3D PRINTED 'NARROWED CHANNEL' MACROENCAPSULATION SYSTEM FOR PANCREATIC ISLET TRANSPLANTATION

A DISSERTATION SUBMITTED

By

TREESA JOY

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DECLARATION

I, Treesa Joy, hereby declare that I had personally carried out the work depicted in the thesis entitled "3D printed 'narrowed channel' macroencapsulation system for pancreatic islet transplantation" under the supervision of Dr. Lynda V. Thomas, Scientist D, Division of Tissue Engineering and Regenerative Technologies, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "3D printed 'narrowed channel' macroencapsulation system for pancreatic islet transplantation submitted by Treesa Joy in partial fulfilment for the degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at the Division of Tissue Engineering and Regenerative Technologies, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram, 695012.

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The Dissertation

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Submitted

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For

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LIST OF ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
ASC	Adult Stem Cells
ATR	Attenuated Total Reflection
BLI	Bioluminscence Imaging
BMSC	Bone Marrow Stem Cells
CIJ	Continuous Inkjet
DEXGEL	Dextran and Gelatin
DM	Diabetes Mellitus
DMEM-HG	Dulbecco's Modified Essential Medium – High Glucose
DMSO	Dimethyl Sulfoxide
DOD	Drop-On- Demand
DSC	Differential Scanning Calorimetry
DTGS	Deuteriated Triglycine Sulphate
EGDMA	Ethylene Glycol Dimethacrylate
ELISA	Enzyme Linked Immunosorbent Assay
ESC	Embryonic Stem Cells
ESEM	Environmental Scanning Electron Microscopy
FBS	Fetal Bovine Serum
FDM	Fused-Deposition Modeling
FITC	Fluorescein Isothiocyanate
FTIR	Fourier Transform Infrared Spectroscopy
GAD	Glutamic Acid Decarboxylase
GDM	Gestational Diabetes Mellitus
GFP	Green Fluorescent Protein

GI	Gastrointestinal
GLP-1	Glucagon-like Peptide-1
HEMA-2	Hydroxyethyl Methacrylate
hES	Human Embryonic Stem cells
IBMIR	Instant Blood-Mediated Inflammatory Reaction
IDDM	Insulin-Dependent Diabetes Mellitus
ILC	Islet Like Clusters
IPN	Interpenetrating Networks
KRBH	Krebs Ringer Bicarbonate HEPES
MCP-1	Monocyte Chemoattractant Protein
NIDDM	Non Insulin-Dependent Diabetes Mellitus
OD	Optical Density
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PU	Polyurethane
SLA	Stereolithography
SLS	Selective Laser Sintering
TGA	Thermogravimetric Analysis
TGF-β	Transforming Growth Factor beta
TNF-α	Tumor Necrosis Factor-α
TMB	3,3',5,5'-Tetramethylbenzidine1

SYNOPSIS

As per the World Health Organisation (WHO) statistics, in 2008 an estimate of 347 million people in the world had diabetes which is continuously and fast growing particularly in the low- and middle income countries. India recorded 69.2 million people who are living with diabetes (8.7% of global statistics) as per the 2015 data. Of these, it remained undiagnosed in more than 36 million people. Pancreatic β islets are responsible for producing the hypoglycemic hormone, insulin. Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin. In an estimated 80 million people worldwide; 5-10% are classified Type I and are dependent on exogenous insulin for life. Despite attempts to control blood glucose levels with diet, exercise and exogenous insulin therapy, however, patients continue to develop serious vascular and neurological complications, including blindness and renal failure highlighting the need for alternate therapy. In the long term, the alternate therapy of pancreatic islet transplantation in humans is limited by relatively low availability of human islets and immune rejection which occurs in the case of allogenic or xenogenic transplantation and also immunosuppression drug therapy. A possible solution might be to transplant xenogenic islets by a permselective synthetic membrane, which isolates transplant from its environment and also allows the low molecular weight insulin and glucose permeate while prevent the entry of immunoglobulins.

Encapsulation of cells within a synthetic 3D printed membrane prior to transplantation is a novel technique to prevent immunorejection of cells and avoid the use of toxic immunosuppressive drugs. The broader clinical use of organ/cell transplantation is hampered by the shortage of human organ donors as well as the need for a permanent immunosuppressive drug therapy inorder to avoid immune rejection. Immunoisolation of cells by 3D printed polymeric membrane which has narrowing pore channels has been conceived with the aim of permitting the transplantation of xenogenic cells, without recourse to immunosuppressive therapy.

In this present study we are attempting to fabricate 3D printed polyurethane membrane for macroencapsulating the islet cells which are microencapsulated in the alginate beads. Polyurethane is a hydrophobic, nonbiodegradable synthetic polymer which was used for 3D printing for the application of macroencapsulation. Alginate is a biodegradable natural polymer which was used for microencapsulation of islet cells. This system was shown to have functional islet cells.

The first chapter gives an overview of diabetes, its types, global statistics, and existing treatments- both pharmacological and non-pharmacological. Also includes islet transplantation and their limitations, immune isolation strategies, fabrication methods for immune isolation bags.

The second chapter describes the various materials and methods used for the fabrication of the immune isolation membrane bags using polyurethane, characterization of the membrane bags by Fourier Transform Infrared Spectroscopy (FT-IR), Environmental Scanning Electron Microscopy (ESEM), Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC),Contact angle measurement, swelling and degradation studies, permeation studies. The procedures for isolation of islets from rabbit, characterization of islets by dithiazone staining, cytotoxicity assays of membrane, cell viability assessment using calcein and ethidium homodimer, glucose challenge assay of the encapsulated islet cells are detailed in this chapter.

The third chapter includes the results and discussion of the study. PU membranes were successfully 3D printed upto 10 layers which has a size of 2.5cm X 2.5cm and alginate beads of 2.61mm size. ESEM images showed the formation of pore channels by the 3D printing and thermal stability was assessed by TGA and DSC. Swelling and degradation values have no significant difference between consecutive time points. Cytotoxicity assay of the membrane indicated that the 3D printed membrane is non-

cytotoxic to cells. The encapsulated islet cells are viable even after 21 days and functionally active till 7 days.

The fourth chapter summarizes the study, conclusion drawn and future perspectives of the study are elaborated in this chapter. PU membrane was successfully printed and islets were encapsulated. Viability and functionality of encapsulated islet cells are maintained which are compared to non-encapsulated cells. This will be a promising strategy in pancreatic islet cell transplantation wherein the narrowed pore channels allows for selective permeation of glucose and insulin and prevent the entry of the higher molecular weight immunoglobulins.

Future studies include functional characterization of islet cells after encapsulation, evaluation of the effectiveness of the 3D printed islet bags in maintaining the functionality for extended time points, real time PCR studies for gene expression are also envisaged. Also the work has to be extended to testing in *in-vivo* models to ascertain the preclinical efficacy and functionality of these 3D printed islet bags.

1. INTRODUCTION AND LITERATURE REVIEW

Diabetes mellitus is one of the rapidly rising threats to human health in the 21st century and it imposes socio economic burden on the society[1]. According to the International Diabetes Federation, in 2011 over 300 million people around the world have diabetes and are expected to rise to 500 million within next 20 years. The global prevalence of diabetes is changing from the developed countries to the developing countries. India recorded 69.2 million people who are living with diabetes (8.7% of global statistics) as per the 2015 data[1]. Of these, it remained undiagnosed in more than 36 million people. This is a chronic metabolic disease characterized by the elevation of glucose levels in blood either due to inherited and/or acquired deficiency in production of insulin by the pancreatic islets, or by the ineffectiveness of the insulin produced. These patients have an increased risk of developing a number of serious problems such as abnormalities in the metabolism of carbohydrate, protein and/or fat which in turn results in micro-vascular/macro-vascular complications and neuropathies. Population based studies shows that a substantial proportion of populations found to have diabetes were not previously diagnosed. Many people remain undiagnosed with diabetes because there are few symptoms of diabetes during the early years of diabetes.

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. It results from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism. Frequent urination, increased thirst, and increased hunger are the common symptoms of high blood glucose level. Acute complications can include diabetic ketoacidosis, nonketotic hyperosmolar coma, or death[2]. Serious long-term complications include heart disease, stroke, chronic kidney failure, foot ulcers, and damage to the eyes. The main cause of diabetes is either that the pancreas is not

producing enough insulin or the cells of the body are not responding properly to the insulin produced. There are three main types of diabetes mellitus:

- Type 1 DM results from the pancreas's failure to produce enough insulin. Previously this is referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes".
- Insulin resistance is the main reason for Type 2 DM. It is a condition in which cells fail to respond to insulin properly. Lack of insulin also increases as the disease progress. This is also known as "non insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". The most common cause is excessive body weight and not enough exercise.
- Sometimes pregnant women without a previous history of diabetes develop high blood sugar levels. This is known as gestational diabetes.

1.1 TYPES OF DIABETES

1.1.1 Type 1 Diabetes (Immune-Mediated Diabetes)

Type 1 Diabetes is prevalent in 5-10% of diabetic patients. This results from cellular mediated autoimmune destruction of β-cells of the pancreas. So this is also called as insulin-dependent diabetes or juvenile- onset diabetes. Destruction of the β-cells are analysed by certain markers which include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65), and autoantibodies to the tyrosine phosphatases IA-2a and IA-2b[3]. These autoantibodies are present when fasting hyperglycemia is detected initially in 85-90% individuals. The rate of β -cell destruction is quite variable in Type 1 diabetes, being rapid in some individuals (mainly infants and children) and slows in others (mainly adults). Ketoacidosis may occur in some patients like children and adolescents as the first manifestation of the disease. At latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Children and

adolescents are most vulnerable to immune mediated disease, but it can occur at any age, even at any time period of life.

Autoimmune destruction of β-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined. Although patients are rarely obese with this type of diabetes, the presence of obesity is not incompatible with the diagnosis. These patients are also prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia.

1.1.2 Type 2 Diabetes

90-95% of patient with diabetes have Type 2 diabetes which is also referred as non–insulin-dependent diabetes, type 2 diabetes, or adult-onset diabetes. The main reason is insulin resistance and also relative (rather than absolute) insulin deficiency. These individuals do not need insulin treatment to survive often throughout their lifetime. There are probably many different causes of this form of diabetes. Autoimmune destruction of β-cells does not occur. Obesity is one of the reason for insulin resistance and most patients with this form of diabetes are obese. Whereas patients with this form of diabetes may have insulin levels that appear normal or elevated, the higher blood glucose levels in these diabetic patients would be expected to result in even higher insulin values had their β-cell function been normal. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Weight reduction and pharmacological treatment of hyperglycemia can improve insulin resistance but is seldom restored to normal. The risk of developing this form of diabetes increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior gestational diabetes and in individuals with hypertension or dyslipidemia, and its frequency varies[3]. It is often associated with a strong genetic predisposition, more so than is the autoimmune form of type 1

diabetes. However, the genetics of this form of diabetes are complex and not fully defined.

1.1.3 Gestational Diabetes Mellitus (GDM)

For many years, GDM has been defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Although most cases resolve with delivery, the definition applied whether or not the condition persisted after pregnancy and did not exclude the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy. This definition facilitated a uniform strategy for detection and classification of GDM, but its limitations were recognized for many years[3]. As the ongoing epidemic of obesity and diabetes has led to more type 2 diabetes in women of childbearing age, the number of pregnant women with undiagnosed type 2 diabetes has increased.

1.2. Current therapeutic approaches

Current therapy for diabetes involves oral antidiabetic drugs and insulin administration. Oral hypoglycaemic agents include sulphonylureas, biguanides, alpha glucosidase inhibitors, meglitinide analogues, and thiazolidenediones. The main objective of these drugs is to correct the underlying metabolic disorder, such as insulin resistance and inadequate insulin secretion. They should be prescribed in combination with an appropriate diet and lifestyle changes. Diet and lifestyle strategies are to reduce weight, improve glycaemic control and reduce the risk of cardiovascular complications, which account for 70% to 80% of deaths among those with diabetes. Diabetes is best controlled either by diet alone and exercise (non pharmacological), or diet with herbal or oral hypoglycaemic agents or insulin (pharmacological). The main side effects are weight gain and hypoglycaemia with sulfonyl ureas, gastrointestinal (GI) disturbances with metformin, weight gain, GI disturbances and liver injury with thiazolidinediones, GI disturbances, weight gain and hypersensitivity reactions with

meglitinides and flatulence, diarrhoea and abdominal bloating with alpha-glucosidase inhibitors. These approaches do not mimic the insulin secretory patterns of native β islets for the regulation of glucose in real-time. Whole pancreas transplantation holds promise towards a cure for diabetes, but this procedure requires major surgery and lifelong immunosuppression to prevent graft rejection. Transplantation of islet cells isolated from a donor pancreas has been shown to control glucose levels successfully. Being less invasive, it is a better alternative to pancreas transplantation yet scarcity of donors, maintenance of islet functions such as cell growth and survival *in vitro*, and concern over the adverse effect of lifelong immunosuppressant used to prevent graft rejection precludes the benefits of islet transplantation from becoming universally acceptable. It is an attractive alternative therapy to conventional insulin treatment or vascularized whole pancreas transplantation for type 1 diabetic patients. It represents a successful example of somatic cell therapy in humans based on complex procedures for islet isolation from whole pancreas. The islets, that are only 1% of the total pancreas tissue, are isolated by two steps method starting with collagenase digestion that operates a rapid dissociation of the stromal component of the gland, while preserving islet anatomical integrity.

Islet cell transplantation is an effectual treatment for improving glycemic condition in diabetic patients thereby reducing the late complications of disease[4]. In the early 1970's, Dr. Clyde Barker at the University of Pennsylvania and the late Paul Lacy at the Washington University in St. Louis were the pioneers in exploring the concept of islet transplantation as a means to cure diabetes. In 1972, Ballinger and Lacy reported amelioration of diabetes in islet recipient rats[5]. In 1973, Reckard and Barker were the first to show that islet transplantation could completely and permanently restore normoglycemia in rodent models of chemically induced diabetes[6].

Human islets isolation procedure is more complex than rodent islets isolation[7]. Ricordi's automated isolation method had given hope for the production of abundant islets for clinical use[8]. Scharp et al. performed the islets transplantation under

immunosuppression in diabetic patients and patients were insulin independent at the period of 22 days[9]. This was followed by several other cases, but success rates continued to be low. In 1999, Bretzel et al reported a markedly improved 3-month islet graft function rate of at least 75% in 24 consecutive patients[11]. In the 1-year follow-up of 37 patients, 24% had achieved insulin independence[10]. Between 1999 and 2005 about 650 patients were treated worldwide. Unfortunately, long-term results did not prove that promising.

The first successful islet allograft was reported in 1990 with steroid free immunosuppressant tacrolimus[12]. The success rate of islet transplantation became outstanding after the Edmonton trial in 2000, which described successful intraportal alloislet transplantation, defined as insulin independence, in 7 consecutive patients with hyperlabile diabetes and frequent episodes of hypoglycemia[13]. The success was partly ascribed to the usage of a steroid free immunosuppressive regimen which was a new combination of immunosuppressive drugs, consisting of sirolimus, tacrolimus and daclizumab, excluding the diabetogenic glucocorticoids and large numbers of donor islets[14].

Several studies have now demonstrated that islet transplant can replace pancreatic endocrine function without major side effects and with liver viability preservation in selected patients affected by long term type 1 diabetes. It can restore endogenous insulin secretion, achieve insulin independence in more than 80% of patients, and recover the metabolism of glucose, protein and lipids. Improved controls of glycated HbA1c, reduced risk of recurrent hypoglycemia and of diabetic complications are also seen as important benefits of islet cell transplantation, irrespective of the status of insulin independence.

Though islet transplantation research has made significant progress, concern over toxicity as well as cost of immunosuppressive therapy still remains. Insulin independence and long term graft survival were achieved for more than three years through a modified immunosuppressive protocol[15] even so the requirement of multiple

donors to obtain 10000 islet equivalents per kilogram of patient's weight remains unsolved. Although insulin independence remains the ultimate goal, today, stabilization of glucose levels and avoidance of hypoglycemia are considered to be the main indications for islet transplantation.

1.3. Alternate sources of pancreatic β cells

The scarcity of donor pancreas for islet transplantation is a major obstacle to the widespread use of islet transplantation which urged the focus towards alternate sources of β cells for future transplants. Several alternative means have been suggested which include use of xenogenic islets and immortalized beta cell lines[16]. Recent advances in the field of stem cell differentiation and regeneration therapy have focused on new ways to generate insulin-producing beta cells that can be used for transplantation. Several candidate cells have been identified including embryonic stem cells (ESC) and adult stem cells or progenitor cells residing in the pancreas or other organs. The differentiated beta cells have shown to regenerate by replication, which opens the possibility to generate novel beta cells *in vitro* and / or *in vivo* from pre-existing beta cells. Additionally, there are reports that show the successful use of liver cells, endocrine cells from the gut, and bone marrow derived stem cells as source to generate islets by cell transdifferentiation.

1.3.1 Xenogenic islets

In a xenogenic approach, islets from different species are used for transplantation purpose. Porcine islets serve as a potential source in view of the fact that porcine insulin differ from human insulin by 1 amino acid. Neonatal porcine islets were also induced to mature endocrine phenotype under *in vitro* and *in vivo* conditions[17]. Xenogenic tissues induce more vigorous rejection than that of allogenic tissue; hence immunosuppressant dosage should be high enough to prevent graft

rejection. Alternately, the cells of xeno origin can be immunoisolated by encapsulation technology to separate the transplanted cells from host immune system.

1.3.2 Stem cells to treat diabetes

Stem cells are non specialized cells which have the ability to self regenerate and differentiate into specialized cell types depending on the niche or external signaling cues[18]. Stem cells offer a limitless supply source for islets as well reduces the graft rejection problems[19]. Ideally stem cells used for cell based therapy should meet the following criteria[20].

- It should be available in abundant quantities (millions to billions of cells),
- harvest procedure should be less invasive,
- Have multilineage differentiation potential and could be efficiently transplanted to the host.

1.3.2.1 Embryonic Stem Cells (ESC)

Embryonic stem cells which are derived from the inner cell mass of pre-implantation blastocysts have gained the attention of researchers due to its pluripotent nature. Human embryonic stem cells (hES) hold promise for research and clinical applications. hES have some unique abilities as compared to all sources of adult cells: 1) the expansion of ESC in the undifferentiated state is nearly unlimited; and 2) ESC can give rise to all cell types including pancreatic insulin-producing beta cells. Many studies have reported the differentiation of mouse and human embryonic stem cells to islet like clusters[78] either by modifying the culture conditions or by genetic manipulation. Manipulation of the culture conditions with various growth supplements like insulin, transferrin, selenium and fibronectin (ITSFn), B27, bFGF and nicotinamide resulted in regulated secretion of insulin. Phosphoinositide kinase inhibitors have been reported to promote the differentiation of larger numbers of ESC towards functional β cells[79].

Though hES are versatile cells, ethical concerns on the use of human hES, and chances of teratoma formation[80] limits their usage. Direct transplantation of embryonic stem cells has reported to culminate in teratoma formation[81] from contaminating undifferentiated ESCs. Safe transplantation of hES could be attempted by viral vector mediated transfection *in vitro*, yet the risks associated with cytomegalovirus promoters in transfection cannot be ruled out.

1.3.2.2 Adult Stem cells

The potential use of adult stem cells offers the advantage of an autologous model whereby a patient's own cells can be used, thereby circumventing immune rejection. Adult stem cells (ASC) are multipotent cells capable of self renewal. They have been reported to be present in almost every tissue like bone marrow, blood, heart, liver, pancreas, adipose tissue and could be transplanted directly without genetic modification or pre-treatments. They exhibit high degree of genomic stability during culture conditions. ASC lack tissue specific characteristics but it could be differentiated to specialized cell types under the influence of appropriate signaling cues[82]. The stem cell microenvironment plays an important role in its differentiation to committed cells[83].The potential of adult human stem cells from various sources to differentiate to insulin producing cells have been explored by various research groups. The relative ease of isolating adult stem cells and their expansion makes it an ideal source for cell based therapy.

