anti-rabbit IgG (H+L)-HRP conjugate was provided by Alpha Diagnostic International (San Antonio, TX, USA). Dako Liquid DAB + substrate chromogen system was purchased from Dako (Glostrup, Denmark). Recombinant mouse IL-1β, TNF-α and IFN-γ (carrier-free) were purchased from Biolegend (San Diego, CA, USA). Alexa Fluor 647-labelled poly-L-lysine and PC-modified chondroitin-4-sulphate was prepared using the previously reported procedures [Zhi ZL et al. *Biomacromolecules* 2010, 11, 610-616]. PC-chitosan conjugate was prepared by the procedure reported by Miyazawa and Winnik (Miyazawa, K., Winnik, F. M. *Macromolecules* 2002, 35, 9536– 9544).

TEM analysis of the encapsulated islets. Encapsulated islet ultrastructure was studied with transmission electron microscopy (TEM). Briefly, the islets were fixed overnight in

2.5% (vol/vol) glutaraldehyde and 0.1 mol/l cacodylate buffer. The islets were post-fixed for 20 min in 1% (wt/vol) OsO_4 dissolved in cacodylate buffer, and then dehydrated in graded series of ethanol. The islets were finally embedded in Agar 100 Resin (Agar Scientific, Stansted, UK). Sections were cut, contrasted, and examined in an Hitachi H7600 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

Fluorescence-labelling for confirmation of the nanocoating. Eight layers were deposited to give an encapsulation with the incorporation of a layer of poly-L-lysine labelled with Alexa Fluor 647 into the 7th layer as a fluorescent marker. The islets were assessed by fluorescence microscopy. The islets were then fixed in 4% (vol/vol) neutral-buffered formalin, dehydrated in graded series of ethanol and xylene, and then paraffin-embedded (at 70 °C); the cooled specimen were sectioned at a thickness of 5 μm and observed by fluorescence microscopy. The deposition of the layers was also assessed by fluorescence microscopy following the incorporation of two layers of PC-chitosan-tagged with FITC into the 5th and 7th layers. However, the histological images can not be obtained due to poor photostability of the dye.

The building-up of the multilayers on individual islets was also monitored by using FITC-labelled chitosan-PC. Eight layers (four bilayers) of FITC-chitosan-PC/alginate were deposited on islets. The fluorescence intensity increases with increasing the number of bilayers on the islets, as is shown in ESM Fig.1 & 5. The fluorescence intensity was obtained by reading the intensity of pixels in the histograms using Photoshop software.

Live-dead cell assay of the encapsulated islets in culture. The long-term cell survival in culture was tested by a two-colour fluorescence live/dead cell test involving the use of PI and FDA (Jones KH & Senft JA. *J Histochem & Cytochem* 1985, 33, 77-79). The staining process involved removing medium from cells, adding working solutions of PI/FDA to cells containing 2 pg of FDA plus 0.6 pg of P1 in 0.2 mL, resuspending the cells, and incubating for 3 min at room temperature. This was followed by removing the staining solution and washing cells with 0.5 mL of PBS three times. The cells were then viewed under a fluorescence microscope with 478−495 nm and 530−560 nm excitation filters and 510−555 nm and 573−648 nm emission filters, respectively. This allowed green (viable) and red (dead) fluorescing cells to be imaged separately. The cell survival experiments were carried out after one and four weeks of culturing. Note this method could not used to distinguish whether the dead or living cells are alpha or beta cells in an islet.

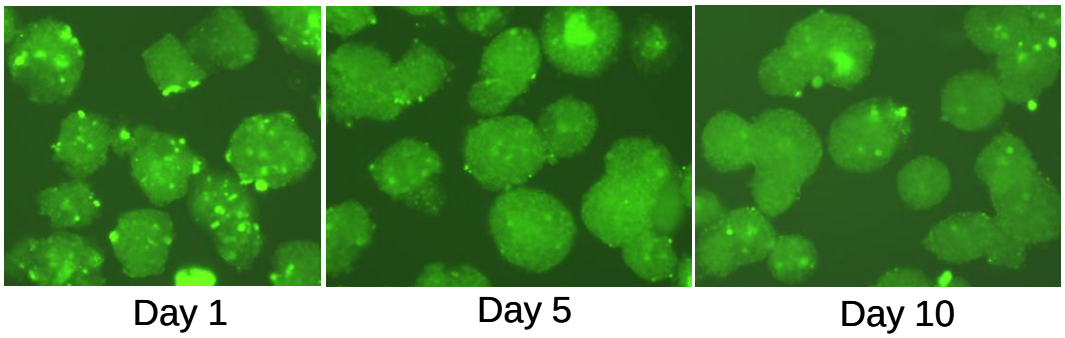
Effect of exposure to the apoptosis-inducing cytokines and complement factors of the nanoencapsulated islets. Islets isolated from BALB/C mice were encapsulated with 8 layers of PC-chitosan/alginate by layer-by-layer deposition. Following the encapsulation, the experimental cells (50 islets in 2 ml medium) were exposed to a mixture of IL-1β (1 ng/ml), TNF-α (5 ng/ml) and IFN-γ (5 ng/ml) in the culture medium or to 50% diluted rabbit serum at 37 °C for 48 h. The incidence of islet cell apoptosis (5 islets and 50 μl medium in each well) was tested using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Southampton, UK) and read using a Spectra Max Gemine EM microwell plate

