IN VITRO AND IN VIVO ASSESSMENT OF ADIPOSE STEM CELL DERIVED ISLET LIKE CELLS ON A NOVEL THREE DIMENSIONAL SCAFFOLD

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IN VITRO AND IN VIVO ASSESSMENT OF ADIPOSE STEM CELL DERIVED ISLET LIKE CELLS ON A NOVEL THREE DIMENSIONAL SCAFFOLD

A THESIS PRESENTED BY

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CERTIFICATE

I, **Rakhi A**, hereby certify that I had personally carried out the work depicted in the thesis entitled, **"In Vitro And In Vivo Assessment Of Adipose Stem Cell Derived Islet Like Cells On A Novel 3D Scaffold",** except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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Dedicated to

GOD ALMIGHTY & MY FAMILY

A C K N O W L E D G E M E N T S

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Rakhi A.

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ABBREVIATIONS

WHO	World health organization
PCL	poly(ε-caprolactone)
PCL-PTHF-	Polycaprolactone-Poly tetra hydrofuran-
PCL	polycaprolactone
ECM	Extra cellular matrix
ILC	Islet like clusters
TZD	Thiazolidinediones
PPAR	Peroxisome proliferator activated receptor
ATP	Adenosine tri phosphate
AG	Alpha glucosidae
SU	Sulphonylurea
GLP-1	Glucagon like peptide-1
ESC	Embryonic stem cells
ASC	Adult stem cells
MSC	Mesenchymal stem cells
AMSC	Adipose derived mesenchymal stem cells
EGF	Epidermal growth factor
FGF	Fibroblast growth factors
PDX-1	Pancreas duodenum homeobox protein -1
IPN	Inter penetrating network
FDA	Food and Drug Administration

PEG	Polyethylene glycol
KRBH	Krebs -Ringer bicarbonate HEPES buffer
ESEM	Environmental scanning electron microscopy
FTIR	Fourier-transform infrared spectroscopy
DSC	Differential scanning calorimetry
XPS	X-ray photoelectron spectroscopy
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
DMEM HG	Dulbecco's Minimal Essential Medium-High glucose
SFM	Serum free media
FBS	Foetal bovine serum
EDTA	Ehylene diamine tetraacetic acid
FITC	Fluorescein isothiocyanate
ISCT	International society for cellular therapy
2D	Two dimensional
3D	Three dimensional

SYNOPSIS

Diabetes mellitus is a metabolic disorder either due to autoimmune-mediated destruction of insulin-producing beta cells in islets of Langerhans or poor insulin directed utilisation of glucose by target tissues due to insulin resistance or both. The resultant action is a derangement of catabolism and anabolism of carbohydrates, proteins and lipids which in turn result in hyperglycemia and leads to micro or macrovascular complications. Worldwide 415 million people suffer from diabetes in 2015 and are expected to rise to 642 million people by 2040.

The main focus of diabetes treatment has been in the control of hyperglycemia. Oral drugs and insulin injections are used to control hyperglycemia and hence control the progression of secondary complications. However, physiological control of insulin release has been an important factor in the tight metabolic regulation of glucose in the body. Failure of current therapy to mimic the physiology pattern of insulin release and metabolic regulation often results in severe hypo/hyperglycemic complications. Islet transplantation is considered as a long term treatment option for diabetes patients that can relieve them from dependence on exogenous insulin injections and secondary complications. However, in practice after transplantation procedure, only 8.2 percent of patients showed exogenous insulin independence for at least a year. Shortage of available donor pancreas, the requirement of more than one cadaveric donor for isolation of the transplantable number of islets and toxicity of immunosuppressive drugs were some of the major limitations of the procedure. In addition, the loss of extracellular matrix during the

isolation of islets by enzymatic digestion of donor pancreas leads to loss of cell viability and hence poor functionality after transplantation.

In this scenario, tissue engineering is a promising strategy, the principles of which propose to develop biological substitutes to restore, maintain, or improve functions of damaged tissues. The concept of tissue engineering for the treatment of diabetes involves the combined use of cells, such as mature islets or beta cells derived from stem cells, with a biomaterial scaffold that provides a suitable environment as the native extracellular matrix (ECM) for cell survival and function. An ideal scaffold mimics the structure and function of ECM components and prevents cell death and loss function of islets. In addition, they permit easy transplantation, monitoring and retrieval in case of graft failure. Therefore, the design strategies of the scaffold should mimic the natural ECM of the tissue (islets) in structure and function. Microarchitecture of the scaffold such as nanofibrous nature and pore size influences the function of the tissue-engineered construct. Beyond providing supporting architecture, ECM components have conserved domains that play an important role in enabling growth factor adhesion and presentation to cells. The integration of these properties of ECM into scaffold design would provide added functional advantage to synthetic scaffolds.