A) Pancreatic stem cells

Pancreatic progenitor/stem cells which are closely related with beta cell lineage represent an attractive source for generation of beta cells[84]. Human pancreatic ductal cells and islet stem cells have been expanded and differentiated to islet like clusters capable of producing insulin *in vitro* which were capable of reversing of diabetes in non obese diabetic mice thus normalizing blood glucose levels for more than 3 months[85]. Even though pancreatic cells seem to be the better source than embryonic

stem cells, the fraction of precursor cells isolated from pancreas is very less and heterogenous. Furthermore the harvest procedure from pancreas is also invasive thus limiting this source being applicable in clinical purposes.

B) Bone marrow stem cells (BMSC)

Bone marrow stem cells were induced to differentiate to mature endocrine pancreatic lineage *in vitro*[86]. The *in vitro* differentiation of human bone marrow stem cells (hBMSC) to endocrine pancreatic cell types were investigated by genetic manipulation using adenovirus coding for mouse transcription factors involved in the early phase of endocrine developmental pathway[87]. The results suggested that bone marrow stem cells shifted towards pancreatic endocrine phenotype with expression of insulin and other transcription factors involved in β cell development. Enhanced green fluorescent protein (GFP) system based genetic approach was utilized to study the differentiation of BMSC to islet like cells.

C) Adipose stem cells

Human subcutaneous adipose tissue, abundant and easily accessible serves as a potential source of adult mesenchymal stem cells. The harvest procedure by lipoaspiration / liposuction is less invasive. Adipose stem cells have been reported to exhibit an increased *invitro* proliferative potential than bone marrow stromal cells[88]. Adipose stem cells release cytokines TGF-β and IL-10 which are responsible for its immunomodulatory properties[89]. The immunosuppressive property of adipose stem cell has been exploited for the treatment of severe graft versus host disease[90]. The differentiation potential of these cells to pancreatic endocrine cells have been investigated by several research groups. Human adipose stem cells induced to islet like cells in serum free differentiation medium for 3 days exhibited an upregulation of pancreatic developmental transcription factors like Isl-1, Ngn3 along with islet hormones such as insulin, glucagon and somatostatin[91]. A novel protocol using taurine designed for islet differentiation generated 47-51% C- peptide positive cells when compared to reports where the yield was only 2-8%[92].

1.4 Limitations of Islet Transplantation

Although the field of islet transplantation has progressed rapidly, the long-term success of allogeneic islet transplantation remains questionable. Patients from the original Edmonton trial had an insulin-independence rate of approximately 10% at five years after transplantation[21]. This rate, based on a recent study, is as high as 50%, but the combination of an optimized immunosuppressive regimen and a sophisticated transplant center is required[22]. The reasons for long-term graft loss can be summarized into the two following categories.

(i) Immunosuppression Associated Factors. Islet recipients must take immunosuppressive medications to prevent allogeneic rejections. Any imperfect immunosuppressive protocol can lead to graft loss. But after long-term usage, even the optimized medications can be toxic to the transplanted islets directly or cause dysfunction of other organs[21]. In addition to the damage allogeneic rejection can cause to the transplanted islets, recurrence of autoimmune attacks on the transplanted islets has also drawn investigator's attention. Histological studies have shown that islet transplantation triggers recurrent autoimmune effects that can cause -cell destruction[23, 24]. Another study has revealed that the presence of pretransplant autoreactivity could lead to strengthened autoimmune reactions targeting β cells[25].

(ii) Non immunosuppressive Associated Factors. Non immunosuppresive factors including insufficient islet mass and poor islet quality can cause the dysfunction of islets in the long term. Islets are transplanted through the portal system and engraft in the liver; this can cause islet graft loss by (1) instant blood-mediated inflammatory reaction (IBMIR) [25;] (2) hypoxia-related islet cell death[26]. It has been reported that approximately 60% of pancreatic islets are destroyed due to IBMIR after intraportal transplantation[27]. This reaction leads to the disruption of islets due to the activation of complement and coagulation systems[28, 29]. Tissue factor together with

monocyte chemoattractant protein (MCP-1) and other inflammatory mediators cause the activation of coagulation and complement system. Poor clinical outcomes of islet transplantation are often associated with increased intensity of IBMIR[30]. In terms of hypoxia-related islet loss, the devascularization caused during the isolation, as well as the implantation of the islets into low oxygen tension within the liver, directly damages the islet cells[26]. The indirect cause of islet loss can be considered as the result of the activation of innate immune system by the hypoxia environment itself. Consequently, the release of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β), damages the islet graft[31].

With the above-mentioned limitations, the field of islet transplantation has been trying to find an alternative strategy to minimize the limitations for both donors and patients. Specifically, two avenues of research are being investigated. First, to find a possible method to decrease islet loss or provide an unlimited source of islet cells for transplantation. Second, to find an alternative approach to avoid the use of immunosuppressive medications. Immunoisolation of pancreatic islets, also known as encapsulation, not only allows for transplantation of cells without immunosuppression but also increases the chance of using cells from a nonhuman origin.

1.5. Immunoisolation strategies for islet transplantation

The principle behind immunoisolation is protection of islets from host immune system using a selectively permeable membrane as a barrier. Low molecular weight substances which include nutrients, oxygen, secretory molecules and cell signaling molecules freely diffuse through the membrane, but passage of immune cells and its products which have high molecular weight is prevented. Immunoisolation mechanism encourages the use of allogenic/ xenogenic sources of islets for transplantation and holds promise towards use of autologous stem cell derived islets in type I diabetic patients. Immunoisolation mechanism includes macroencapsulation and microencapsulation[32] of cells.

1.5.1.1 Microencapsulation

Microencapsulation is the encapsulation of single islets or small groups of islets. These capsules are usually spherical in shape[39]. Microcapsules offer the advantage of increased oxygen and nutrient transport due to the large surface area to volume ratio. Microencapsulation strategy is advantageous due to several reasons like greater surface to volume ratio and ease of implantation. The spherical shapes contributes better diffusion capacity and are mechanically stable. The primary drawback of microencapsulation is the difficulty in removing the implants if necessary. Moreover the implantation could be achieved by simple injection procedure[40]. Porcine islets microencapsulated in polylysine- alginate transplanted to diabetic monkeys could achieve normoglycemia without immunosuppression for more than 800 days[41]. Human and rat islets encapsulated in alginate gels when transplanted in mice survived for 7 months[42]. Xenogenic islets immobilized in microcapsules fabricated from alginate-PLL when implanted into peritoneum of non immunosuppressed diabetic rats remained in excellent condition for more than 40 weeks[43]. Despite these advantages some authors have reported reduced functionality of microencapsulated islets in response to glucose challenge[44].

1.5.1.2 Macroencapsulation

Macroencapsules contain a large mass of islet cells within a diffusion chamber, which are usually formed from spun coat membranes or spun drawn hollow fibers. The advantages of macrocapsules are they could be easily retrieved when required and can be shaped in required geometries such as tubes or discs. Two approaches such as intravascular and extravascular have been tried out in macroencapsulation. Intravascular approach utilizes the principle of perfusion chambers which consists of microporous tubular structures perfused with blood and enclosed within another tube. Islets were seeded in the space between the hollow fibers and the device is anastomised to the host vasculature[45]. Polyacrylonitrile and polyvinylchloride copolymers have been chosen as materials for creating artificial microcapillaries.

Results from implantation of intravascular macrocapsules of islets have shown restoration of normoglycemia in various animal models[46]. Due to the direct contact of device with the blood, intense anticoagulation is required to prevent thrombus formation, consequently the material chosen should be highly blood compatible and thromboresistant. These concerns have shifted the attention toward extravascular devices.

Extravascular devices are based on the principle of diffusion chambers which does not require anastomosis to host vasculature. The geometry could be planar in the form of flat or hollow fiber model[47]. This approach does not pose severe biocompatibility issues and risks to the patient as that of intravascular devices. Extravascular devices can be implanted to different sites such as peritoneal cavity[43], subcutaneously or under kidney capsules[48] with minimal surgical risks. Most commonly used biomaterials for macrocapsule fabrication are nitrocellulose acetate, 2-hydroxyethyl methacrylate (HEMA), acrylonitrile, polyacrylonitrile and polyvinylchloride copolymer, and alginate.

The biocompatibility of immunoisolation membrane depends on several factors like geometry of the device, implantation site and material chosen. Hollow fiber geometry is preferred because of its reduced surface area of contact with the host per islet and reduced foreign body response. Higher density of islet cells often results in reduced viability and necrosis at the center due to nutrient limitation. Smooth outer surface and hydrogels have been reported to improve the biocompatibility by the absence of interfacial tension, thus reducing protein adsorption, cell adhesion and fibrosis[49]. Nair et al studied the effect of degree of hydrophilicity on tissue response of polyurethane interpenetrating networks (IPN)[50].The results indicated that an increase in hydrophilicity of polyurethane-polyvinyl pyrrolidone IPN's elicited an inert tissue response.

George et al., [51], Nair[52] demonstrated the use of non porous polyurethane membranes and porous polyurethane IPN macrocapsules as islet immunoisolative

matrices. Islet cell morphology remained intact and insulin secretion ability was also retained within the immunoislation membranes. Membranes allowed diffusion of glucose and insulin while retained transplant rejection factors like antibodies, immunoglobulins and immune cells. Reduced protein adsorption and cell adhesion on polyurethane membranes contributed to improve the biocompatibility which made them ideal for immunoisolation. The IPN macrocapsules also served as an *in vivo* bioreactor cum immunoisolation device permitting immature islet like clusters derived from a variety of stem cell sources to mature completely and control glycemic levels of streptozotocin induced diabetic animal models without immunosuppression for periods upto 3 months[53, 54, 55, 56].Hybrid systems involving macro and microencapsulation have also been fabricated and analyzed for its efficiency in immunoisolation. Chitosan/gelatin hydrogel system was used as an immune isolative matrix to protect the microencapsulated islet cells from recipient's immune system in xenotransplantation. Mouse insulinoma /agarose microspheres macroencapsulated in chitosan/gelatin hydrogel reversed diabetes in rats. The study suggests that this could be applied as a cell carrier for injectable bioartificial pancreas after certain modifications[57].

1.6. Fabrication methods used for immunoisolation of islets

1.6.1 Development of hydrogels

Biomaterials for hydrogels are used or designed to elicit specific cellular functions and to direct cell-cell interactions both in implants that are initially cell-free and may serve as matrices to contribute to tissue regeneration, and in implants to support cell transplantation[33]. Materials for hydrogels can be classified according to their source as natural or synthetic. Their biocompatibility and biodegradability are essential to their application in tissue engineering, and will therefore be given greater attention. Cross-links in hydrogels are formed by covalent or ionic bonds. Weaker forces such as van der Waals forces and hydrogen bonds can also serve as cross-links,

resulting in the formation of swollen networks that will behave as hydrogels. Finally, semicrystalline, uncross-linked hydrophilic polymers may form hydrogels upon swelling since the crystallites act as physical cross-links and do not dissolve in water[34]. They are called "physical" or "reversible" gels when the network is formed by molecular entanglement or by non-covalent force, and called "permanent" or "chemical" gels when a covalently cross-linking network is present[35].

A) Preparation of Hydrogels by Chemical Cross-Linking

Cross-links can be formed by means of chemical reaction initiated by heat, pressure, change in pH, or radiation[36]. As previously mentioned, the contact lens is a classic chemical cross-linking hydrogel developed by Wichterle and Lim, based on copolymerization of hydroxyethyl methacrylate (HEMA) with the crosslinker ethylene glycol dimethacrylate (EGDMA)[37]. This hydrogel is created by free radical chain polymerization which can be initiated by light, heat, or redox[35].

B) Preparation of Hydrogels by Physical Cross-Linking

Physical hydrogels are not homogeneous, because clusters of molecular entanglements, or hydrophobically- or ionically-associated domains, can create inhomogeneities. Free chain ends or chain loops also represent transient network defects in physical gels[35]. Electrostatic between a polyelectrolyte and a multivalent ion of the opposite charge accounts for the formation of a physical hydrogel known as "ionotropic" hydrogel[35]. For example, alginates are naturally derived anionic polysaccharides and used as hydrogels by adding calcium ions as the opposite charge[93]. Divalent cations like Ca^{2+} cooperatively bind between the G blocks of adjacent alginate chains, creating ionic interchain bridges which cause gelling of aqueous alginate solutions[94].

1.6.2 Electrospinning

Electrospinning is a technique used widely for fabricating fibers with diameters in the nanoscale (1000 nm) or microscale (0.1 μm) range[58]. In the electrospinning process, a syringe pump, a high voltage source, and a collector are needed. Firstly, a very high voltage is applied to a capillary tube filled with polymer solution or melt, which is held at the tip of the capillary via surface tension. Secondly, a mutual charge repulsion caused by application of an electrical field is induced within the polymer solution or melt, which directly opposes the surface tension of the polymer solution. When the intensity of the electrical field is increased, the charge repulsion will overcome the surface tension to form a jet. Finally, the ejected polymer solution and melt repel each other and the solvent evaporates to form fibers as the jet travels to the collector. Fibers ranging from nanometers to micrometers in size can be formed by regulating parameters including intrinsic solution properties, eg, viscosity, conductivity, and surface tension, and operational conditions, including the hydrostatic pressure in the capillary tube, strength of the electrical field applied, and distance between the tip and collector. A variety of synthetic polymers, eg, poly(lactic acid) [PLLA], poly(glycolic acid), poly(lactic-co-glycolic acid) [PLGA] and polycaprolactone, and natural polymers (eg, collagen, chitosan, silk fibroin, and chitin) have been fabricated as three-dimensional nanofibrous scaffolds using the electrospinning method for tissue engineering[59, 60].Fibrous polyurethane (PU) membranes were manufactured with different fiber diameters using an electrospinning setup. The planar macroencapsulation device was fabricated by welding two layers of the electrospun membranes[95]. The electrospun membrane or cell-laden devices were then implanted subcutaneously in SD rats or C57BL/6 mice. The host Foreign Body Reactions to membranes were analyzed by histological study. Noninvasive bioluminscence imaging (BLI) was performed to monitor the cells within the device. The results showed that the nanofibrous membrane not only possesses superior biocompatibility, but also can act as cell barrier to prevent cell invasion.

Although electrospinning is a quick and simple approach to fabrication of various types of nanofibrous scaffolds, it is still a big challenge to engineer scaffolds with complex structures for many tissue engineering applications. Moreover, generation of scaffolds containing a homogeneous distribution of pores will also need to be addressed.

1.6.3. Phase separation

The phase separation process can be induced thermally or by a non solvent, and has been utilized to fabricate porous membranes or foams for filtration and separation purposes[61]. Induction of the phase separation process using a non solvent commonly results in scaffolds with a heterogeneous pore structure which is not suitable for fabrication of tissue engineering scaffolds, which generally need a uniform pore structure[62]. The thermally induced phase separation process takes place when a homogeneous polymer solution becomes thermodynamically unstable under certain temperature conditions and tends to separate into a multiphase system domains, comprising a polymer-lean phase (with a low polymer concentration) and a polymer-rich phase (with a high polymer concentration)[63, 64].

Subsequently, the polymer-rich phase solidifies to form a matrix while the polymer-lean phase turns into pores as a result of solvent removal. Thermally induced phase separation can be divided into solid-liquid phase separation and liquid-liquid phase separation. The solid-liquid phase separation process is used to induce solvent crystallization from a polymer solution by lowering the temperature, which leads to formation of pores after removal of solvent crystals. In the liquid-liquid phase separation process, polymer solutions with an upper critical temperature form a bicontinuous structure (both polymer-lean phase and polymer-rich phase) [65, 66].

Compared with the electrospinning approach, phase separation holds great potential for fabrication of three-dimensional nanofibrous scaffolds with uniform pore

structures through dual or multiple phase separation processes. In addition, phase separation can engineer three-dimensional shapes via several techniques, including solid free-form fabrication, rapid prototyping, and computer-assisted design and manufacture. However, limitations such as limited material selection and inadequate resolution still exist.

1.6.4. Freeze-drying

Freeze-drying has emerged as a drying process for converting solutions of labile materials into solids of sufficient stability for distribution and storage in applications such as food science, pharmaceuticals, and enzyme stabilization[67]. Freeze-drying involves three major steps: the solution is frozen at a low temperature (−70°C to −80°C); the frozen sample is located in a chamber in which the pressure is lowered (to a few millibars) through a partial vacuum, known as the primary drying process, in which ice in the material is removed by direct sublimation; and most of the unfrozen water in the material is removed by desorption in a secondary drying process. In the last two decades, the freeze-drying method has been widely investigated for the fabrication of three-dimensional porous scaffolds for tissue engineering[68].A three-dimensional (3D) biodegradable scaffold comprised of natural polymers dextran and gelatin (DEXGEL) synthesized by freeze drying for differentiation of adipose stem cells to islet-like clusters (ILCs). Adipose stem cells derived from subcutaneous fat of New Zealand white rabbits were differentiated to ILCs on DEXGEL scaffold and two-dimensional (2D) culture plates via three stage protocol using cocktail of growth factors[113].

Although there are several advantages of the freeze-drying method, including use of water and ice crystals instead of an organic solvent in the scaffold fabrication process, which is more suitable for biomedical applications, it is still a big challenge to engineer scaffolds with hierarchical structures (eg, vascularized systems) using this approach.

1.6.5. Self-assembly

The self-assembly process, defined as an autonomous organization of components into patterns or structures without human intervention, has been utilized for fabrication of various nanofibers[69,70]. Such self-assembly of biological molecules can be induced by noncovalent bonds or weak covalent interactions, including electrostatic, van der Waals, hydrophobic interactions, ionic, hydrogen, and coordination bonds. A typical example of molecular self-assembling into an ordered structure in nature is phospholipids, which are important components of the cell membrane and can self- assemble into several higher order structures such as vesicles, tubules, and micelles in aqueous solution due to their natural amphiphilic structures[71]. In addition, various nanoscale filaments of proteins (eg, peptides) can be assembled into nanofibers with a high aspect ratio, which can mimic the physical microenvironment of cells in vivo. In the body, these nanofibers could wrap around cells covering long distances over their surfaces and act as cables that connect neighbor cells and mechanically support them by creating three-dimensional networks.[72]

The formation of nano-thin, poly(ethylene glycol) (PEG)-rich functional conformal coatings on individual islets via layer-by-layer assembly technique. The surface of the islets is modified with biotin-PEG-N-hydroxysuccinimide (NHS), and the islets are further covered by streptavidin (SA) and biotin-PEG-peptide conjugates using the layer-by-layer method. An insulinotropic ligand, glucagon-like peptide-1 (GLP-1), is conjugated to biotin-PEG-NHS. The insulinotropic effect of GLP-1 is investigated through layer-by-layer encapsulation of islets using the biotin-PEG-GLP-1 conjugate. The effect of islet surface modification using the biotin-PEG-GLP-1 conjugate on insulin secretion in response to glucose challenge is compared via static incubation and dynamic perifusion assays. The results show that islets coated with the functional PEG conjugate are capable of secreting more insulin in response to high glucose levels compared to control islets. Finally, the presence of SA is confirmed by

indirect fluorescent staining with SA-Cy3, and the presence of PEG-peptide on the surface of the islets after treatment with biotin-PEG-GLP-1 is confirmed by indirect fluorescent staining with biotin-PEG-fluorescein isothiocyanate (FITC) and separately with an anti-GLP-1 antibody. This demonstrates the feasibility of treating pancreatic islets with reactive polymeric segments and provides the foundation for a novel means of potential immunoisolation[96].

Although the self-assembly approach can form various types of nanofibers and nanoscale networks upon adjustment of sample parameters, such as the pH and ionic concentration of the aqueous solution, it is still a challenge to form stable three-dimensional geometrical structures due to their poor mechanical properties and the fact that the engineered peptide nanofibers can be fragmented and are susceptible to endocytosis[73]. Moreover, the high cost of synthesis of biomaterials limits their applications in tissue engineering and regenerative medicine.