reader (Molecular Devices, Wokingham, UK). Islet cell viability was also assessed separately by the CellTiter-Blue Cell Viability assay (Promega).

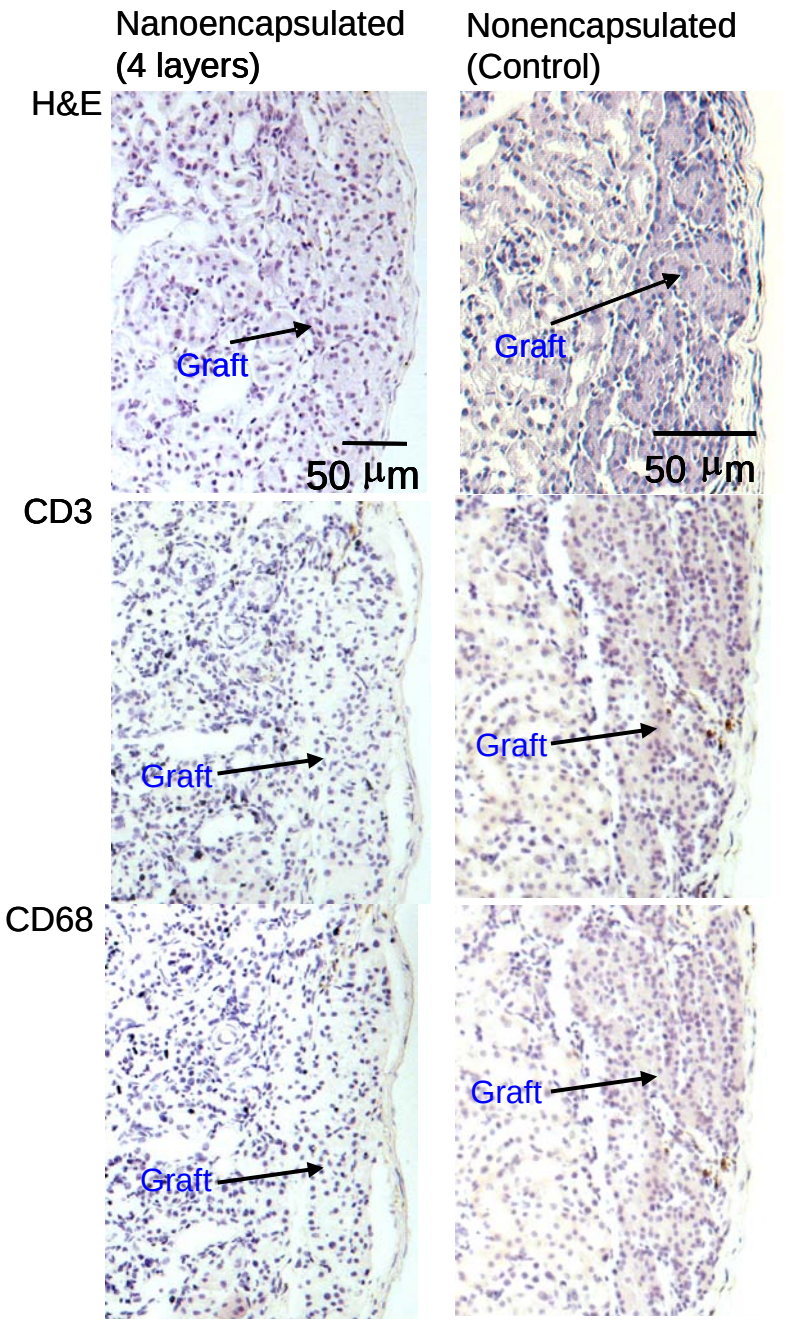
Antibody Exclusion Assay. An antibody exclusion assay with FITC-labeled antimouse major histocompatibility antigens (MHC) Class II antibody (eBioscience, Hatfield, UK) was used to test the permeability of the nanocoating to large immunologically relevant molecules. Twenty of the naked and nanoencapsulated islets (live cells without fixing) were added to 100 μl of the blocking buffer (2% BSA and HBSS buffer), and then 1 μl of antibody-FITC solution was added. The solution was then incubated at 37 °C for 30 min. The islets were then rinsed three times with HBSS-0.1% Tween (0.5 mL) to remove all unbound dye. The islets were then transferred to a coverslip for observation with a fluorescence microscope. As shown in ESM Fig. 6, a bare islet showed clear binding of the antibody-FITC probe to the surface of some individual cells on the islet face. The image of an 8-layer-coated islet shows exclusion of the antibody probe, indicating a complete nanolayer coating. Low levels of fluorescence were detected around the surface of the capsules surrounding the islet, suggesting some nonspecific antibody adsorption on the surfaces.