One aspect of tissue engineering is combining cells such as islets or islets differentiated from stem cells with the designed scaffolds that may help to overcome the shortage of donor pancreas. Embryonic and adult stem cells can be used as a source for differentiating ILCs. Embryonic stem cells have the potential to be a limitless source of differentiated islets however achieving glucose responsiveness and terminal differentiation of these cells poses a setback to the use of these cells in

clinical settings. Adult stem cells from autologous sources may provide a better outcome in the clinical scenario as sustained immunotoxic therapy can be avoided. In recent publications differentiated ILCs from adult mesenchymal stem cells have been applied in diabetic animal models with varying degrees of success.

The main objective of the study was to develop a nanofibrous three-dimensional porous scaffold that can support the viability and function of islets and differentiation of adipose stem cells to viable, functional islet-like clusters, which upon implantation in diabetic rat model can decrease hyperglycemia. The thesis entitled “In *vitro* and *in vivo* assessment of adipose stem cell-derived islet-like clusters on a novel 3d scaffold" is divided into six chapters. Introduction and background to the study are elaborated in chapter 1. In this chapter, pathophysiology and different types of diabetes mellitus are explained. The current treatment methods of using insulin injections, oral drugs and islet transplantation are discussed with their advantages and limitations. This chapter introduces the promising strategy of tissue engineering and the requirements of scaffolds and cells for the construction of a functional islet construct. The study hypothesis " three-dimensional biomimetic nanofibrous scaffolds incorporated with specific ECM proteins could enhance stem cell differentiation to islet-like clusters and maintain its survival and functionality when transplanted *in vivo"*, is put forward in this chapter. To prove the hypothesis major objectives were identified and put forward as follows.

- Develop a 3D nanofibrous polymer scaffold for tissue engineering of the pancreas.
- Develop a tissue-engineered pancreatic construct with adipose-derived MSC on the 3D nanofibrous scaffold.

- Immobilise ECM components on the nanofibrous polymer scaffold to make it biomimetic.
- Evaluate the potential of the tissue-engineered construct to reverse hyperglycemia in the diabetic rat model.

In the second chapter, the literature is reviewed in detail on the current status of islet tissue engineering. It details the function of pancreatic islets, different types of diabetes and its pathophysiology, the current treatment methods and its limitations. Tissue engineering aspects of pancreatic islets is reviewed with emphasis on the different types of design strategies used for scaffold development, the alternative cell sources used for islet tissue engineering, and different ECM cues used for enhancing the function of islets. The site of implantation and their impact on the function of the tissue-engineered construct is explained in this chapter.

In Chapter 3, the experimental design adopted in the study is elaborated. Materials used and experiment protocols are described in this chapter. First, the fabrication method of the scaffold is described. The electrospinning method used for developing a three-dimensional microporous scaffold is described. Further, physicochemical characterisation of the developed scaffold by ESEM, Micro-CT, FT-IR to evaluate the functional groups, tensile testing, contact angle and surface wettability, scaffold crystallinity using DSC, Cyto-compatibility using direct contact, viability staining and MTT assay, cell penetration and cell proliferation in the scaffold is described. Next, the procedure for the isolation of islets from rat pancreas and characterised by immunostaining is described. In the next section, mesenchymal stem cell isolation from rat adipose tissue, its characterisation by immunostaining,

multilineage differentiation and flow cytometry is described. The method of differentiating rat adipose stem cells to islet-like clusters are described and the formed islets are characterised by immunostaining and *invitro* insulin release to different concentration of glucose which is further evaluated by insulin ELISA. In the next section, adipose stem cells were differentiated to islet-like clusters (ILC) on 2D culture systems and scaffold which serve as a 3D culture. ILC protein expression was analysed by immunophenotyping. The viability and function of ILC were assessed by live-dead assay and insulin release quantification. The next section describes diabetic induction in the rat model, *in vivo* implantation of ILC and mature Rat islets in the omental pouch of the diabetic rat model is described. Comparison of fasting blood glucose level of the implanted rats with diabetic controls, *in vivo* glucose challenge assay and serum insulin level assessment is described. The sectioning for histology and staining of the retrieved implant is also described in this chapter. In the last section, the coating of the electrospun scaffolds with ECM components and their characterisation by FTIR, XPS, Picosirius red staining, differentiation of adipose MSCs into Islet like Clusters (ILC's) in these coated scaffolds and their functional analysis by glucose challenge assay and Insulin ELISA.