1.6.6. Three-Dimensional printing

Additive manufacturing, otherwise known as three-dimensional (3D) printing, is driving major innovations in many areas, such as engineering, manufacturing, art, education and medicine. Three-dimensional (3D) printing[97] or additive manufacturing enables the fabrication of near-net-shaped complex 3D parts without expensive molds or tools in short periods of time, based on 3D computer-aided design (CAD) data. 3D printing is expected to revolutionize the manufacturing of components. While several 3D printing systems are available[98], printing based on fused-deposition modeling (FDM) using thermoplastics is particularly widespread because of the simplicity and potential applicability of the method. However, the mechanical properties of products fabricated by conventional FDM 3D printing are inherently poor because of the thermoplastic resins used, although the optimization of processing parameters, such as the lamination direction and laminate thickness, has been investigated for improving the mechanical properties of thermoplastic resin parts[99] .Recent advances have enabled 3D printing of biocompatible materials, cells and supporting components into complex

3D functional living tissues. 3D printing is being applied to regenerative medicine to address the need for tissues and organs suitable for transplantation. Compared with non-biological printing, 3D bioprinting involves additional complexities, such as the choice of materials, cell types, growth and differentiation factors, and technical challenges related to the sensitivities of living cells and the construction of tissues. Addressing these complexities requires the integration of technologies from the fields of engineering, biomaterials science, cell biology, physics and medicine[74].

3D printing was first described in 1986 by Charles W. Hull. In his method, which he named 'sterolithography', thin layers of a material that can be cured with ultraviolet light were sequentially printed in layers to form a solid 3D structure[75]. This process was later applied to create sacrificial resin molds for the formation of 3D scaffolds from biological materials. The development of solvent-free, aqueous-based systems enabled the direct printing of biological materials into 3D scaffolds that could be used for transplantation with or without seeded cells[76]. A related development was the application of 3D printing to produce medical devices such as stents and splints for use in the clinic[77].

Although it is possible to print the same material using multiple printing techniques “ink formulation”,i.e., the form and composition of the printable material, varies significantly. 3D printing technologies are classified under four main groups : extrusion-based methods, particle fusion-basedmethods, light induced (photopolymerization) methods and inkjet printing. Each of these categories contains subgroups that use slight mechanical or chemical variations on each technique, which affect the material properties required for successful design and printing of the “ink”material.

A) Extrusion-Based 3D Printing Methods.

Extrusion-based 3D printing methods, such as fused deposition modeling (FDM) and direct ink writing (DIW), are some of the most widespread methods to

fabricate devices and scaffolds for tissue engineering applications[100]. The idea behind extrusion based additive manufacturing is that an ink is forced through a nozzle as a viscous liquid or melts to form individual lines that solidify onto a build plate. As the material is extruded, the nozzle follows a predefined path determined by a computer model to build up a 3D object layer-by-layer. For traditional FDM, theink is in the form of a solid filament (typically, 1.50 or 1.75 mm in diameter). The ink is rolled into a hot nozzle (typically temperatures up to 200°C) where it is melted (to become flowable) at the nozzle, and extruded using a motorized pinch roller system. This requires materials, generally polymers thatcan be formed into a filament with sharp solid-to-melt transition (i.e.,flow and solidify readily upon melting and cooling, respectively).

B) Particle Fusion-Based 3D Printing Methods.

Particle fusion printing methods, consisting of selective laser sintering (SLS) and particle binding (PB), have found significant applications in industrial prototyping due to the ability to print polymers, ceramics, metals and composites of these into unique and complex geometries[101]. SLS uses a directed laser beam, traditionally from a CO2 laser, to raise the temperature of the polymer or metal particles to above their melting temperature, causing the particles to fuse together[102]. The beam is patterned over the cross sectional area of the computer-modeled object to create a single layer, at which point a new layer of articles is applied over the top, and the process is repeated. Therefore, the ink materials suitable for SLS should be processable into a fine powder form (range from 10 to 100μm), and must have an attainable melting temperature, and bind together when heated (above Tm). It is also important that the particles possess good particle flow dynamics within the bed system, which may require surface functionalization to eliminate electrostatic forces[103]. Generally, SLS machines are slow, bulky, expensive, and require a large amount of material. These methods have been used to create devices for hard-tissue engineering applications, such as orthopedics and oral surgeries.

C) Light-Assisted 3D Printing Methods

Light-assisted 3D printing, also known as stereolithography (SLA), is considered the original additive manufacturing method after Charles Hull first developed and commercialized the process of curing specific areas of polymer resins in the mid-1980s. SLA involves patterning a beam of light (UV or laser) over a bath of photopolymerizable (viscous) liquid polymer to create a single hardened polymer layer. After polymerization, the build stage lowers further into the solution, allowing new resin to flow over the printed surface, and the next layer is polymerized on top of the previous. Recent advances in the development of more efficient light sources and refined mirror-lens systems have drastically improved SLA regarding both its speed and resolution. However, SLA has been limited in its biomedical applications by the harsh nature of UV-based cross-linking, extensive postprocessing, inadequate mechanical properties, trapping of liquid resin within the end product and most importantly the lack of available biocompatible and biodegradable materials suitable for SLA. Recent developments in both natural and synthetic biodegradable, cross-linkable polymers, as well as higher-resolution machines have started to open SLA to a wider array of applications, especially in tissue engineering.

D) Inkjet Printing

Inkjet printing enables disposition of very small volumes (1−100 picoliters)[104] of individual droplets from a nozzle to a printing surface with the goal of forming structures postsolidification. Multinozzle inkjet print heads containing several hundred individual nozzles have been developed to accelerate the printing process. Inkjet printers are classified into two groups based on the droplet generation mechanism: continuous inkjet (CIJ) printing and drop-on- demand (DOD) inkjet printing[104].In CIJ printing continuous stream of drops (around 100μm in diameter) are produced and unused ink is recycled. In DOD inkjet printing, individual drops (in the range of 25 to 50μm in diameter) are generated when required. DOD type printers are commonly used for tissue engineering applications. There are three important stages in inkjet

printing that define and constrain printable ink formulations including drop generation, drop/ substrate interaction, and drop solidification[105]. The mechanism of drop formation defines the ink (fluid) properties that are required for a given polymer solution to be printable. The most important properties of the ink are the viscosity and the surface tension. The viscosity of the ink should be suitably low usually below 10 cP (mPa s), under high shear rates, between 1×10^{5} and $1\times10^{6}s^{-1}$. The surface tension determines the shape of the drop emerging from the nozzle and the shape of the drop on the substrate. Surface tension values of the inks generally range from 28 to 350 mN m^{-1}. The resolution and accuracy of the printed object are determined by the interaction between adjacent drops (coalescence)[106] and between individual drops and the substrate (such as surface tension and wettability)[107]. The liquid-to-solid phase trans- formation (i.e., solvent evaporation, temperature controlled transition, or gelling of a precursor solution) controls the final shape and size of the printed objected.

The ability of polymeric bioinks materials to function in many printing technologies with high biocompatibility and good mechanical properties has made them a common base for many formulations. Future development of printable polymeric biomaterials will need to build on these properties while addressing concerns with degradation, brittleness, and cell compatibility.

1.7 Polyurethane membranes for islet cell encapsulation

Zondervan et al. crosslinked an aliphatic polyurethane with dicumyl peroxide in- order to retard degradation of the polymer following implantation[108]. A porous material was fabricated by salt leaching. Results with encapsulated islets of langerhans showed a good insulin response to glucose, although the speed of response was slower than that of free cells. A pore size of 0.3-0.7 μm offered protection against the invasion of granulocytes. Ward et, al. fabricated polyurethanes into hollow fibres by a dip casting method[109]. The polymer was synthesized from an aromatic, diamine extended hard segment, and an alkylene oxide soft segment. *In vitro* culture of

pancreatic cells was maintained for six months. Seeded tubes were implanted in mice; a low level of rejection was observed. The surrounding tissue was vascularized, nonfibrous, and not strongly attached to the implant. The membrane was permeable to glucose and insulin, but not immunoglobulins. The permeability characteristics were believed to be governed by activated diffusion.

1.8 Alginate polymers for microencapsulation of islet cells

Transplantation of encapsulated living cells is a promising approach for the treatment of a wide variety of diseases. During cell encapsulation, viable cells are suspended in a biomaterial designed to serve as a transport barrier, allowing nutrients and oxygen to diffuse in and waste products to diffuse out while providing a barrier to larger objects such as antibodies and immune cells. This concept can be utilized to prevent graft rejection of non-autologous cell transplants and has been studied extensively for the delivery of islets as a treatment for type 1 diabetes. Islet transplantation is being evaluated as a therapy for some patients whose blood glucose levels are difficult to control, despite intensive insulin therapy[110]. The goal of this procedure is to achieve normal blood glucose levels and to reduce or ultimately eliminate the need for daily insulin injections that are associated with the risk of hypoglycemic shock. Furthermore, long-term complications such as retinopathy, neuropathy, nephropathy, and cardiovascular problems could be prevented through reduction in hyperglycemic events .Microencapsulation has been shown to result in prolonged survival and function of islet grafts in chemically induced and autoimmune diabetic animal models (rodents, dogs, and monkeys)[111]. A variety of synthetic and natural biomaterials have been explored for cell encapsulation. The most investigated material for islet encapsulation is alginate, which has reached the level of clinical trials for encapsulation of islets from both allogeneic and xenogeneic sources. However, capsule stability, biocompatibility, and reproducibility of these systems are a

significant concern. Animal studies often provide a limited ability to predict the long-term stability required for ultimate clinical success[112].

1.9. Hypothesis

A 3D printed hydrophobic membrane with narrowing channel pores enables efficient macro encapsulation of islet cells which are micro encapsulated within alginate beads with selective permeation of insulin and glucose but prevents the permeation of immunoglobulins into the system thus providing a novel strategy for islet transplantation with a combined macro and microencapsulation of islet cells.

1.9.1 Objectives

- To fabricate an immunoisolation bag made of polyurethane using 3D printing to encapsulate islets cells for effective transplantation.
- To characterize the physicochemical properties of the immunoisolation bag and to study the permeability of the membrane towards glucose, insulin and immunoglobulins.
- To prepare alginate beads for the microencapsulation of islets cells.
- To isolate primary pancreatic islet cells from rabbit.
- To study the diffusion of insulin from the microencapsulated alginate beads through the immunoisolation bags and also to study the viability of the cells after a period of 7 days.

1.9.2 Significance

Although islet transplantation offers a promising method to treat patients with type 1 diabetes, the major challenges include the islet donor shortage and the requirement for lifelong immunosuppression. Hence an encapsulation strategy that can prevent the rejection of transplanted islets or stem cell derived allogenic islets can help to overcome these disadvantages. The present study is based on a combined macro and microencapsulation strategy for effective islets cell transplantation for patients

suffering from type 1 diabetes. Here we have fabricated a novel immunoisolation bag using nonbiodegradable synthetic polymer polyurethane using the most modern technique 3D printing. The immunoisolation bag fabricated has shown the survival of islet cells and diffusion of insulin and glucose from the bag through the narrowed pore channels created by specific layering approach of the different layers. This strategy also helps to prevent the entry of immune cells. In future this can be effectively used for islet cell transplantation.

2. MATERIALS AND METHODS

2.1 MATERIALS

Polyurethane (Miku Traders Tecoflex EG-60D), Chloroform, Calcium chloride, Sodium Alginate, HEPES buffer were purchased from Merck, s d fine-CHEM limited, Sisco research laboratories respectively. ELISA Kit (Mercodia Rat Insulin), BCA protein assay Kit (Thermo Scientific), Glucose Assay Kit (Enzyme technologies). The chemicals were used as received without further purification. For cell culture Dulbecco's Modified Essential Medium - High Glucose (DMEM-HG), Fetal Bovine serum (FBS), Antibiotic -Antimycotic were procured from Invitrogen, USA. Collagenase V (125 CDU/mg solid) from Sigma Aldrich for islets isolation.

2.2 METHODS

2.2.1 Viscosity of polyurethane solution

The concentration of the PU solution was determined using the viscosity measurements. The viscosities of different concentrations (10%, 13%, 15%, 20%) were measured using Brookfield Viscometer. The rpm used were 140, 90, 45, and 30 respectively. Small sample holder was used and the spindle used was with the size S31. The constant value in CPS when the torque is between 60-70 is taken as the viscosity of the particular concentration.

2.2.2 Fabrication of immunoisolation membranes

PU solutions were prepared by dissolving PU in chloroform by continuous stirring overnight in a magnetic stirrer. The solutions were then transferred into a 10 ml BD syringe and 3D printed on to aluminium foil using 3D printer (Maker city, USA). Based on the ease of printability of the different solutions, 3D printing parameters were standardized using the 13% solution which showed good printability pattern (Fig 1). The standardized parameters as shown in the table 1.

PARAMETERS- PRINT SETTINGS	
Layer height	0.25mm
First layer height	0.25mm
Fill density	20%
Fill patern	Rectilinear
Fill angle	45
Solid infill threshold area	$70mm^2$
SPEED FOR PRINT MOVES	
Perimeters, Small perimeters ,External perimeters, infill, bridges, first layer speed	8mm/s
Speed for non print moves (travel)	130mm/s
PRINTER SETTINGS (Extruder)	0.25mm

Table 1: Standardized parameters for 3D printing

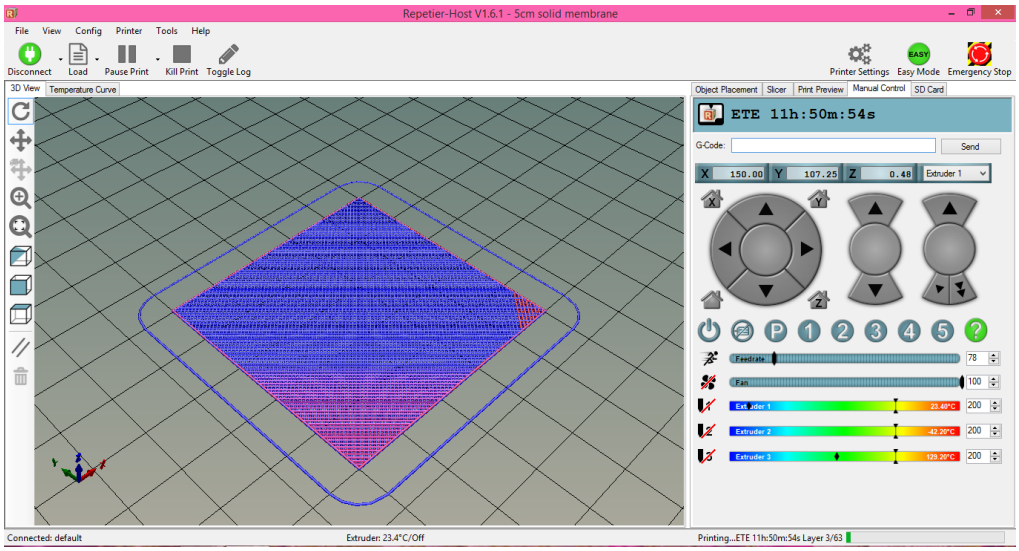


Figure 1: Print preview of the expected 3D membrane

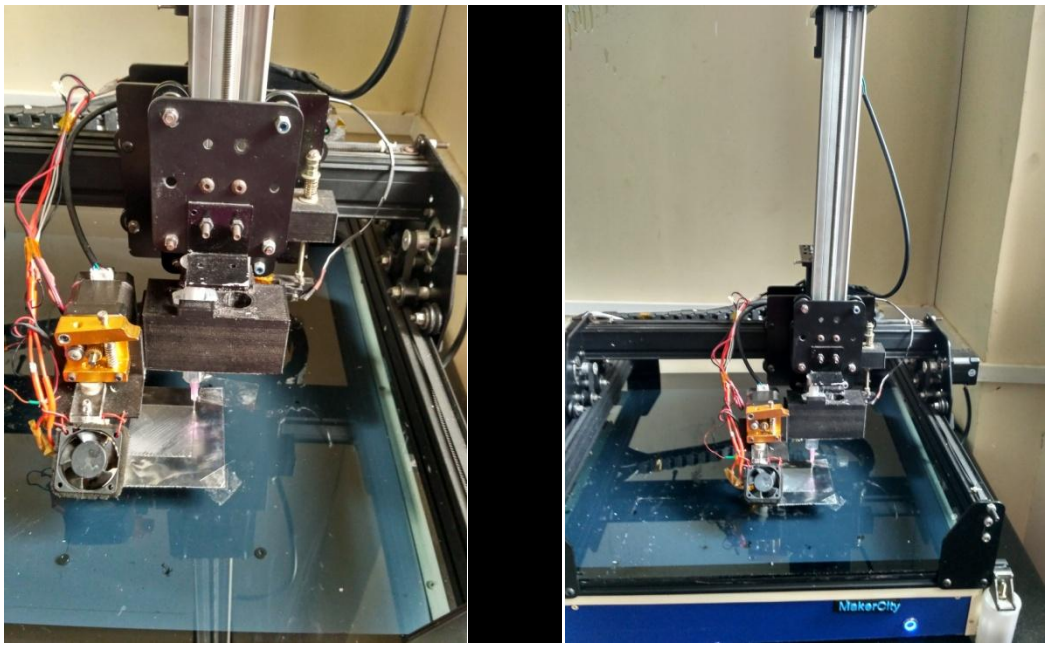


Figure 2: 3D printing of the PU (13%) membrane

2.2.3 Permeation studies of glucose, insulin and immunoglobulin

After the membranes were prepared, permeation of glucose, insulin and immunoglobulins (IgG) were studied using a diffusion cell. The diffusion cells were used side-by-side cells consisting of donor and receptor half cells Amount of known concentrations (5ml) of solution containing the solute of interest (450µg/ml, 10U/ml, 25µg/ml) were placed in the donor compartment. The pure solvent was kept in the receptor compartment. Periodically the contents of the receptor cell was removed and replaced by fresh solvent. The aliquots removed were analyzed using the respective methods for glucose estimation, insulin estimation via ELISA at 450nm and IgG estimation using BCA kit at 640nm in ASYS UVM340. The solute permeability coefficient P was determined using the following equation:

$$\ln(2Co/Ct-l) = 2Apt/Vl$$

In this expression, Co is the initial concentration of the donor cell, Ct is the solute concentration in the receptor cell at time t, V is the volume of each half cell, l is the swollen membrane thickness, and A is the effective area of permeation. A plot of Vl/2A ln(2Co/Ct-l) versus t yielded a slope from which the value of permeability coefficient was calculated.

2.2.4 Characterization of the membrane

2.2.4.1 FT-IR analysis

Fourier Transform Infrared (FTIR) Spectroscopy provides information about the specific chemical groups of the materials. FTIR with Attenuated Total Reflection (ATR) was the sampling tool used for analyses within the outermost atomic layer from 100 nm to 1µm. The FTIR-ATR spectra of 1.5 mm thick film samples were recorded at room temperature in the range of 4000-400 cm^{-1} region using a NICOLET 5700 FTIR spectrophotometer with DTGS (Deuteriated triglycine sulphate) detector (Thermo Corporation, USA) and Diamond ATR (Attenuated Total reflectance)

accessory. Fifty scans were recorded per collection and the spectrum was analyzed using OMNIC software.

Polyurethane membrane sheets were subjected to Fourier Transform Infrared (FTIR) spectroscopy analysis using Thermo Nicolet 5700 FT-IR with Diamond ATR Accessory. Films were pressed against the crystal provided with the ATR cell of the FTIR (Nicolet) Spectrophotometer. The spectra were taken in the frequency range of 4000-400cm^{-1} and the resultant peaks obtained were analysed.

2.2.4.2 Surface topography of Scaffold

The microstructure of the 3D printed membrane sheets of different layers (2,5,7,10) were characterized using environmental scanning electron microscopy (ESEM), Quanta FEI, Hillsboro, USA. The pattern of printing, pore size was analyzed from ESEM micrographs using image analysis software (Image J, National Institutes of Health, USA). Pore size is defined as void spaces bound by fibers and their diagonal axis were measured and averaged to give pore size measurement.

2.2.4.3 Contact angle measurement

The surface wettability of the materials were analysed with static water contact by sessile drop method. 3D printed membrane sheets are prepared for contact angle measurements. Glycerol was dispensed vertically onto the membranes and the images of the droplet on the film were visualized and contact angle measurements were obtained through the software (OCA15 plus, Optical Contact Angle System; Data Physics).