Graft morphology, histology and immunohistochemistry. After unilateral nephrectomy at the end of the study, graft-bearing kidneys were fixed in 4% (vol/vol) neutral-buffered formalin and later paraffin-embedded, the specimen was sectioned at a thickness of 5 μm. The paraffin sections were re-hydrated by sequential 5 min incubations in xylene (twice), 100% ethanol, 95% ethanol and then 70% ethanol. The re-hydrated sections were

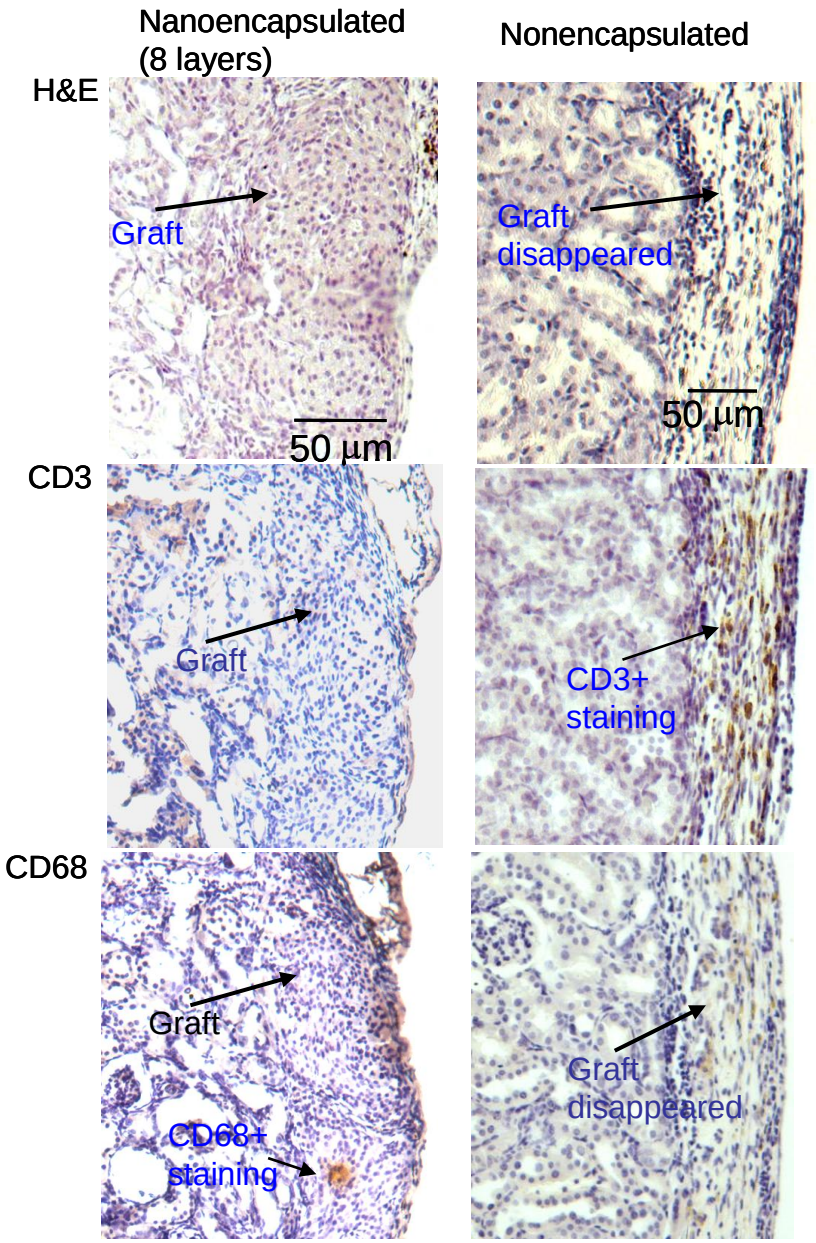
stained with haematoxylin or haematoxylin/eosin for routine morphology studies (ESM Fig 2 &3). The graft sections were further evaluated by immunohistchemical staining of the kidney sections with antibodies targeting insulin and two types of immune cells (CD3+ and CD68+). No further study is performed in the CD68+ staining to clarify whether the infiltrated macrophages (CD68+) belong to proinflammatory 'M1' phenotype that exacerbate islet cell damage, or to anti-inflammatory 'M2c/suppressor' one that promote epithelial and vascular repair (Anders HJ, Ryu M *Kidney Intern* 2011, 80: 915-25). Haematoxylin/eosin staining was also shown. The de-waxed sections were microwave-heated in 10 mmol/l citrate buffer (pH 6.0) with 0.05% Tween at 100 °C for 20 min to retrieve the antigen (the microwave was initially set at 700 Watts, after boiling, it was adjusted to 120 Watts to keep boiling minimal). The samples were then blocked using 1% BSA in PBS buffer for 10 min and incubated with primary antibody (diluted with the same blocking buffer) for 1 h followed by horseradish peroxidase-conjugated rabbit anti-rabbit IgG (H+L) secondary antibody. Bound peroxidase was colour-developed with the liquid DAB and substrate chromogen system containing 3,3'-diaminobenzidine. Sections were then counterstained with haematoxylin or haematoxylin/eosin to identify the presence of nuclei of the cells in the section.