Chapter 4 comprises of the results from the study represented as figures, tables and graphs. In the first section, the results deal with the enzymatic isolation, characterization of rat pancreatic islets and the isolation, characterization of mesenchymal stem cells from adipose tissue of rats. The islets were positive for insulin, glucagon and somatostatin expression. The mesenchymal stem cells were positive for cytoskeletal markers such as actin and vimentin and surface markers as

CD105, CD90 and negative for CD34/45. The cells showed multilineage differentiation potential to chondrocytes, adipocytes and osteocytes.

The second section elaborates on the process of electrospinning 3D scaffolds of different pore sizes and results of the physicochemical characterisation of three-dimensional electrospun scaffolds compared to the normal electrospun membrane. An environmental scanning electron micrograph reveals the morphology of the scaffolds designed. MicroCT analysis proved that the scaffolds had different pore sizes in the range of 24μm to 250 μm for large lattice scaffold and 24 μm to 190μm in small lattice scaffold and cell penetration studies show that cell penetration depth in influenced by the pore sizes of the scaffolds. Contact angle measurement shows the scaffolds are highly hydrophilic and the 3D scaffolds have a high swelling ratio which enables efficient nutrient and media uptake of the scaffold. *In vitro* cytotoxicity results confirm the noncytotoxicity of the scaffold.

In the next section, the islets are cultured on large lattice 3D scaffolds and 2D culture plates. The viability and insulin release function of islets decreases in 2D compared to the 3D scaffold. In the fourth section, mesenchymal stem cells were differentiated to islet-like clusters in 3D small lattice scaffold, large lattice and electrospun membranes. Depending on the pore size of the scaffold small and large ILCs were formed on the scaffolds. They showed insulin expression. Insulin release at high glucose concentration was significantly higher for large lattice scaffold.

In the fifth section, the 3D ILC construct was implanted in diabetic rat models and compared to mature rat islets and diabetic controls. ILC constructs were capable of blood glucose reduction when compared to diabetic controls, however mature rat islets group showed better results. In the sixth section, the large lattice scaffolds were

covalently coated with ECM molecules as collagen IV, vitronectin and fibronectin and all three to evaluate the insulin release potential. XPS analysis showed the presence of nitrogen on the surface of the scaffolds and Picosirius red staining was positive for collagen iv. The scaffold coated with all three ECM components showed a better insulin release from the 3D cultured ILCs.

In chapter 5 the findings of the study are discussed and analysed in comparison with the literature. It is shown that microarchitectural characteristics of scaffold such as pore size and three-dimensional architecture and presence of ECM molecules influence the cell differentiation and formation of functional islet-like clusters.

In chapter 6 the results are summarised and the conclusions are drawn. The limitations of the study are identified and future perspectives for developing an improved tissue-engineered islet construct is proposed.

CHAPTER 1

INTRODUCTION

1.1 Diabetes Mellitus

Diabetes incidence is increasing worldwide to be one of the leading non-communicable causes of morbidity and mortality. Diabetes mellitus is not a single disorder but a syndrome with different forms of diabetes having different etiology. Diabetes mellitus can be defined as a clinically and genetically heterogeneous group of disorders characterised by high blood glucose levels or hyperglycaemia. Diabetes is a chronic metabolic disorder caused either by loss of function of insulin-producing beta cells of the islets or along with insulin resistance and subsequent increase in blood glucose level(Association, 2012). Pathogenesis of diabetes mellitus can either be due to autoimmune destruction of pancreatic beta cells located in the islets of Langerhans that secretes insulin hormone or defects in one or more points in the complex pathway of insulin action. This results in the abnormalities in carbohydrate, protein and fat metabolism in the body.