2.2.4.4 Swelling studies

In vitro swelling profile of the 3D printed membrane sheets were studied by measuring the change in weight as a function of time. The dry 3D printed PU membrane sheets of known weight were dipped into 1 ml of Phosphate Buffered

Saline (PBS) (pH – 7.4) at room temperature. At predetermined time intervals (7days, 14 days, 21 days, 28days, 35 days) the samples were withdrawn from the PBS buffer, excess PBS was blotted and wet weight of the scaffolds were measured. The swelling ratio was calculated using the formula:

Swelling ratio = (Wet Weight – Dry Weight)/Dry Weight

2.2.4.5 Degradation studies

Degradation studies of the PU membrane were studied by incubating in phosphate buffer saline (PBS) at 37^0C. Each sample was kept in 10 ml PBS and incubated at 37^0C. After 24h, the PBS was removed and the sample weight was taken. Fresh PBS was added after each weighing and again incubated at 37^0C. The procedure was continued till the end of the experiment. The experiment was done in triplicate. Percent weight loss is the weight lost divided by the initial weight of the sample multiplied by 100.

$$\text{Weight loss ratio} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

2.2.4.6 Thermal analysis

The thermal stability of the samples was determined by Thermogravimetric Analysis as per ASTM E1131-03. The thermograms were recorded using a simultaneous TGA-DTA instrument (model SDT 2920 TA Instruments Inc., NewCastle, DE). Approximately 10mg of the samples were heated from room temperature to 800ºC at a heating rate of 10° C/min in a N2 atmosphere.

2.2.4.7 Differential scanning calorimetry

The glass transition temperatures of the samples were determined by Differential Scanning Calorimetry as per ASTM E1356-03. The thermograms were

recorded in the range of -50°C to 200°C on a TA Instrument, DSC 2920 with TA 4000 controller at a heating rate of 10°C/min in a N2 atmosphere.

2.3 Preparation of Alginate beads

The alginate solutions were prepared of different concentrations (1.5%, 2.5%, 5%) by diluting sodium alginate in distilled water with continuous stirring using magnetic stirrer. Alginate beads are prepared by adding sodium alginate drop-wise to a solution of 100mM $CaCl_2$ (Sigma- Aldrich) with 10mM HEPES (Sigma-Aldrich) using a 1 ml pipette.

2.3.1 Characterization of alginate beads by e-SEM

The microstructure of the alginate beads (1.5%, 2.5%, 5%) were characterized using environmental scanning electron microscopy (ESEM), Quanta FEI, Hillsboro, USA. The internal structure of gelled beads was examined by an Environmental Scanning Electron Microscope. Characterization of surface morphology and bead size were analyzed using ESEM images.

2.4 Isolation of islets from rabbit pancreas

Islets were isolated from pancreas of rabbit after obtaining consent from Institute Animal Ethics Committee. The whole pancreatic tissue was minced into small pieces and was digested with 1% collagenase V (125 CDU/mg solid) for 10 minutes at 37ºC. The reaction was then stopped using Dulbeccos Modified Essential Medium –High Glucose (DMEM-HG) medium with 10% FBS and 1% Ab/Am and islets were separated from other cells and tissue debris by centrifugation at 2500 rpm for 10 minutes followed by filtration with 40micron filters. The islets were then cultured in DMEM-HG medium with 10% FBS and 1% Ab/Am medium in a humidified incubator at 37 ºC with 5%CO_2.

2.5 Dithiazone staining of rabbit islets

Dithiazone (Sigma Aldrich Bangalore) stock was used as a confirmatory stain to confirm the presence of islet β cells. It was prepared at a concentration of 39mM in dimethyl sulfoxide (DMSO) (Sigma Aldrich, Bangalore) and briefly stored at 4ºC. Dithiazone working solution was prepared fresh by mixing 100mL dithiazone stock, 10mL Krebs Ringer Bicarbonate buffer (pH 7.4), and 10mM HEPES (Gibco). Rabbit islets were washed with PBS and incubated with filtered dithiazone working solution for 20min at 37ºC. The stained cell clusters were visualized with phase contrast microscope (Olympus IX71). Dithiazone staining will give brick red colour to islet β cells.

2.6 Macro and microencapsulation of islet cell clusters.

Microencapsulation of islet cell clusters was done using alginate beads. The cell clusters were mixed with 1 ml sodium alginate and added dropwise to the 100mM $CaCl_2$ containing 10mM HEPES. The 2-3 beads with islet clusters were macroencapsulated in PU immunoisolation membranes. The edges of the membrane were sealed using sterile forceps.

2.7 Cytotoxicity analysis using L929 cell lines

2.7.1 Direct contact test

To assess the cell compatibility of the membrane, L929 cells were seeded at a cell density of 10^5 cells/ml onto a 6 well culture plate and cultured until the cells became confluent. Polyurethane membrane sheets were placed on the confluent cell monolayer and observed for 7 days in culture. Cells grown on cell culture treated plates were taken as control. Images were taken using phase contrast microscope (Olympus lX71).

2.7.2 Test on extract (MTT assay)

The cell viability was evaluated by colorimetric MTT assay. 24 h extract of PU immunoisloation bags were prepared by incubating with DMEM HG containing 20% FBS at 37ºC. L929 were seeded in 96-well cell culture plate at a density of 10^4 cells/ml and was incubated for 24 h at 37ºC with 5% CO_2. On day 2, the medium was replaced with the scaffold extracts and incubated for further 24 h. The control cells were incubated with normal culture medium. On day 3 the scaffold extracts was removed and the cells were incubated with 20µl of 5 mg/ml MTT solution for 3 h and the formazan crystals formed were dissolved in Dimethyl Sulphoxide. Optical Density (OD) was measured at 540 nm with the use of a microplate reader (ASYS UVM 340). Percentage cell viability was calculated using the formula:

$$\text{Percentage cell viability} = \left(\frac{\text{Absorbance of test extract treated cells}}{\text{Absorbance of Control cells}} \text{ X } 100 \right)$$

2.7.3 Live-dead staining of islets and L929 cells in membrane bags

The viability of both islets and L929 cells encapsulated in alginate beads and PU immunoisolation bags were qualitatively assessed using live-dead staining. The encapsulated islet cells in membrane bags were incubated with 4mM calcein and 2mM ethidium homodimer (in DMEM) and incubated for 15 minutes in dark. Then the samples were washed with PBS and observed under A1R si Nikon confocal microscope. Calcein stains live cells and ethidium homodimer stains dead cells.

2.7.4 *Invitro* Glucose challenge assay

Quantification of the protein level expression of insulin secretion was carried out for RIN-5F rat cell lines encapsulated in alginate beads and membrane bags of PU.

Samples were pre incubated for 1 h in glucose free Krebs Ringer Bicarbonate HEPES (KRBH) buffer, followed by incubation with KRBH containing 2 and 20 mM glucose for an additional 30 min respectively. RIN-5F (Rat islets celllines) used as controls were also challenged with similar concentrations of glucose. The same experiment was repeated using rabbit islets. The supernatants were collected and stored at -20ºC till the assay was performed. Insulin assay was performed by ELISA method, according to the manufacturer's instruction (Mercodia Rat ELISA Kit). It's a solid-phase two-sit enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase conjugated anti-insulin antibodies and anti-insulin antibodies bound to microplate wells. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate was detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction was stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450nm.

2.6 Statistical analysis

The quantitative results are represented as mean ± standard deviation and were statistically assessed using two - way analysis for cell proliferation assay and one-way ANOVA for MTT. A value of $p<0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

Intensive monitoring of blood glucose followed by insulin injection is the current therapy to treat diabetes. But it is not a permanent cure because over or under treatment can lead to life threatening complications such as coma, diabetic retinopathy, neuropathy, nephropathy, vasculopathy, etc. Islet transplantation offers a promising method to treat patients with type 1 diabetes. Reports highlight that normal blood glucose can be achieved by transplanting the whole pancreas, pancreatic islets, insulin secreting cell lines and stem cell derived insulin producing cells[115]. The major challenges are inability to maintain the functionality of islets *in vivo* for long periods after transplantation which then results in insulin dependence, the islet donor shortage and the requirement for lifelong immunosuppression. Hence an encapsulation strategy that can prevent the rejection of transplanted islets or stem cell derived allogenic islets can help to overcome these disadvantages. The principle behind immune isolation is protection of islets from host immune system using a selectively permeable membrane as a barrier. Low molecular weight substances which include nutrients, oxygen, secretory molecules and cell signaling molecules freely diffuse through the membrane, but passage of immune cells and its products which have higher molecular weight is prevented. Immunoisolation mechanism encourages the use of allogenic/ xenogenic sources of islets for transplantation and holds promise towards use of autologous stem cell derived islets in type I diabetic patients[43].

Human and rat islets encapsulated in alginate gels when transplanted in mice survived for 7 months[42]. Xenogenic islets immobilized in microcapsules fabricated from alginate-PLL when implanted into peritoneum of non immunosuppressed diabetic rats remained in excellent condition for more than 40 weeks[43]. Microencapsulation provides for a larger volume to surface area ratio which increases the diffusion properties and is stable and easy to construct. Despite these advantages some authors have reported reduced functionality of microencapsulated islets in

response to glucose challenge[44]**.** Extravascular devices are based on the principle of diffusion chambers. The geometry could be planar in the form of flat or hollow fiber model[47]. This approach does not pose severe biocompatibility issues and risks to the patient as that of intravascular devices. Most commonly used biomaterials for macrocapsule fabrication are polyurethane, nitrocellulose acetate, 2-hydroxyethyl methacrylate (HEMA), acrylonitrile, polyacrylonitrile and polyvinylchloride copolymer, and alginate.

The present study is based on a combined macro and microencapsulation strategy for effective islets cell transplantation. Here we have fabricated a novel immune isolation bag using nonbiodegradable synthetic polymer polyurethane using the most modern technique of 3D printing. The polyurethane that we have used here is a medical grade aliphatic polyether based thermoplastic polyurethane. The immunoisolation bag fabricated has shown the survival of islet cells and diffusion of insulin and glucose from the bag through the narrowed pore channels created by specific layering approach of the polyurethane through the technique of 3 D printing. This strategy also helps to prevent the entry of immune cells and hence holds a promising future for islet cell transplantation therapy.

3.1 Fabrication of immunoisolation bags and its characterization

3.1.1 3D printing of PU immunoisolation bag

Different concentrations (10%, 13%, 15%, and 20%) of polyurethane dissolved in chloroform were evaluated using Brookfield viscometer (Brookfield, USA) to find the flowable viscosity required for effective printing. Spindle size 31 was used for the study and the viscosity was noted when the torque was between 60-70% which is within the acceptable range.

Table 2. shows the viscosity of different polyurethane concentrations.

Polyurethane concentration	Temperature (°C)	Torque (%)	RPM	Viscosity(CPS)
10%	28.4	69.1	140	131.5 ± 2.05
13%	26.8	67.9	90	226.3 ± 2.82
15%	26.5	63.3	45	421.9 ± 1.95
20%	26.3	66.2	30	661.9 ± 3.46

Table 2. Viscosity of different concentrations of PU

3D printing was done using these solutions of different concentrations. Viscosity of the ink used plays a major role in 3D printing. Properties that facilitate handling and deposition by the printer may include viscosity, gelation methods[114]. On printing the 10% PU solution seemed to spread widely and rigid layers could not be obtained as shown in Fig 3. The overlaying layers were fused together since the viscosity of the solution was low and the solvent (chloroform) seemed to volatilize at lower rate. Similar problem was seen with the higher concentrations (15% and 20%) (Fig 3b & c) which were seen to be dropping from the nozzle rather than printing and the print was not continuous. However the 13% PU solution was observed to be print well and maintain the solid structure.

The print parameters were also optimized using the 13% PU solution were in 10 layers were printed using 0.25mm ID (30 gauge size) needle(Fig 4a & b**)**. Thin layers could be printed by using this small gauge sized needles. 8 mm/s was the speed used for the printing. The first layer almost dried up completely before the printing of consecutive layers with a drying rate 100mm/s. preventing fusing of layers. Since the

resolution of the printer is low with the large gauge needle used for the printing, the individual layer moved at a distance of approximately 203.3 $\pm$ 2.01µm in one side with each layer which resulted in the decrease in the channel size of the membrane as the layers were built up. This narrowing of the pore channel from the first to last layer prevents the high molecular weight molecules from diffusing into the membrane and allows the diffusion of low molecular weight molecules like glucose and insulin. The concept of pore narrowing using this technology is depicted in the schematic figure 5 which shows how the layers built up and narrows down the pores. The exploded view of the cross-section also shows how the pore channels get tapered as the layers are built up.

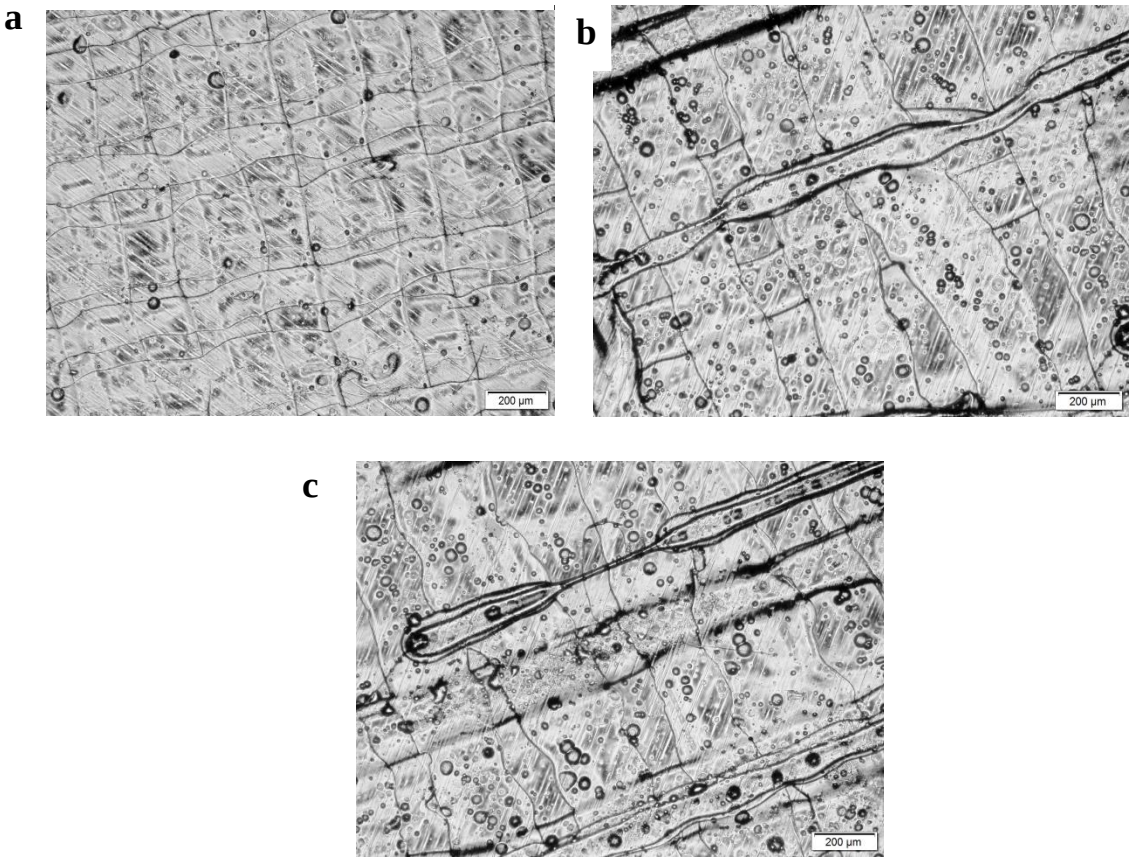


Figure 3: Optical image of the 3D printed PU membrane using a) 10% b)15% c) 20% volume concentration.

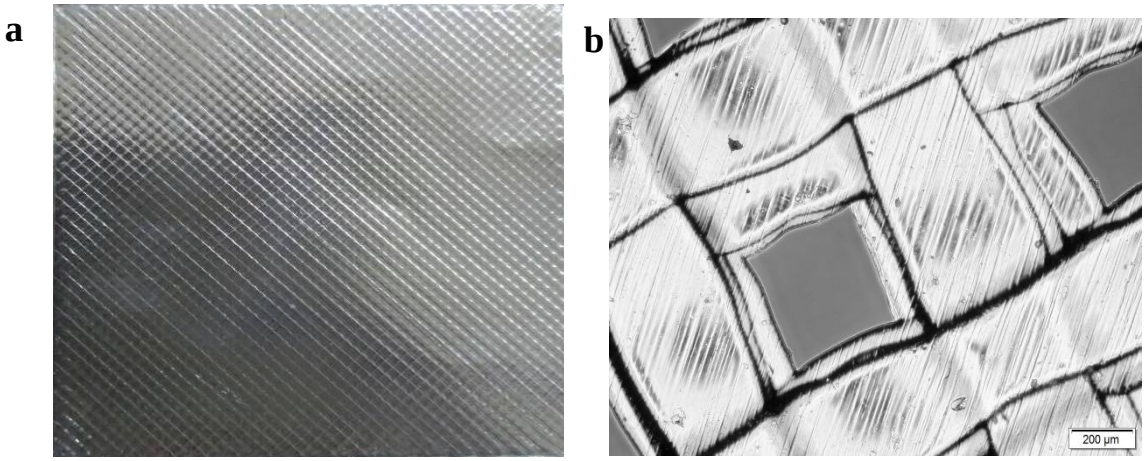


Figure 4: Photograph of the 3D printed 10 layered polyurethane membrane b)optical image after 4 layers

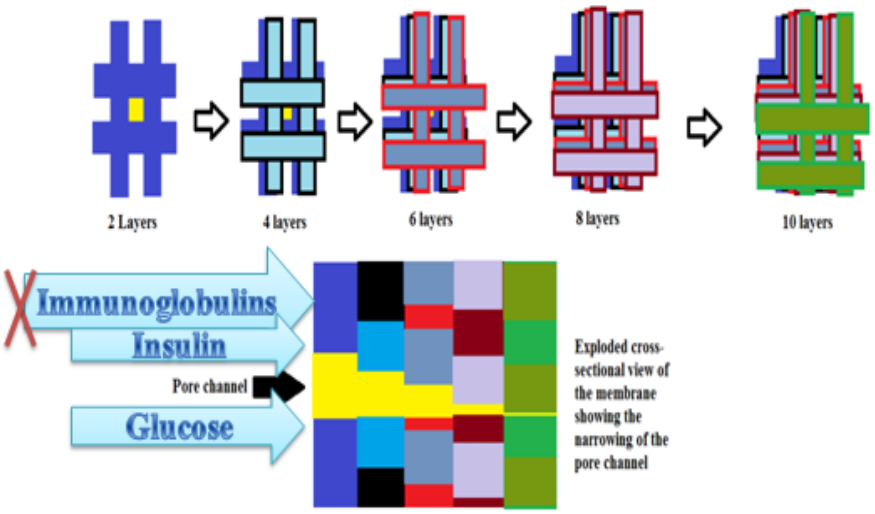
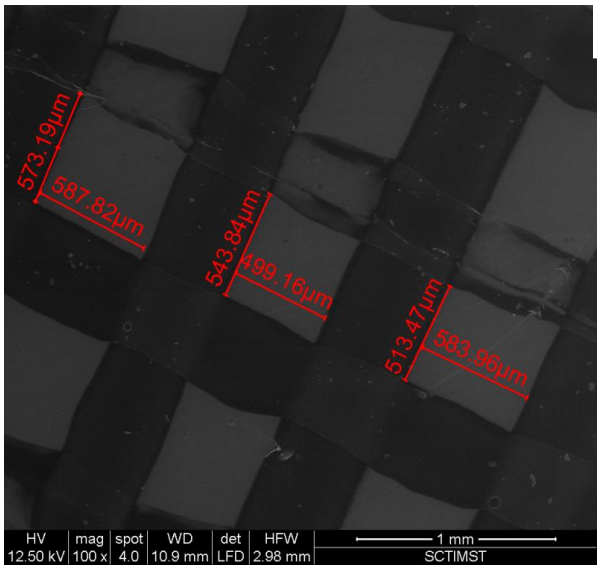


Figure 5: Cross-sectional view of the membrane showing the narrowing of the pore channel