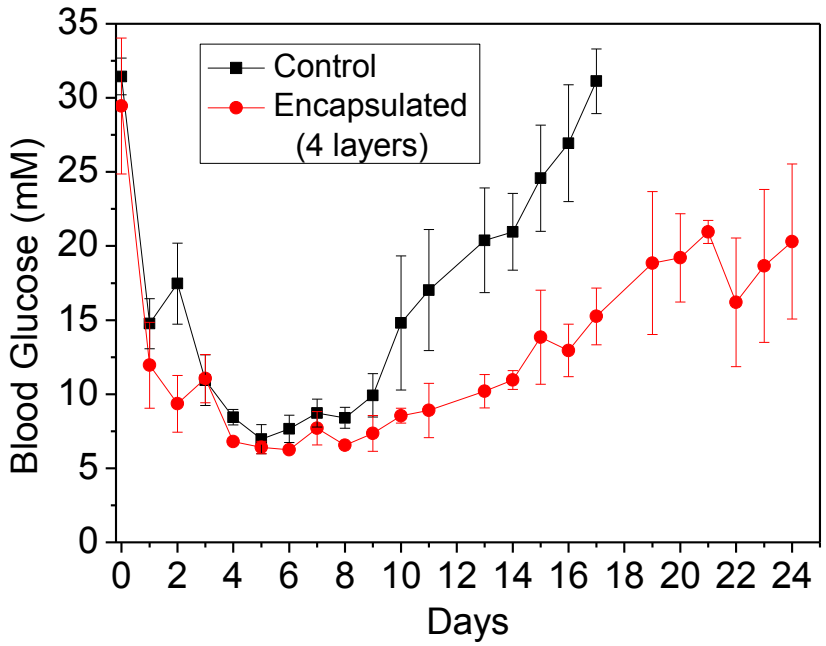
ESM Fig 1. Time course monitoring of the stability of chitosan/alginate multilayers deposited on islets in culture. Fluorescence was generated by incorporation of two layers of PC-chitosan-tagged with FITC into the 5th and 7th layers. Note the real fluorescence intensity may decrease with time. The exposure time of the camera thus increased accordingly to get clear images.



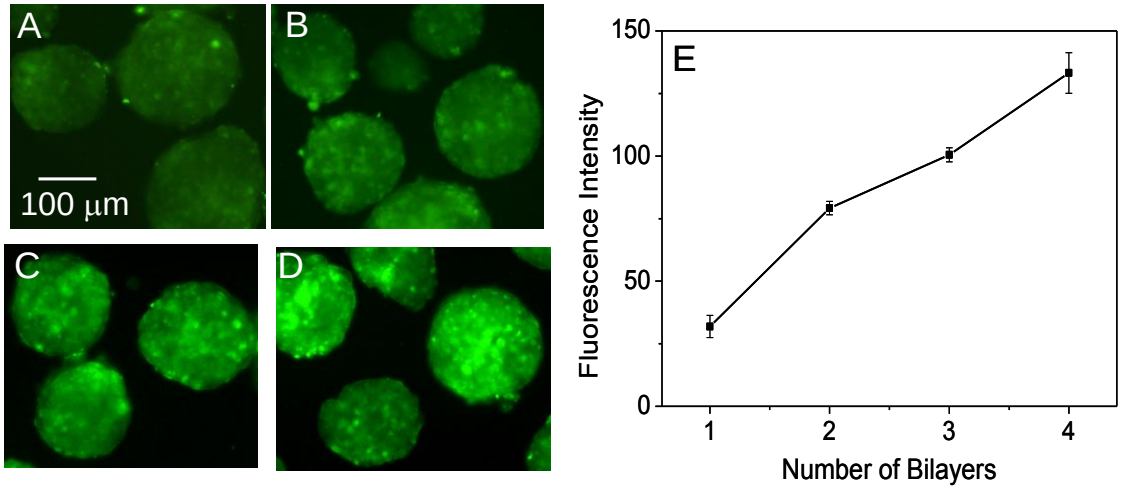
ESM Fig. 2. Immunohistochemical analysis of cross sections of the kidney capsules of C57BL/6 mice transplanted with the nanoencapsulated and bare syngeneic C57BL/6 islets. Kidney sections were stained for the presence of immune cells (CD3+ and CD68+ cells). Haematoxylin/eosin staining was also shown.



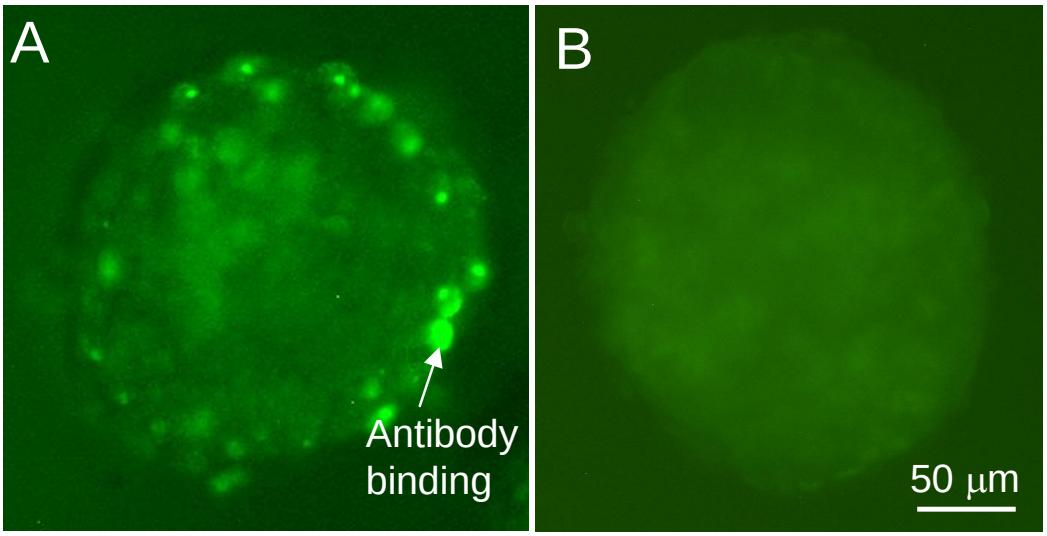
ESM Fig. 3. Histochemical and immunohistochemical analysis of the cross-sections of the kidney capsules of C57BL/6 mice allotransplanted with the nanoencapsulated (8 layers) and bare Balb/C islets. Kidney sections were stained for the presence of immune cells (CD3+ and CD68+ cells).



ESM Fig. 4 Allogeneic transplantation of non-encapsulated (control) and 4-layer-encapsulated (PC-chitosan/alginate/PC-chitosan/PC-condroitin-4-sulphate) Balb/c islets reversed STZ-induced hyperglycaemia in diabetic C57BL/6 mice. Average blood glucose concentrations of five individual animals are shown.



ESM Fig. 5 Monitoring of the stepwise build-up of the polysaccharide layers on mouse islets. Chitosan-PC (Ch-PC) conjugated with FITC was incorporated into the multilayers by alternative deposition with alginate (Alg) on the islet surfaces. Bright spots may due to the density difference of either cells or extracellular matrix. A, FITC-Ch-PC/Alg; B, $(\text{FITC-Ch-PC/Alg})_2$; C, $(\text{FITC-Ch-PC/Alg})_3$; D, $(\text{FITC-Ch-PC/Alg})_4$; E. Fluorescence intensity measured on islet surfaces increases with the increase of number of bilayers (Ch-PC/Alg).



ESM Fig. 6 IgG recognition of the uncoated and coated islets (live cells). A: binding of anti-MHC II-FITC to the individual cells in an islet. B: exclusion of the antibody binding by the nanocoating.