The size of the pore at the second print layer was 556.98 ± 50.11µm X 543.5 ± 29.86.µm (fig 6 a). The pore size was found to be decreased to 484.13 ± 14.51µm X 264.34 ± 21.23µm and 117.73 ± 2.19µm X 109.13± 5.55µm after five and seven

layers respectively (fig 6b & 6c). This was due to the shifting of each layer by 203.3 ± 2.02µm (fig 6d). At the end of 10 layers the pore size narrowed rapidly and pore size could not be determined from the SEM images. Selective diffusion takes place in the case of insulin and glucose. The macroencapsulation bag was prepared with the inner layer facing the interior of the bag with the more open channels and the outer layer having the narrowed end. These bags could be easily and effectively sealed by thermal compression.

a



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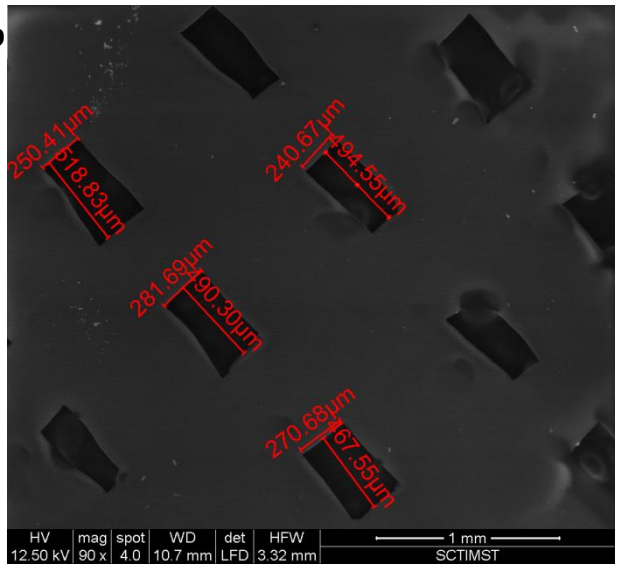
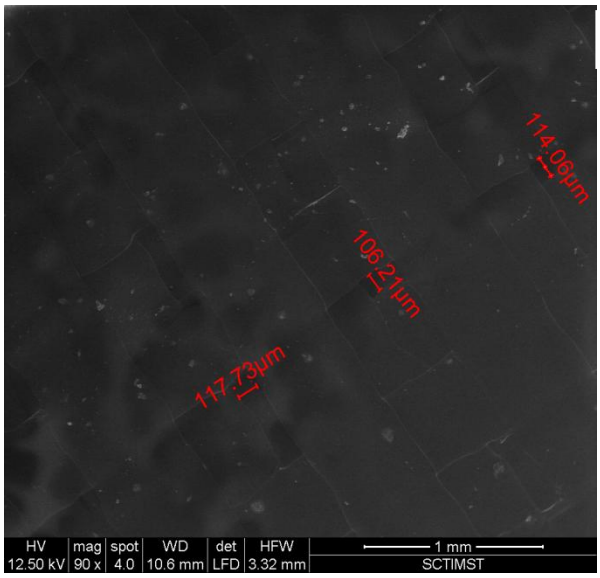


c



d

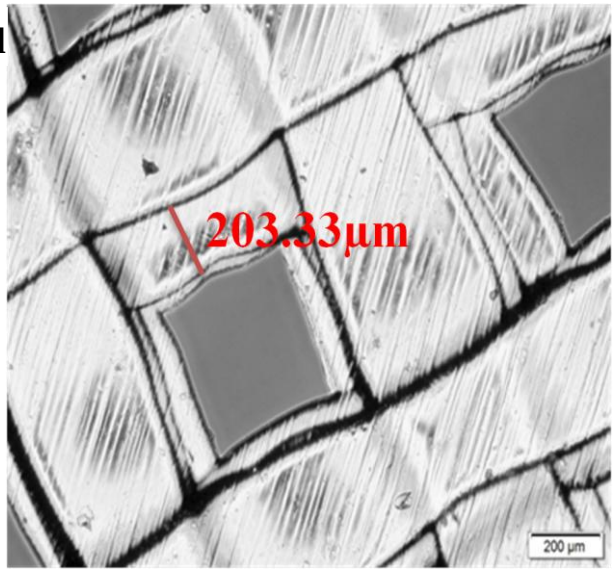


Figure 6: ESEM image of a) Pore size of two layered membrane b) Pore size of five layered membrane c) Pore size of seven layered membrane d) Optical image shows the shift occurred during every layer

McCall and Shapiro reported that Polyurethane (PU) is an elastomer polymer with wide-spread biomedical applications. They used it to create macrocapsules in the geometry of hollow fibers for the encapsulation of pancreatic islets[119]. To facilitate cell growth in PU hollow fibers, the cell suspension was usually embedded in a matrix of collagen or alginate and subsequently injected into the hollow fibers[120]. The advantages of using polyurethane membranes in the above studies are less wall thickness, which improves oxygen and nutrient transport[119]. Moreover these are heat sealable and bags of various sizes can be made with this concept. This concept can also be tailored in such a way wherein vasculogenesis can also be introduced throughout the system. To study the physical and chemical properties of the bag further physicochemical characterization studies were carried out.

3.1.2 FTIR analysis of 3D printed membranes

IR spectroscopy is one of the widely employed techniques to understand the molecular structure of polymers. Diamond ATR spectroscopy couples the analytical

method of IR spectroscopy with the physical phenomenon of total internal reflection to enable the molecular vibrations within the surface regions of materials to be studied.

The IR spectroscopic images had shown the characteristic peaks of polyurethanes (fig. 7). The peak at 3318.1cm^{-1} is an indication of -NH stretch. The sharp peaks at 2922.2 cm^{-1} and 2851.4 cm^{-1} are associated with $-CH_2$ stretching, while other modes of $-CH_2$ vibrations are identified by the bands at 1447.9cm^{-1}, 1366.8cm^{-1}, and 1318.4cm^{-1}.The peak at 1689.7cm^{-1} indicates –C=O group.

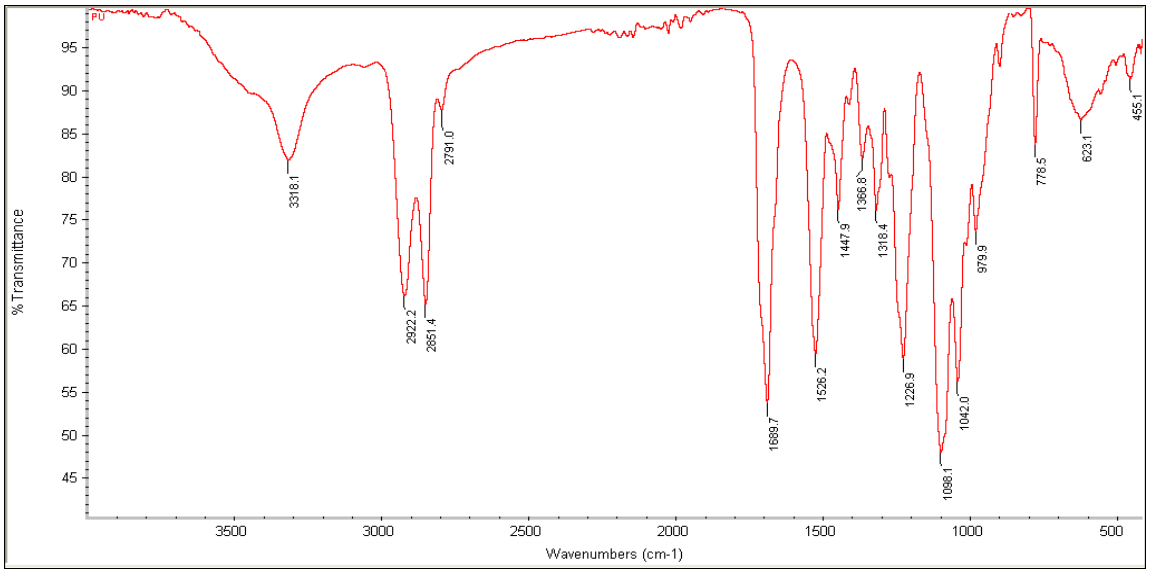


Figure 7: FT-IR spectra of Polyurethane membrane

3.1.3 Thermal analysis

3.1.3.1 DSC

Glass transition temperature of a polymer is the point in which there is a transition from a hard brittle condition to a viscoelastic or rubber elastic condition. The greater the amorphous portion the lower will be the glass transition temperature and the higher the glass transition temperature the more brittle condition is observed.

The glass transition temperature of polyurethane is found to be in the range of 35.14ºC which falls within the range of Tg values for thermoplastic polyurethanes

(fig 8). This nature imparts better processability of the PU which also imparts good pliability to the membranes.

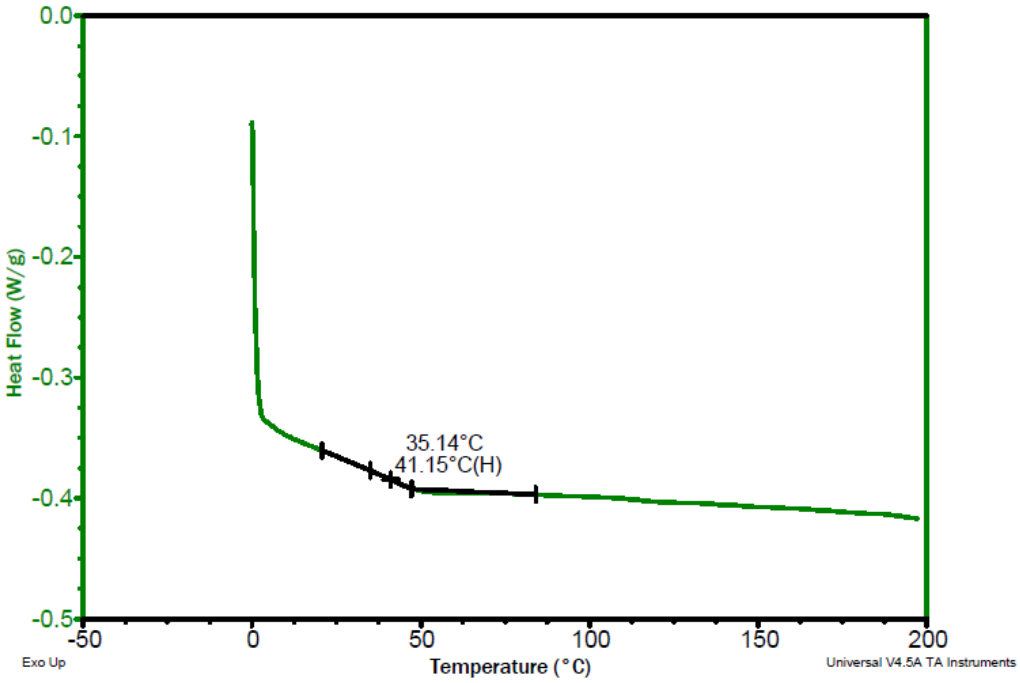


Figure 8: DSC thermogram of Polyurethane

3.1.3.2 TGA

The technique of TGA involves the study of thermal stability and decomposition of the material. Fig.9 shows the representative thermogram of PU. The thermogram shows that the point at which thermal decomposition starts was 268.97^{0}C. 50% of decomposition is complete when the material reaches at the temperature at 355.66^{0}C and final decomposition at 572.46^{0}C. At 800^{o}C about 2% of residual PU was observed. This study proved that the material is thermally stable.

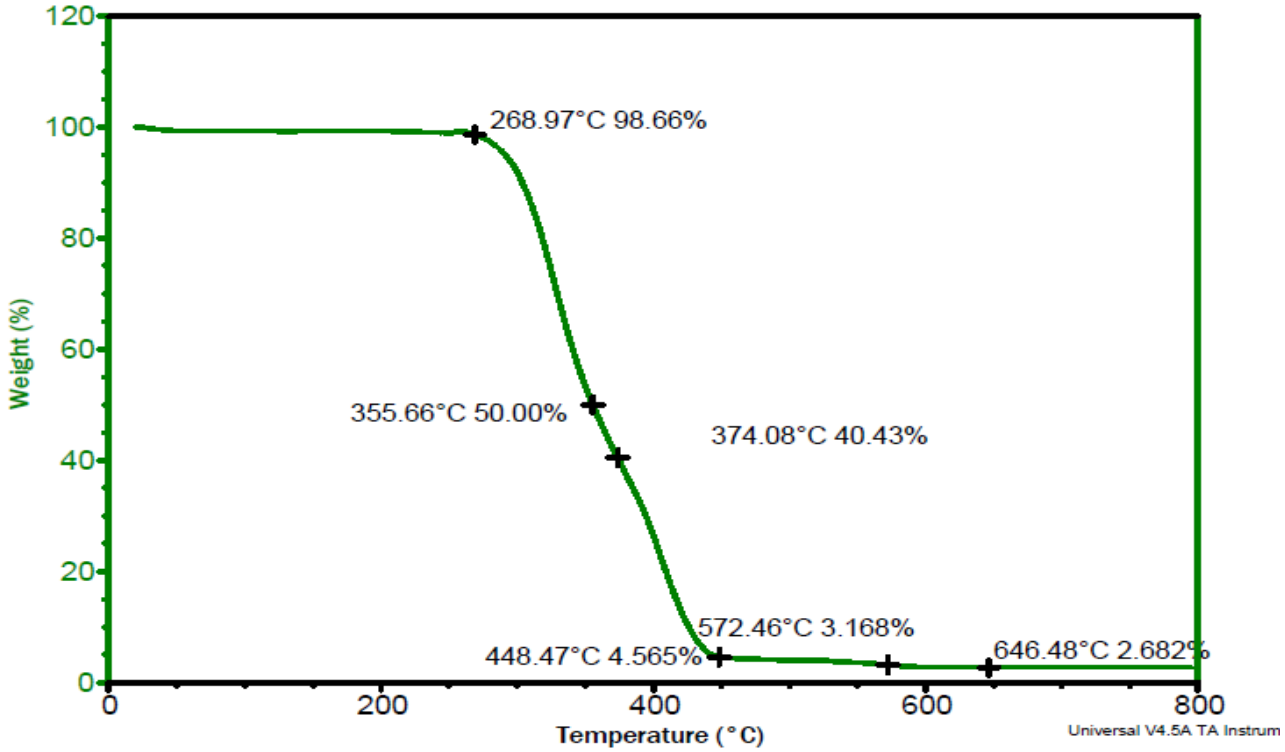


Figure 9: Thermogram of PU membrane.

3.1.4 Contact angle studies

To further study the surface property of the material contact angle was measured which is also an indication of the hydrophilicity of the material. The water in air contact angle was measured where a higher contact angle indicates that the material is hydrophobic and lower contact angle indicate that the material is hydrophilic. The contact angle value for the PU membrane was found to be 84.35 $\pm$ 2.55^{o} which showed that the PU membrane maintained its hydrophobicity. This was found to be advantageous in our study as the hydrophobic membranes would prevent blood protein adsorption and also prevent the adhesion of the membrane with body fluids and tissue. The representative image of contact angle measured on PU membrane was shown in fig 10.

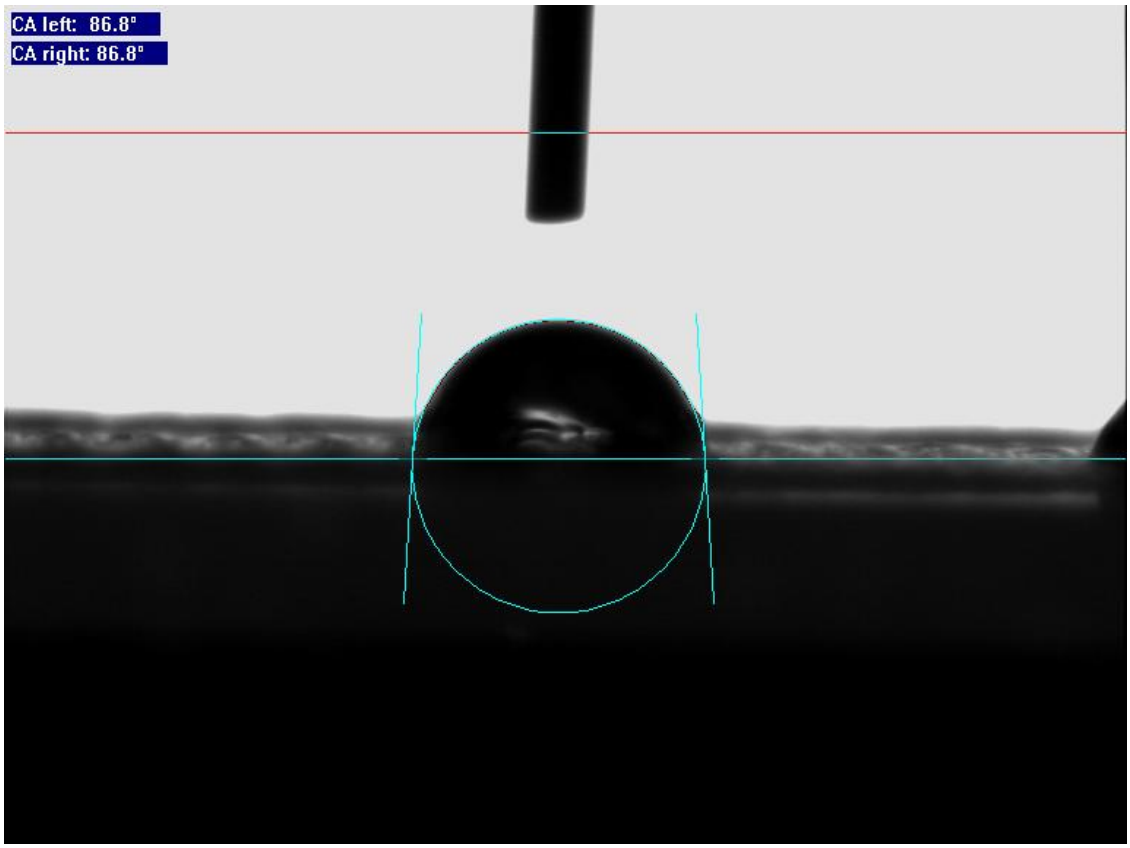


Figure 10: Contact angle of PU membrane

3.2 Diffusion studies using 3D printed PU membrane

Membrane permeability was measured using a two chamber diffusion cell. The permeation coeifficient was determined. The model compounds used for diffusion study were glucose, insulin, and immunoglobulin. Diffusion studies were done using 3D printed membranes placed between diffusion cells. Diffusion of glucose, insulin and immunoglobulin (IgG) were studied different time intervals (7, 14 Days). It was observed that cumulative diffusion levels of glucose and insulin on 7^{th} and 14^{th} day were 19.7 $\pm$ 1.52µg/L and 38.5 $\pm$ 2.08µg/L respectively as shown in the graph (Fig 11). While in the case of insulin, 7^{th} day release was 1.31 $\pm$ 0.351 µg/L and 2.6 $\pm$ 3.05µg/L was the cumulative amount released on 14^{th} day. A steady release was observed for both insulin and glucose from 7^{th} day to 14^{th} day. Immunoglobulin IgG

showed really negligible or no diffusion through the membrane since the membrane channels are too narrow to prevent the immunoglobulins (Table 3).

Robert et al., has reported that the nonporous polyurethane membranes were permeable to both glucose and insulin but impermeable to immunoglobulins[127]. In our study the pore channels also will help in diffusion along with the nonporous regions. The permeation coefficient of glucose and insulin are $0.03cm^2/s$ and $0.8cm^2/s$ respectively. From this result we can say that the permeation of both insulin and glucose is similar and in a controlled fashion.

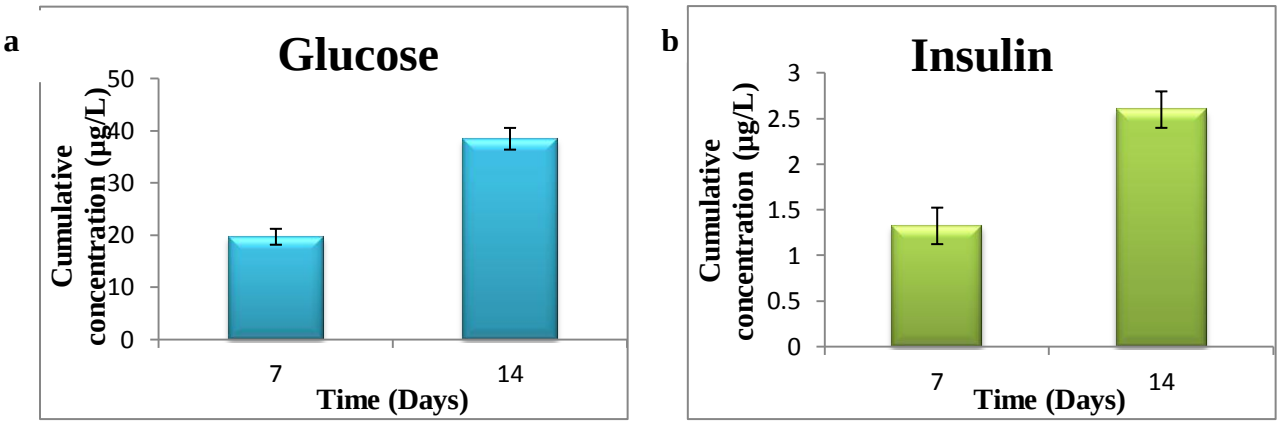


Figure 11: Graph showing a) the amount of glucose diffused b) the amount of insulin diffused

Time	Glucose(µg/L)	Insulin (µg/L)	IgG(µg/L)
7^{th} Day	**19.7 ± 1.52**	**1.31 ± 0.351**	**0**
14^{th} Day	**38.5 ± 2.08**	**2.6 ± 3.05**	**0.0001 ± 0.01**

Table 3: Amount of glucose, insulin and Immunoglobulin diffused through 3D printed membrane

3.3 Swelling studies

Fluid uptake is an important parameter which influences the chemical and physical characteristics of the membrane. Fig 12 represents the swelling percentage of 3D printed PU membrane at different time periods (7, 14, 21, 28, 35 days). It was observed that only 2% swelling occurs in 7 days which tends to equilibrate to 17% in 35 days. There is no significant difference ($p < 0.05$) in fluid uptake between consecutive time points and hence the pore size in the channels is preserved. The swelling behavior is an important property because it relates also to the diffusion of signaling molecules and nutrients. From the clinical aspect, over load swelling percentage of the immunoisolation bags may cause pressure to the surrounding tissues. Moreover when swelling increases the pore channels make tend to widens and may allow the immune cells to enter the membrane and destroy the islets. Our membrane shows a lower percentage of swelling will help to avoid the increase in the pore channels and hence protect the encapsulated islet cells.

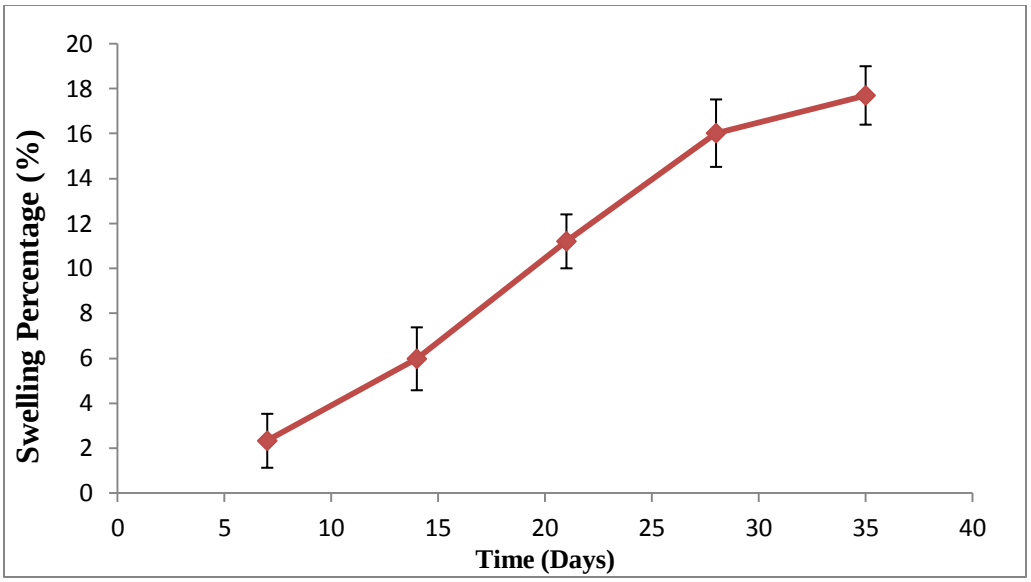


Figure 12: Graph representing the swelling percentage of 3D printed PU membrane

3.4 Degradation studies

The biodegradation test of PU membrane was carried out in physiological saline (pH 7.4) at 37ºC in triplicates. The biodegradation was monitored by the weight loss of the material for 35 days. This study gives the measurement of leachants that may harm the encapsulated islet cells. The biodegradation study results were given as weight loss versus time in days and the plot of percentage weight loss versus time (in days) were plotted as in fig 13. Only 8% degradation or weight loss was observed in 7 days which increased to only about 23% in 35 days. There is no significance difference in biodegradation percentage between consecutive time points. Polyurethanes are widely used in medical implants as they are inert and show resistance. Apart from this they show higher elasticity and toughness, and resistance to tear, oxidation and humidity[122]. Further confirmation on the degradation was also assessed using the cytotoxicity test on extract of the sample.

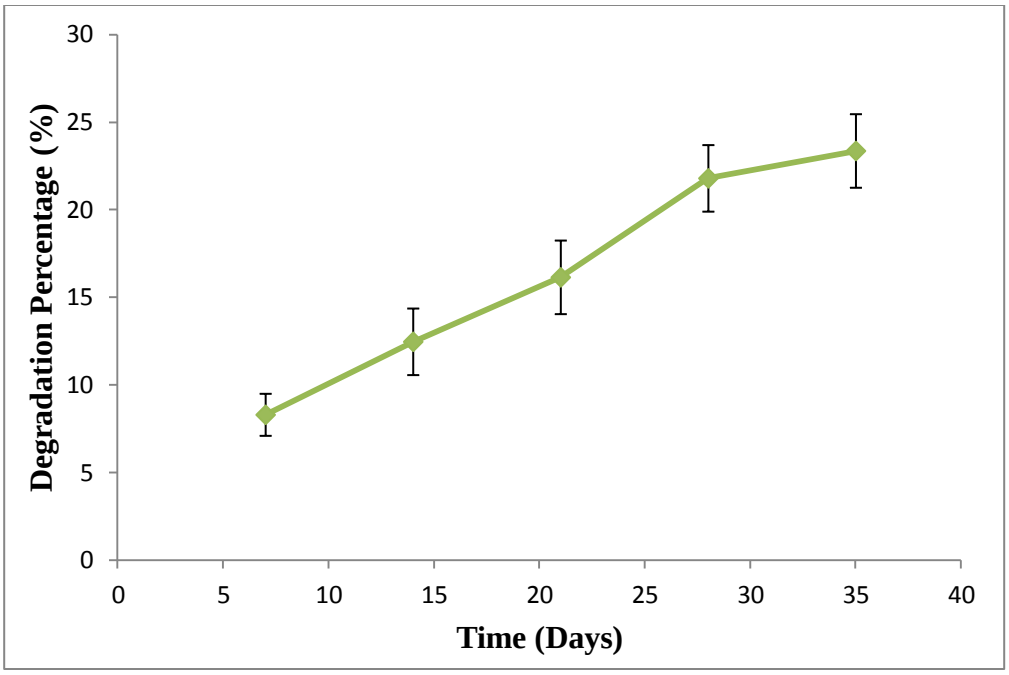


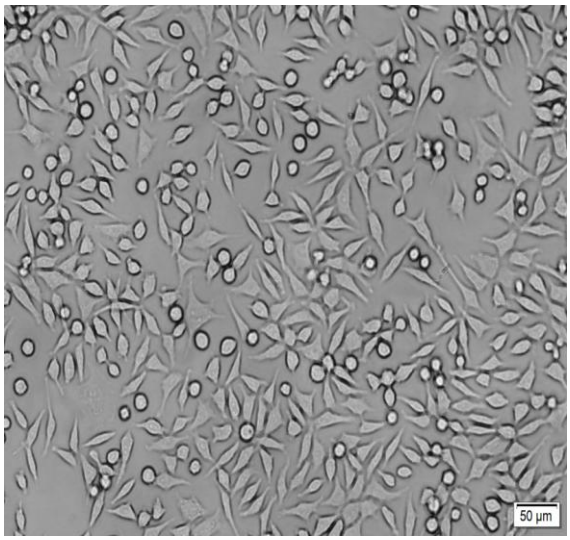
Figure 13: Graph representing the biodegradation percentage of 3D printed PU membrane

3.5 *In vitro* cell cytotoxicity studies

3.5.1 Direct contact test

Cellular response to a biomaterial can be impacted by both the crosslinked material and the soluble monomers that may leach out[116]. Direct contact test was performed to evaluate the cytotoxicity of the membrane when placed in direct contact on a monolayer of L929 cells. After 24h of incubation with PU membrane on 70% confluent layer of cells optical images were taken using Olympus IX71 microscope. Fig 14 indicates no changes in cell viability, morphology or detachment of cells. The cells were seen to maintain their spindle shaped morphology which indicated that little or no cytotoxic response was imparted by the polyurethane membranes

a



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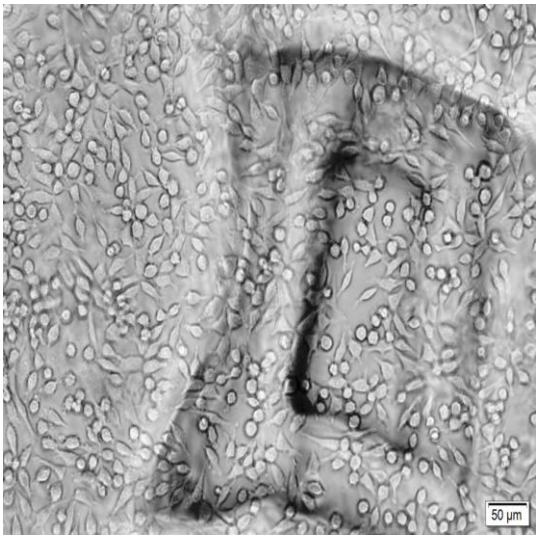


Figure 14: Phase contrast images of Direct Contact test a) Control b) Cells with 3D printed membrane

3.5.2 MTT assay

Cell viability was determined by MTT assay. L929 cells were cultured with 24h extract of polyurethane membrane in culture media. After 24h, the medium was replaced with MTT solution. MTT (thiazol blue) was converted from yellow coloured salt to purple coloured formazan crystals by cleavage of the tetrazolium ring by the mitochondrial succinate dehydrogenase of the live cells, the activity of which is linear to the live cell number. Fig 15 represents the percentage viability of L929 when treated with the extracts of PU membrane. The cells were around 85% viable in the test group as compared to the control. There was no significant difference between the viability of cells in control and the test group.

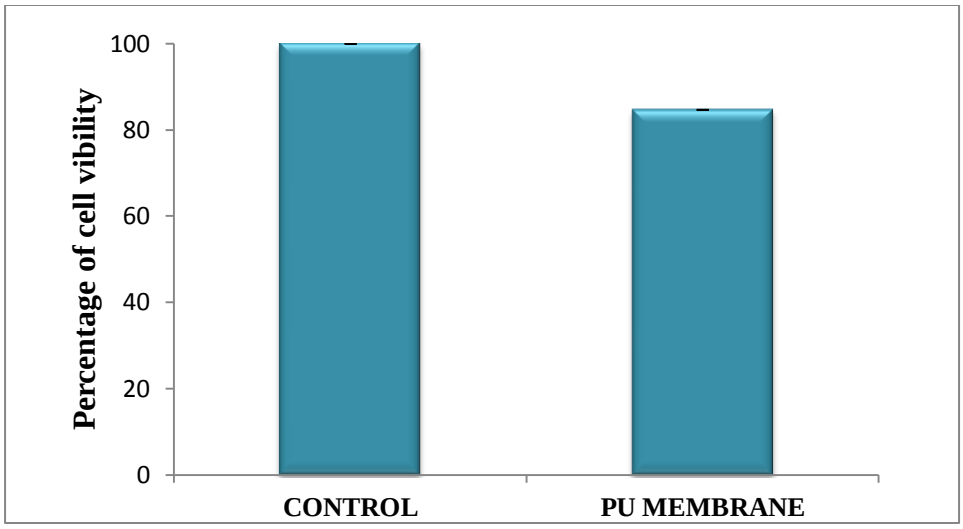


Figure 15: Graph showing percentage cell viability with 3D printed PU membrane

3.6 Preparation and characterization of alginate beads

Alginate beads were prepared using different concentration (1.5%, 2.5%, 5%) of sodium alginate solution. It was observed that the morphological consistency of beads prepared from 5% solution was more stable compared to other concentrations as it is evident from SEM images (Fig 16). Hence the 5% concentration was selected for the bead preparation in the remaining studies. These beads are spherical in shape and act as a scaffold for microencapsulation of islet cells. The spherical shape of the beads was confirmed from the photographic image as shown in the figure 17.

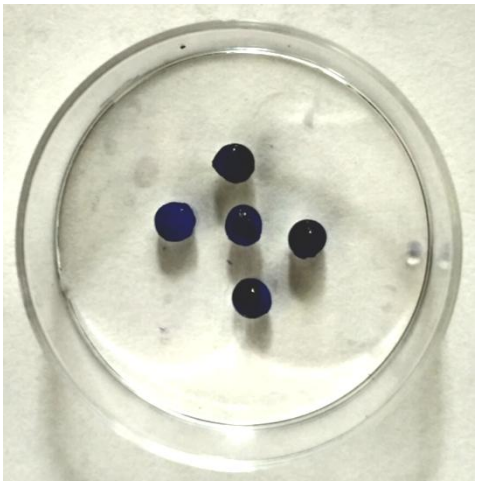


Figure 17: 5% Alginate spherical beads using toluidine blue dye

Characterization of surface morphology and bead size were analysed using ESEM. It was observed that on increasing the concentration of alginate solution from 1.5- 5 % the size of the beads decreased and the beads were found to be more stable having micropores for effective media and nutrient diffusion. The size of the bead prepared from 1.5 % was 3.18$\pm$ 0.022mm which is reduced to 2.61 $\pm$ 0.0134mm. Berit et.al., reports clinical transplantation of human islets in alginate-based capsules in four small trials and are in general recognized it as safe substrate for encapsulation. Also very recent positive findings regarding increased size of capsules and chemical modifications of the alginate where reduced fibrosis is seen in monkeys also makes an optimistic view of the future of encapsulated islet transplantation[117] using this polymer.

Microcapsules allow for the fast exchange of therapeutic molecules and have been shown to closely mimic the release of insulin and glucose. Because of this beneficial property of microcapsules, the majority of research groups have concentrated on the development of microcapsules that provoke low or no inflammatory responses for the cure of endocrine diseases[125]. de Vos et.al.,has also commented on the purity of alginate polymer solution[126] in his study.

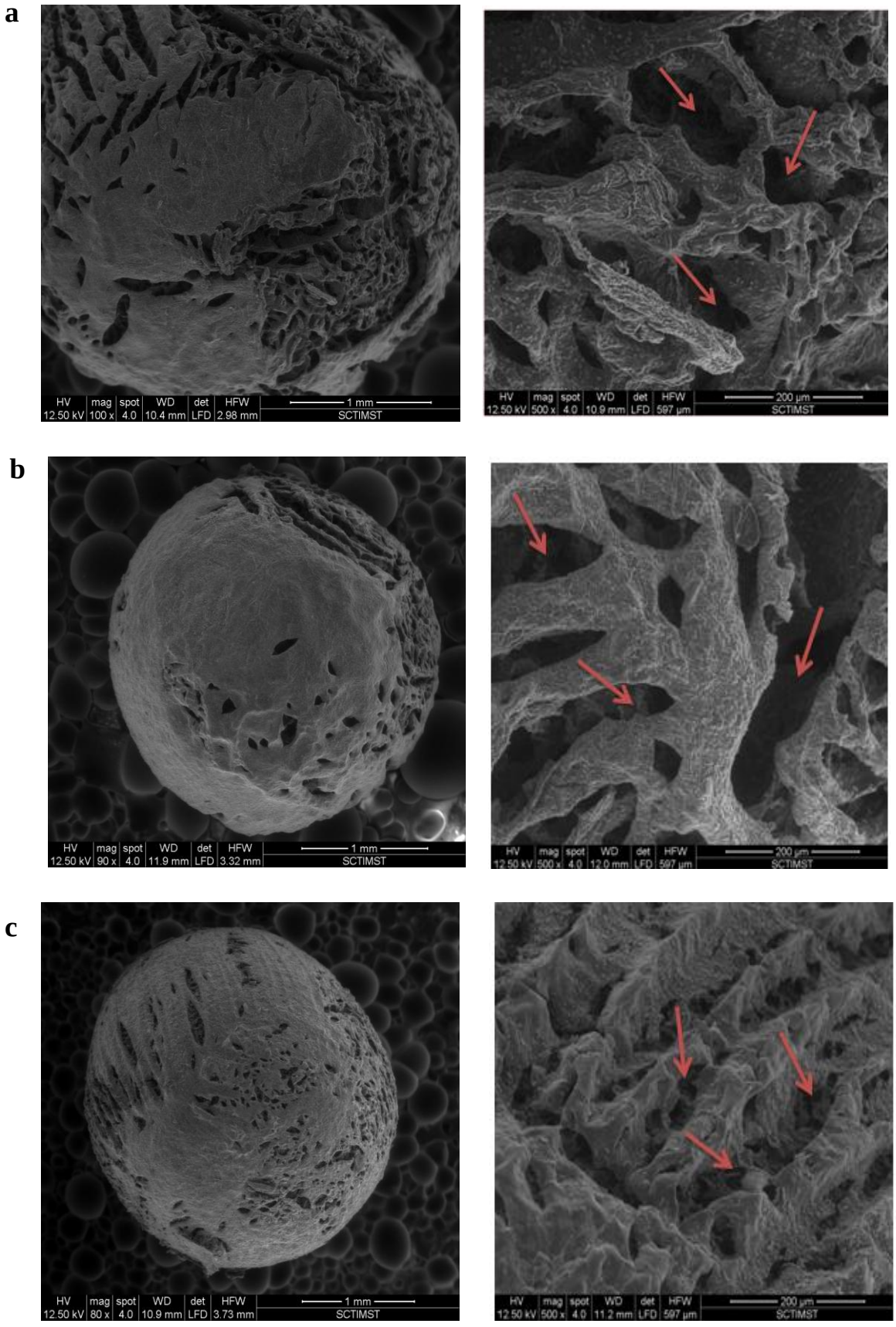


Figure 16 : ESEM images of alginate beads made of a) 1.5% alginate b) 2.5% alginate and c) 5% alginate (Red arrows shows the pores in the beads)

3.7 Isolation and characterization of islets from rabbit

The islet clusters were isolated (fig 18) from rabbit pancreas by 1% collagenase V enzymatic digestion as per references[128]. Islets were observed under phase contrast microscope after the isolation at day 0 where the cells exhibited cluster morphology with the size of clusters ranging from 50-200µm. A multitude of islets (50-100 clusters) were obtained on day 0.

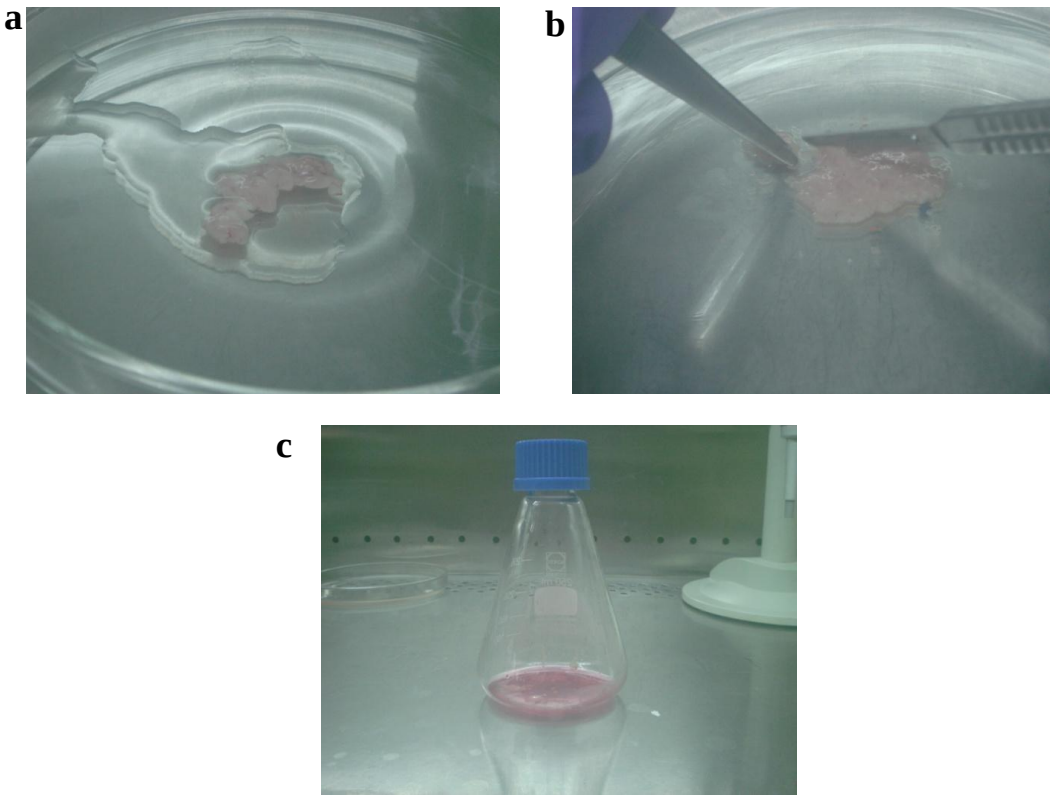
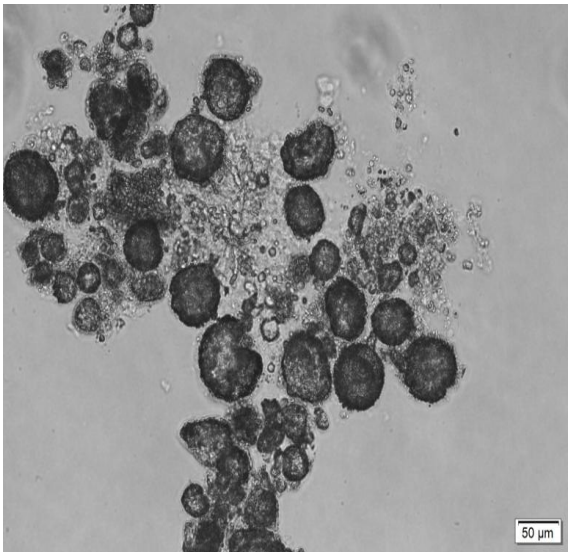


Figure 18: Isolation of pancreatic islets from rabbit using collagenase V a) isolated pancreas b) chopping of pancreas into fine pieces c) digestion of the chopped tissue using collagenase V

Presence of β cells were confirmed by performing dithizone staining (fig 19). Dithizone stained positive cells which appeared crimson red as it is a zinc chelating agent which binds to zinc granules in beta cells.

a



b

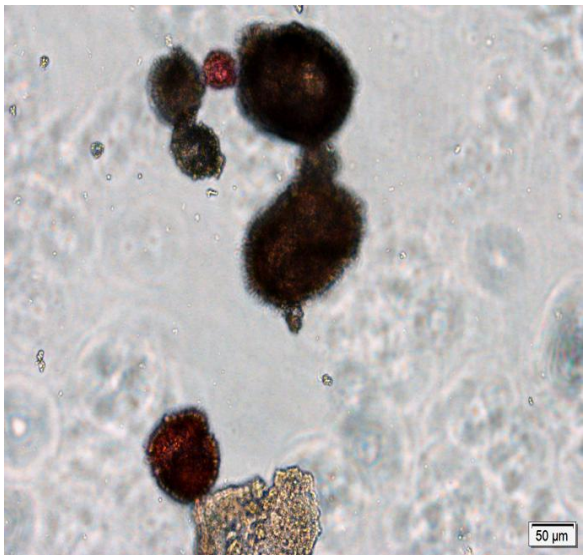


Figure 19: Characterization of rabbit islets a) Phase contrast image of islet after 0 day of isolation b) Dithizone staining of islets

3.8 Macroencapsulation studies

After the islet cell isolation on day 2, the islet clusters were then encapsulated in the alginate beads. These were then simulataneously macroencapsulated within PU immuno-isolation bags. 2-3 beads containing 6-8 clusters of islets were macroencapsulated in the PU immunoisolation bags which had a size of 2.54 X 2.5cm and it was thermally sealed. As per the study by Kollmer et.al. , encapsulated islets in alginate substrate can be protected from the host's immune system and remain viable and functional following transplantation. However, the long-term success of these therapies requires that alginate microcapsules maintain their immunoprotective capacity and stability in vivo for sustained periods[124].

3.8.1 Live dead assay performed on islet bags

To assess the viability of encapsulated cell within the 3D printed islet bags live dead assay was performed after 21 days. Initial viability studies were performed using L929 mouse fibroblast cells followed by an experiment using rabbit islet cells. The assay was performed using calcein and ethidium homodimer. Live cells were

distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein was well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. It was observed that the cells were viable within the PU membrane bags.

Fig 20 depicts that L929 and islets were alive in PU membrane bags after 21 days respectively.

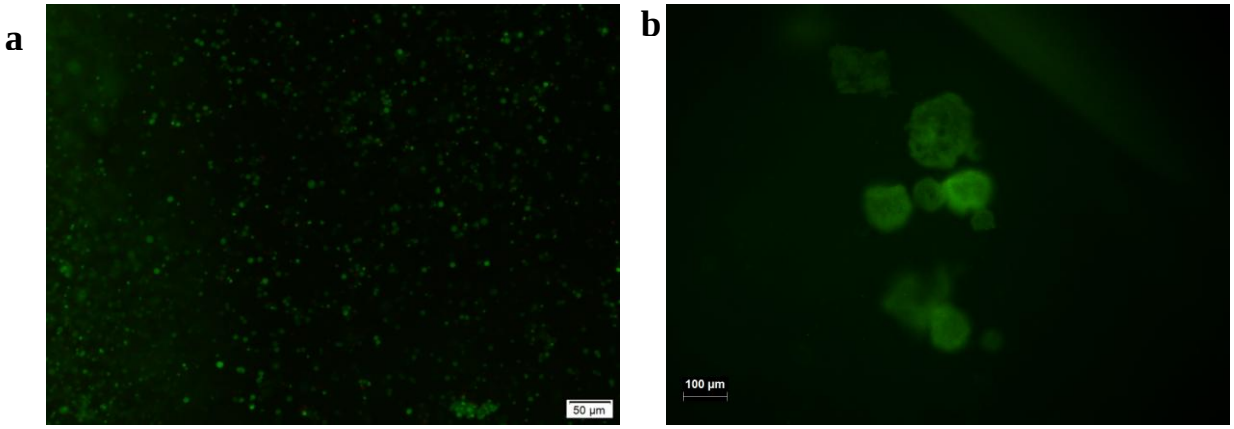


Figure 20: Fluorescent images of live and dead staining of a) L929 cells and b) islet cells within the alginate gel beads which are encapsulated in 3D printed membrane bags after 21 days post culture.

3.8.1 *Invitro* glucose challenge assay

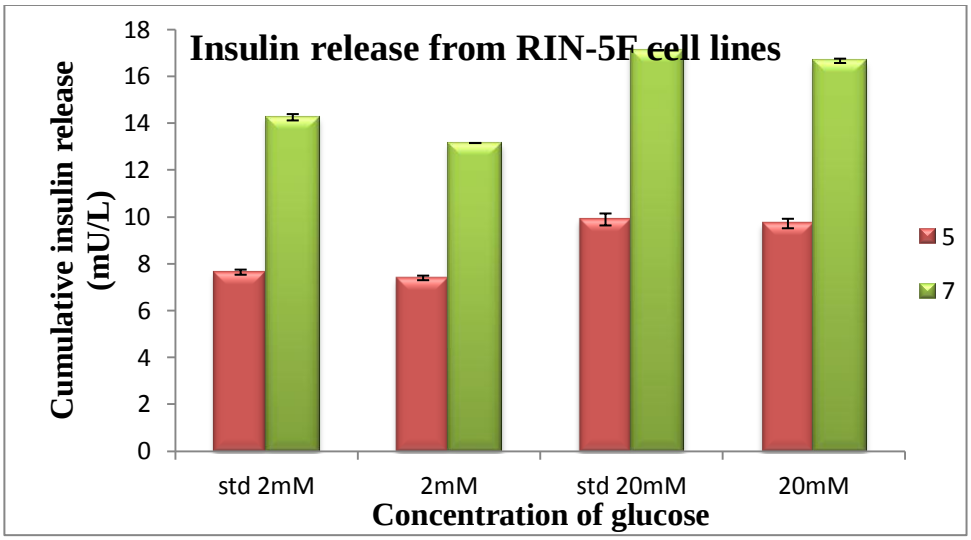
Finally to assess the functionality of these microencapsulated islet cells i*nvitro* glucose challenge assay was done using both RIN-5F rat islet cell lines and primarily isolated rabbit islets. These cells were microencapsulated within alginate beads and macroencapsulated in 3D printed PU membrane bags. Glucose challenge of 2mM (low) and 20mM (high) were given on 5^{th} and 7^{th} day of culture. On providing 2mM

glucose challenge it was observed that a significant difference of insulin release was observed from the 5 day and 7 day macroencapsulated islets when compared to the non encapsulated islet cells. The 5^{th} day secretion of insulin by rat cell lines was seen to be 6.61 $\pm$ 0.52 mU/L which increased to 7.3 $\pm$ 0.63mU/L on day 7 when treated with 2mM glucose for nonencapsulated islet cells. A similar increase can be seen in the case of encapsulated islets. It was observed that the amount of insulin released on 5^{th} day was 5.75 + 0.56 mU/L which is increased to 7.3 $\pm$ 0.81mU/L (fig 21). Similarly when the encapsulated and nonencapsulated islets treated with high glucose (20mM) an increase of insulin release from 6.94 $\pm$ 0.76mU/L to 9.72 $\pm$ 0.65mU/L which is very similar to the insulin release of nonencapsulated islets.

When compared between RIN-5F rat islet cell lines and primary isolated rabbit islets the insulin release was similar as compared to the control. The insulin release was compared with the work of Stephen Harrington et.al., in which in vitro studies using the HA-COL gel had diffusion characteristics that would allow small molecules such as glucose and insulin to enter and exit the gel, whereas larger molecules (70 and 500 kDa dextrans) were impeded from diffusion. Islets encapsulated in HA-COL hydrogel showed significantly improved in vitro viability over unencapsulated islets and retained their morphology and glucose sensitivity for 28 days[123].

Similarly our strategy of microencapsulating the islet cells in alginate beads and then macro encapsulating them in 3D printed narrowed channel membrane bags enabled maintenance of cell morphology as well as glucose sensitivity or functionality for 5 and 7 days respectively. This study confirmed that the islet cells are functionally active with a consistent diffusion through the 3D printed bags. This concept provided for a better encapsulation strategy which can pave the way for effective pancreatic islet transplantation.

a



b

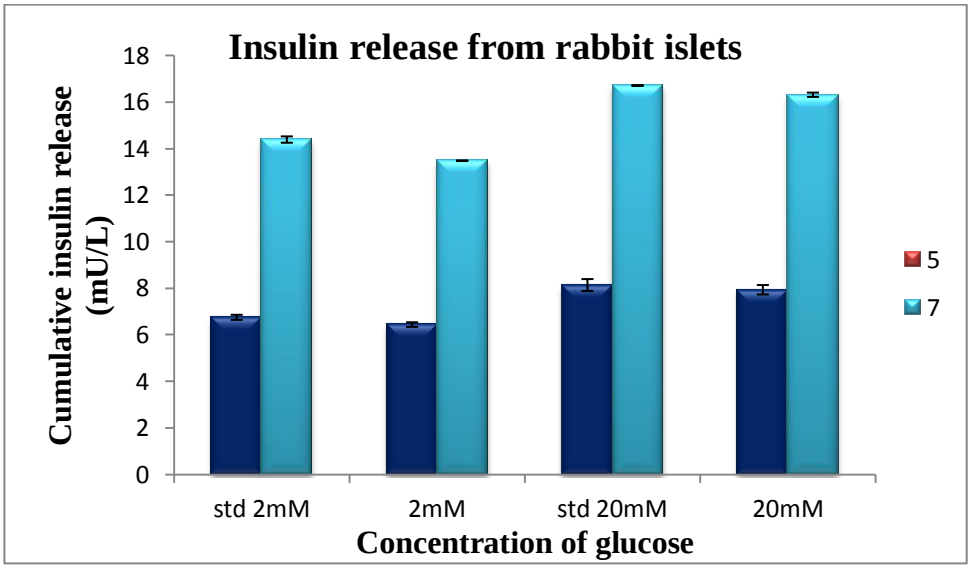


Figure 21: Graph showing a) insulin release from RIN-5F cell lines b)insulin release from rabbit islets.

4. CONCLUSION AND FUTURE PERSPECTIVES

Diabetes mellitus is a fast growing chronic metabolic disease caused either due to insulin insufficiency or its resistance resulting in lifelong dependency on insulin has become one of the most significant non-communicable diseases globally. The complications associated with the disease include diabetic neuropathy, nephropathy, cardiovascular diseases etc. Diabetes is mainly treated by administration of oral hypoglycemic drugs or insulin injection. However, these approaches cannot mimic the physiological oscillating pattern of insulin release to achieve normoglycemia thereby cannot prevent the long term complications associated with diabetes and hypoglycemic shock. Transplantation of pancreatic islets aims to achieve euglycemia by following the insulin release pattern as in physiological manner. Immunosuppression is one of the major risk in using allogenic and xenogenic islets for transplantation. Islet transplantation has been sought as strategies for overcoming lifelong immunosuppression, which can provide a physical immune barrier by keeping out high molecular weight immune system components, while still allowing low molecular weight oxygen, insulin and nutrients to pass through.

The study was an attempt to do a combined approach of micro and macro encapsulation for effective islet transplantation. Microencapsulation was done using alginate beads. Alginate is one of the effective methods for encapsulating islet cells. The application of 3D printing in making macro immunoisolation bags is a novel strategy wherein the diffusion property can be controlled. Since PU is a hydrophobic polymer it would not adhere to the body fluids when transplanted. The main benefit in using the 3D printing is that we could generate narrowed pore channels which could allow permeation of low molecular weight molecules such as glucose and insulin but prevent the diffusion of high molecular weight immunoglobulins. The physico chemical characterizations of the membranes were conducted and the membrane was found to be highly stable. Also not much swelling or degradation of the membranes

were observed which was also supported from the cytotoxicity assays. The encapsulated islet cells released insulin at a constant rate and the functionality was confirmed by glucose challenge assay. The cells were found functionally active when live dead assay was done. Hence this study is a promising starting point for exploring the potential advantages of 3D printing in developing narrow pored channel membranes or bags for islet encapsulation and the use of this combined micro and macro encapsulation strategy for further scale up technologies in the area of pancreatic islet transplantation. However, moving forward more future studies are warranted to assess the safety and efficacy of this system over longer periods in culture and in the in vivo system.

Future Perspectives

Microencapsulation in alginate beads and macroencapsulation in 3D printed membrane would help to diffuse, media, insulin and glucose through the pore channel made by 3D printing. The cells were functionally active after the encapsulation and also able to release insulin when glucose challenge occurs. However further study are warranted to evaluate the effectiveness of the 3D printed islet bags in maintaining the functionality for extended time points. Future studies that need to be done in this area include:

- More functional characterization of islet cells after encapsulation by immunostaining for specific markers and gene expression studies.
- To study the islet cell functionality over extended time points.
- To extend the study to in vivo preclinical animal models to ascertain the efficacy and functionality of these 3D printed islet bags.
- To look at scale up potential using this strategy.

REFERENCES

1. Dall TM, Zhang Y, Chen YJ, Quick WW, Yang WG, Fogli J (2010) The economic burden of diabetes. *Health Affairs* 29(2): 297–303.

2. Kitabchi AE, Umpierrez GE, Miles JM, Fisher JN (2009) Hyperglycemic crises in adult patients with diabetes. *Diabetes Care* 32 (7): 1335–43.

3. Saydah SH, Miret M, Sung J, Varas C, Gause D, Brancati FL (2001) Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 37(1).

4. Shapiro AMJ (2003) Islet Transplants and Impact on Secondary Diabetic Complications: Does C-Peptide Protect the Kidney. *Journal of the American Society of Nephrology* 14(8): 2214–2216.

5. Ballilnger WF and Lacy PE (1972) Transplantation of intact pancreatic islets in rats. *Surgery* 72(2): 175-186.

6. Reckard CR and Barker CF (1973) Transplantation of isolated pancreatic islets across strong and weak histocompatibility barriers .*Transplantation Proceedings*. 5(1): 761-763.

7. Gray DW, McShane P, Grant A, and Morris PJ (1984) A method for isolation of islets of Langerhans from the human pancreas. *Diabetes* 33(11): 1055-61.

8. Ricordi C, Lacy PE, Finke EH, Olack BJ, and Scharp DW (1989) Automated method for isolation of human pancreatic islets. *Diabetes* 37(4): 140-142.

9. Scharp DW, Lacy PE, Santiago J (1990) Insulin independence after islet transplantation into type I diabetic patient. *Diabetes* 39(4): 515-518.

10. Bretzel RG (2000) Current status and perspectives in clinical islet transplantation. *Journal of Hepato-Biliary-Pancreatic Surgery* 7(4): 370-373.

11. Bretzel RG, Brandhorst D, Brandhorst H(1999) Improved survival of intraportal pancreatic islet cell allografts in patients with type-1 diabetes mellitus by refined peritransplant management. *Journal of Molecular Medicine* 77(1): 1432-1440.

12. Tzakis AG, Ricordi C, Alejandro R, Zeng Y, Fung JJ, Todo S, Demetris AJ, Mintz, DH and Starzl TE (1990) Pancreatic islet transplantation after upper abdominal exenteration and liver replacement. *Lancet* 336(8712): 402-405.

13. Shapiro AMJ, Lakey JRT, Ryan EA, Korbutt GS, Toth EL, Warnock GL, Kneteman NM and Rajotte RV (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *The New England Journal of Medicine* 343(4): 230-238.

14. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert- Margolis V, Bluestone J and Lakey JR (2006) International trial of the Edmonton protocol for islet transplantation. *The New England Journal of Medicine* 355(13): 1318-1330.

15. Bellin MD, Kandaswamy R, Parkey J, Zhang HJ, Liu B, Ihm SH, Ansite JD, Witson J, Bansal-Pakala P, Balamurugan AN, Papas K, Sutherland DE, Moran A and Hering BJ (2008) Prolonged insulin independence after islet allotransplants in

recipients with type 1 diabetes. *American Journal of Transplantation* 8(11): 2463-2470.

16. Narushima M, Kobayashi N, Okitsu T, Tanaka Y, Li SA, Chen Y, Miki A, Tanaka K, Nakaji S, Takei K, Gutierrez AS, Rivas-Carrillo JD, Navarro-Alvarez N, Jun HS, Westerman KA, Noguchi H, Lakey JR, Leboulch P, Tanaka N and Yoon JW (2005) A human beta cell line for transplantation therapy to control type 1 diabetes. *Nature Biotechnology* 23(10): 1274 – 1282.

17. Korbutt GS, Ao Z, Flashner M and Rajotte RV (1997) Neonatal porcine islets as a possible source of tissue for humans and microencapsulation improves the metabolic response of islet graft post transplantation. *Annals of the New York Academy of Sciences* 831(1): 294-303.

18. Smith A (2006) A glossary for stem-cell biology *Nature* 441(7097): 1060.

19. Street CN, Sipione S, Helms L, Binette T, Rajotte RV, Bleackley RC and Korbutt, GS (2004) Stem cell-based approaches to solving the problem of tissue supply for islet transplantation in type 1 diabetes. *The International Journal of Biochemistry & Cell Biology* 36(4): 667–683.

20. Gimble JM (2003) Adipose tissue-derived therapeutics. *Expert Opinion on Biological Therapy* 3(5): 705-713.

21. EA Ryan, BW Paty, PA Senior (2005) Five-year follow-up after clinical islet transplantation *Diabetes* 54(7): 2060–2069.

22. MD Bellin, FB Barton, A Heitman (2012) Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *American Journal of Transplantation* 12: 1576–1583.

23. AA Rossini (2004) Autoimmune diabetes and the circle of tolerance. *Diabetes* 53(2): 267–275.

24. G Worcester (2006) Human Islet Transplantation Autoimmunity after islet-cell allotransplantation. *The New England Journal of Medicine* 355: 1397–1399.

25. P Carlsson, F Palm, A Andersson, and P Liss (2001) Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes* 50(3): 489–495.

26. R Hilbrands, VAL Huurman, P Gillard (2009) Differences in baseline lymphocyte counts and autoreactivity are associated with differences in outcome of islet cell transplantation in type 1 diabetic patients. *Diabetes* 58(10): 2267–2276.

27. NR Barshes, S Wyllie, and JA Goss (2005) Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. *Journal of Leukocyte Biology* 77(5): 587–597.

28. W Bennet, B Sundberg, C Groth (1999) Incompatibility between human blood and isolated islets of langerhans: a finding with implications for clinical intraportal islet transplantation. *Diabetes* 48(10): 1907–1914.

29. L Özmen, KN Ekdahl, G Elgue, R Larsson, O Korsgren, and B Nilsson (2002) Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction

triggered by isolated human islets: possible application of the thrombin inhibitor Melagatran in clinical islet transplantation. *Diabetes* 51(6): 1779–1784.

30. H Johansson, A Lukinius, L Moberg (2005) Tissue factor produced by the endocrine cells of the islets of langerhans is associated with a negative outcome of clinical islet transplantation. *Diabetes* 54(6): 1755–1762.

31. A Rabinovitch and WL Suarez-Pinzon (1998) Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. *Biochemical Pharmacology* 55(8): 1139–1149.

32. Narang AS and Mahato RI (2006) Biological and Biomaterial Approaches for Improved Islet Transplantation. *Pharmacological Reviews* 58(2):194–243.

33. Hubbell JA (1995) Biomaterials in tissue engineering. *Nat. Biotechnol* 13: 565–576.

34. Huglin MR (1986) *Hydrogels in Medicine and Pharmacy*; CRC Press: Boca Raton, FL, USA.

35. Hoffman AS (2002) Hydrogels for biomedical applications. *Adv. Drug. Deliv. Rev 54*: 3–12.

36. Peppas NA, Hilt JZ, Khademhosseini A, Langer R (2006) Hydrogels in biology and medicine: From molecular principles to bionanotechnology. *Adv. Mater 18*: 1345–1360.

37. Wichterle O, Lim D (1960) Hydrophilic gels for biological use. *Nature 185*: 117–118.

38. Mohan N, Nair PD and Tabata Y (2010) Growth factor-mediated effects on chondrogenic differentiation of mesenchymal stem cells in 3D semi-IPN poly(vinyl alcohol)– poly(caprolactone) scaffolds. Journal *of Biomedical Materials Research Part A* 94(1): 146–159.

39. Chang TM (1964) Semipermeable microcapsules. *Science* 146: 524-525.

40. De Vos P, Hamel AF and Tatarkiewicz K (2002) Considerations for successful transplantation of encapsulated pancreatic islets. *Diabetologia* 45(2): 159- 173.

41. Sun Y, Ma X, Zhou D, Vacek I and Sun AM (1996) Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. *The Journal of Clinical Investigation* 98(6): 1417–1422.

42. Schneider S, Feilen PJ, Brunnenmeier F, Minnemann T, Zimmermann H, Zimmermann U and Weber MM (2005) Long-term graft function of adult rat and human islets encapsulated in novel alginate-based microcapsules after transplantation in immunocompetent diabetic mice. *Diabetes* 54(3): 687–693.

43. Lanza RP, AM Beyer and Chick WL (1999) Xenogenic humoral responses to islets transplanted in biohybrid diffusion chambers. *Transplantation* 57(9): 1371-1375.

44. Sandler S, Andersson A, Eizirik DL, Hellerstrom C, Espevik T, Kulseng B, Thu B, Pipeleers DG and Skjak-Braek G (1997) Assessment of insulin secretion in vitro from microencapsulated fetal porcine islet like clusters and rat, mouse and human pancreatic islets. *Transplantation* 63(12): 1712-1718.

45. Chick WL, Like AA and Lauris V (1975) Beta cell. culture on synthetic capillaries:an artificial endocrine pancreas. *Science* 187(4179): 847-849.

46. Sun AM, Parisius W, Healy GM, Vacek I and Macmorine HG (1977) The use, in diabetic rats and monkeys, of artificial capillary units containing cultured islets of Langerhans (artificial endocrine pancreas). *Diabetes* 26(12): 1136–1139.

47. Scharp DW, Mason NS and Sparks RE (1984). Islet Immunoisolation:the use of hybrid artificial organs to prevent islet tissue rejection. *World Journal of Surgery* 8(2): 221-229.

48. Siebers U, Zekorn T and Bretzel RG (1990) Histocompatibility of semipermeable membranes for implantable diffusion devices (bioartificial pancreas). *Transplantation Proceedings* 22(2): 834-835.

49. Burczak K, Gamian E and Kochman A (1996). Long-term in vivo performance and biocompatibility of poly(viny1 alcohol) hydrogel macrocapsules for hybrid type artificial pancreas. *Biomaterials* 17(24): 2351-2356.

50. Nair PD, Mohanty M, Rathinam K, Jayabalan M amd VN Krishnamurthy (1992). Studies on the effect of degree of hydrophilicity on tissue response of Polyurethane interpenetrating polymer networks. *Biomaterials* 13(8): 537-542.

51. George S, Nair PD, Risbud, MV. and Bhonde, RR (2002) Nonporous Polyurethane Membranes as Islet Immunoisolation Matrices - Biocompatibility Studies. *Journal of Biomaterial Applications* 16(4): 327-340.

52. Nair PD (2009) A process for the preparation of a biocompatible polymeric composition of an inter-penetrating polymeric network (IPN), Indian patent 230740.

53. Kadam S, Muthyala S, Nair PD and Bhonde R (2010) Reversal of experimental diabetes in mice by transplantation of neo-islets generated from human amnion—derived mesenchymal stromal cells using immuno-isolatory macrocapsules. *Cytotherapy* 12(8): 982-991.

54. Kadam S, Muthyala S, Nair P and Bhonde R (2010) Human Placenta-Derived Mesenchymal Stem Cells and Islet-Like Cell Clusters Generated From These Cells as Novel Sources for Stem Cell Therapy in Diabetes. *The Review of Diabetic Studies* 7(2): 168-182.

55. Phadnis SM, Joglekar MV, Dalvi MP, Muthyala S, Nair PD, Ghaskadbi SM, Bhonde RR and Hardikar AA (2011) Human bone marrow-derived mesenchymal cells differentiate and mature into endocrine pancreatic lineage in vivo. *Cytotherapy* 13(3): 279-293.

56. Muthyala S, Bhonde RR and Nair PD (2010) Cytocompatibility studies of mouse pancreatic islets on gelatin - PVP semi IPN scaffolds in vitro: Potential implication towards pancreatic tissue engineering. *Islets* 2(6): 357-366.

57. Yang KC, Wu CC, Lin FH, Qi Z, Kuo TF, Cheng YH, Chen MP and Sumi S (2008) Chitosan/gelatin hydrogel as immunoisolative matrix for injectable bioartificial pancreas. *Xenotransplantation* 15(6): 407–416.

58. Reneker DH, Chun I (1996) Nanometre diameter fibres of polymer, produced by electrospinning. *Nanotechnology* 7: 216–223.

59. Li XY, Liu HC, Wang JN, Li CJ (2012) Preparation and characterization of PLLA/nHA nonwoven mats via laser melt electrospinning. *Mater Lett* 73: 103–106.

60. Park JS, Choi JB, Jo SY (2012) Characterization and structure analysis of PLGA/collagen nanofibrous membranes by electrospinning. *J Appl Polym Sci* 125: 595–603.

61. Nolsoe H, Imer S, Hultin HO (2007) Study of how phase separation by filtration instead of centrifugation affects protein yield and gel quality during an alkaline solubilisation process – different surimi-processing methods. *Int J Food Sci Technol* 42:139–147.

62. Guillen GR, Pan YJ, Li MH, Hoek EMV (2011) Preparation and characterization of membranes formed by nonsolvent induced phase separation: a review. Ind *Eng Chem Res* 50: 3798–3817.

63. Nam YS, Park TG (1999) Biodegradable polymeric microcellular foams by modified thermally induced phase separation method. *Biomaterials* 20: 1783–1790.

64. Shao JD, Chen C, Wang YJ, Chen XF, Du C (2012) Early stage structural evolution of PLLA porous scaffolds in thermally induced phase separation process and the corresponding biodegradability and biological property. *Polym Degrad Stabil.* 97: 955–963.

65. O'Brien FJ (2011) Biomaterials and scaffolds for tissue engineering. *Mater Today* 14: 88–95.

66. Ma PX (2004) Scaffolds for tissue fabrication. *Mater Today* 7: 30–40.

67. Pisano R, Barresi AA, Fissore D (2011) Innovation in monitoring food freeze drying. *Dry Technol* 29: 1920–1931.

68. Pisano R, Barresi AA, Fissore D (2011) Innovation in monitoring food freeze drying. *Dry Technol* 29: 1920–1931.

69. Whitesides GM, Grzybowski B (2002) Self-assembly at all scales. *Science* 295: 2418–2421.

70. Zhang SG, Gelain F. Zhao XJ (2005) Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures. *Semin Cancer Biol* 15: 413–420.

71. Ma PX (2008) Biomimetic materials for tissue engineering. *Adv Drug Deliv Rev* 60:184–198.

72. Stupp SI (2010). Self-assembly and biomaterials. *Nano Lett* 10: 4783–4786.

73. Zhao J, Han W, Chen H (2011) Preparation, structure and crystallinity of chitosan nano-fibers by a solid-liquid phase separation technique. *Carbohydr Polym* 83:1541–1546.

74. Murphy SV, Atala A (2014) 3D bioprinting of tissues and organs. *Nat Biotechnol*. 32(8):773-85.

75. Hull CW (1986) Apparatus for production of three-dimensional objects by stereolithography. US 4575330 A.

76. Nakamura M, Iwanaga S, Henmi C, Arai K and Nishiyama Y (2010) Biomatrices and biomaterials for future developments of bioprinting and biofabrication. *Biofabrication* 2: 014110.

77. Zopf DA, Hollister SJ, Nelson ME, Ohye, RG and Green GE (2013) Bioresorbable airway splint created with a three-dimensional printer. *N. Engl. J. Med*. 368: 2043–2045.

78. Segev H, Fishman B, Ziskind A, Shulman M and Itskovitz-Eldor J (2004) Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* 22(3): 265–274.

79. Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD and Kim SK (2002) Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proceedings of the National Academy of Sciences of the USA* 99(25): 16105–16110.

80. Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T and Petersen BE (2005) Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *The American Journal of Pathology* 166(6): 1781–1791.

81. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H and Murry CE (2007) Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *The FASEB Journal* 21(7): 1345–1357.

82. Barry FP and Murphy JM (2004). Mesenchymal stem cells: clinical applications and biological characterization. *The International Journal of Biochemistry & Cell Biology* 36(4): 568–584.

83. Barry FP and Murphy JM (2004) Mesenchymal stem cells: clinical applications and biological characterization. *The International Journal of Biochemistry & Cell Biology* 36(4): 568–584.

84. Serup P, Madsen OD and Mandrup-Poulsen T (2001) Islet and stem cell transplantation for treating diabetes. *British Medical Journal* 322(7277): 29 –32.

85. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB and Cornelius JG (2000) Reversal of insulin-dependent diabetes using islet cells generated *in vitro* from pancreatic stem cells. *Nature Medicine* 6(3): 278 –282.

86. Sun Y, Chen L, Hou XG, Hou WK, Dong JJ, Sun L, Tang KX, Wang B, Song J, Li H and Wang KX (2007).] Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells *in vitro*. *Chinese Medical Journal* 120(9): 771–776.

87. Karnieli O, Izhar-Prato Y, Bulvik S and Efrat S (2007) Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation. *Stem cells* 25(11): 2837-2844.

88. De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J and Hedrick MH (2003).

89. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla, L and Blancher A (2005) Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. *British Journal of Haematology* 129(1): 118-129.

90. Yanez R, Lamana ML, Garcia-Castro J, Colmenero I, Ramirez M and Bueren JA (2006) Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. *Stem Cells* 24(11): 2582–2591.

91. Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, Muller, B and Zulewski H (2006) Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochemical and Biophysical Research Communications* 341(4): 1135-1140.

92. Jiang J, Au M, Lu K, Eshpeter A, Korbutt G, Fisk G and Majumdar AS (2007) Generation of insulin producing islet like clusters from human embryonic stem cells. *Stem cells* 25(8): 1940-1953.

93. Smidsrød O, Skjak-Brk, G (1990) Alginate as immobilization matrix for cells. *Trend. Biotech 8*: 71–78.

94. Rowley JA, Madlambayan G, Mooney DJ (1999) Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials 20*: 45–53.

95. Wang K (2015) The paracrine effects of adipose-derived stem cells on neovascularization and biocompatibility of a macroencapsulation device. *Acta Biomater.* 15: 65-76

96. Kizilel S, Scavone A, Liu X, Nothias JM, Ostrega D, Witkowski P, Millis M (2010) Encapsulation of pancreatic islets within nano-thin functional polyethylene glycol coatings for enhanced insulin secretion. *Tissue Eng Part A* 16(7): 2217-28.

97. Obregon F, Vaquette C, Ivanovski S, Hutmacher DW, Bertassoni LE (2015) Three-Dimensional Bioprinting for Regenerative Dentistry and Craniofacial Tissue Engineering. *J. Dent. Res* 94(9): 143S−152S.

98. Wong JY (2015) Ultra-Portable Solar-Powered 3D Printers for Onsite Manufacturing of Medical Resources. *Aerospace Medicine and Human Performance* 86(9): 830−834.

99. Fedorovich NE, Alblas J, Hennink WE, Oner F C, Dhert WJA (2011) Organ printing: the future of bone regeneration. *Trends Biotechnol* 29(12): 601−606.

100. Turner BN, Strong R, Gold SA (2014) A review of melt extrusion additive manufacturing processes: I. *Process design and modeling. Rapid Prototyping Journal* 20(3): 192−204.

101. Hirazi SFS, Gharehkhani S, Mehrali M, Yarmand H, Metselaar HSC, Kadri NA, Abu Osman NA (2015) A review on powder-based additive manufacturing for tissue engineering: selective laser sintering and inkjet 3D printing. Sci. *Technol. Adv. Mater* 16(3): 033502.

102. Kruth JP, Mercelis P, Van Vaerenbergh J, Froyen L, Rombouts M (2005) Binding mechanisms in selective laser sintering and selective laser melting. *Rapid Prototyping Journal* 11(1): 26−36.

103. Yap CY, Chua CK, Dong ZL, Liu ZH, Zhang DQ, Loh LE, Sing SL (2015) Review of selective laser melting: Materials and applications. *Appl. Phys. Rev* 2(4): 041101.

104. Derby B (2010) Inkjet Printing of Functional and Structural Materials: Fluid Property Requirements, Feature Stability, and Resolution. *Annu. Rev. Mater. Res* 40: 395−414

105. Yoo H, Kim C (2015) Experimental studies on formation, spreading and drying of inkjet drop of colloidal suspensions. *Colloids Surf., A* 468: 234−245.

106. Sprittles JE, Shikhmurzaev YD (2014) The coalescence of liquid drops in a viscous fluid: interface formation model. *J. Fluid Mech* 751: 480−499.

107. Ahn BY, Lewis JA (2014) Amphiphilic silver particles for conductive inks with controlled wetting behavior. *Mater. Chem. Phys* 148(3): 686−691.

108. Zondervan GJ, Hoppen HJ, Pennings AJ, Fristchy W, Wolters G, and Schilfgaarde RV (1992) Design of a polyurethane membrane for the encapsulation of islets of Langerhans. *Biomaterials* 13:136.

109. Ward RS, White KA, Wolcott CA, Wang AT, Kuhn RW, Taylor JE, and John JK (1993) Development Of a hybrid artificial pancreas with a dense polyurethane membrane. *ASAIO J*.: M261.

110. McCall M, and Shapiro AM (2012) Update on islet transplantation. *Cold Spring Harb Perspect Med* 2: a007823.

111. Sun Y, Ma X, Zhou D, Vacek I, and Sun AM (1996) Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. *J Clin Invest* 98: 1417.

112. Jacobs-Tulleneers-Thevissen D, Chintinne M, Ling Z, Gillard P, Schoonjans L, Delvaux G, Strand BL, Gorus F, Keymeulen B, Pipeleers D (2013) Beta Cell Therapy Consortium EU-FP7. Sustained function of alginate encapsulated human islet cell implants in the peritoneal cavity of mice leading to a pilot study in a type 1 diabetic patient. *Diabetologia* 56: 1605.

113. Alosyius Neena and Prabha D Nair (2014) Enhanced survival and function of islet-like clusters differentiated from adipose stem cells on a three dimensional natural polymeric scaffold: an *invitro* study. *Tissue Engineering part A* 20(9-10): 1508-1522.

114. Sean V Murphy and Anthony Atala. (2014)3D bioprinting of tissues and organs. *Nature Biotechnology* 32.

115. Brunett CM, Hsu SL, and Macknight WJ (1982). *Macromolecules* 15: 71.

116. Wang MO (2013) Evaluation of the in vitro cytotoxicity of cross linked biomaterials. *Biomacromolecules* 14: 1321-1329.

117. Berit L Strand, Abba E Coron, Gudmund, Skjak-Braek (2017) *Stem cells translational medicine* 6: 1053–1058

118. RP Lanza, D Ecker, WM KÅhtreiber, JE Staruk, J Marsh, WL Chick (1995) Current and Future Perspectives on Alginate Encapsulated Pancreatic Islet. A simple method for transplanting discordant islets into rats using alginate gel spheres, *Transplantation* 5: 1485–1487.

120. RH Li (1988). Materials for immunoisolated cell transplantation. *Adv. Drug Deliv. Rev* 33: 87–109.

121. Marchant RE (1992). Biodegradability of biomedical polymers. In: Hamid SH, Amin MB, Maadhah AG (eds) Handbook of polymer degradation. Marcel Dekker, Inc, New York, 617–631.

122. Dombrow BA (1957) Polyurethanes. Reinhold Publishing Corporation, New York

123. Stephen Harrington, Janette Williams, Sonia Rawal, Karthik Ramachandran, and Lisa Stehno-Bittel (2017) Hyaluronic Acid/Collagen Hydrogel as an Alternative to Alginate for Long-Term Immuno-protected Islet Transplantation. *Tissue Engineering: Part A.*

124. Melanie Kollmer, Alyssa A Appel, Sami I Somo, and Eric M Brey (2015) Long-Term Function of Alginate-Encapsulated Islets *TISSUE ENGINEERING: Part B.*22(1):34-46.

125. P de Vos, M. Bucko, P Gemeiner, M Navratil, J Svitel, M Faas, BL Strand, G Skjak-Braek, YA Morch, A Vikartovska, I Lacik G Kollarikova, G Orive, D Poncelet., JL Pedraz, MB Ansorge-Schumacher (2013) Polymers in cell encapsulation from an enveloped cell perspective. *Adv. Drug Deliv. Rev.*67(68): 15-34.

126. P de Vos, M Bucko, P Gemeiner, M Navratil, J Svitel, M Faas, BL Strand, G Skjak-Braek, YA Morch, A Vikartovska, I Lacik, G Kollarikova, G Orive, D Poncelet, JL Pedraz, MB Ansorge-Schumacher (2009) Multiscale requirements for bioencapsulation in medicine and biotechnology. *Biomaterials* : 2559–2570.

127. Robert S Ward, Kathleen A White, Cary A Wolcott, Albert Y Wang, Robert W Kuhn, Julie E Taylor, and Judith K.John (1993) *ASAIO Journal*: M261.

128. Ricordi C, Lacy PE, Finke EH, Olack BJ and Scharp DW (1988) *Diabetes* **37**: 413-420.