Symptoms of diabetes include polyuria, polyphagia, polydipsia. Diabetes can be diagnosed by the presence of these classic symptoms and unequivocal elevation of blood plasma glucose level also known as hyperglycaemia. Any individual along with the classic symptoms has a fasting glucose concentration of venous whole blood more than 120mg/dL (6.7mmol/L) and more than 180mg/dl (10.0mmol/L) 2 hours after intake of 75g glucose dose is diagnostic of diabetes. Uncontrolled hyperglycaemia can lead to life-threatening conditions as ketoacidosis or non-ketotic

hyperosmolar syndrome. Long term presence of diabetes can lead to secondary complications such as retinopathy, nephropathy, peripheral neuropathy that leads to foot ulcers, Charcot joints and autonomic neuropathy that leads to cardiovascular and sexual dysfunction.

Worldwide 415 million people suffer from diabetes in 2015 and are expected to rise to 642 million people by 2040 (7 th edition IDF). Increased consumption of refined food and reduced physical activity are the main reason for the increased burden of diabetes worldwide. In India prevalence of diabetes is increasing at an alarming rate of 20% in urban areas and the diabetic population has increased to 65.1 million in 2013 from 50.8 million in 2010 as estimated by International Diabetes Federation. International diabetes atlas, 2015 estimates that India has the second-highest population of children with type I diabetes in the world. Furthermore, it estimates that in India one million deaths in 2015 could be attributed to diabetes. A growing population of diabetic patients imposes a huge economic burden on health care costs. A study suggests that approximately USD 2.2 billion would be required to treat all cases of type II diabetes in India.

1.2 Types of Diabetes

The heterogeneity of diabetes mellitus syndrome is vast with over 30 distinct, mostly rare disorders characterised by hyperglycaemia, in addition, there is ethnic variability in prevalence and clinical features of the disease. This heterogeneity of the disease makes it difficult to assign a patient to one class of diabetes and it largely depends on the circumstances at the time of diagnosis. For clinicians and patients understanding the pathogenesis of hyperglycaemia and selection of treatment methods is more important. Diabetes mellitus is classified into four types based on

the etiology of the disease. They are, Type I diabetes, Type II diabetes, Gestational diabetes and other specific types of diabetes.

1.2.1 Type I Diabetes:

This class of diabetes results from autoimmune destruction of pancreatic beta cells. The pancreas predominantly infiltrated with CD8+ T cells, macrophages, CD4+ T cells, B lymphocytes. The insulitis (islet infiltrate) destroys the insulin-producing beta cells leading to insulin deficiency and hyperglycaemia. Serological analysis of the patients also shows the presence of autoantibodies reactive to insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated autoantigen 2 (IA2A), and zinc transporter 8 (ZnT8A) of the pancreatic beta cells. These autoantibodies appear months or years before the symptoms of the disease and hence has high diagnostic importance in this disease(Atkinson et al., 2014).

1.2.2 Type II Diabetes

This type of diabetes is the most prevalent and constitutes 90-95% of all diabetic cases. The type II diabetes patients produced insulin in response to nutrient absorbed but are insulin insensitive due to insulin resistance leading to excessive production of glucose by the liver and decreased uptake of glucose by muscle and adipose tissues. This insulin resistance leads to a decrease in insulin production and apoptosis of pancreatic beta cells. The risk of developing type 2 diabetes increases with age, obesity and lack of physical activity (Association, 2012).

1.2.3 Gestational Diabetes

Gestational diabetes is defined as glucose intolerance developed during pregnancy and reverts to normal glucose homeostasis after childbirth. These affected women show a high risk of developing type 2 diabetes in the future. Increase in

estrogen and progesterone hormones during gestation causes a delay in gastric emptying and increases appetite leading to increased postprandial glucose concentrations and decreased tissue sensitivity to insulin. In women developing gestational diabetes, beta cells fail to increase the production of insulin to override insulin resistance. This may trigger increased insulin production in the foetus and result in excess growth of foetus referred to as a macrosomic foetus (Reece et al., 2009).

1.2.4 Other types of diabetes

These diabetes forms are associated with specific mutations of genes in beta cells or in pathways of insulin action. Diabetes can be caused by diseases of the exocrine pancreas such as pancreatitis, infection or pancreatic carcinoma. Other endocrinopathies as Cushing's syndrome, acromegaly also lead to diabetes. Drugs and hormones such as nicotinic acid, glucocorticoids, alpha interferons can impair insulin action and lead to diabetes(Association, 2012).

1.3 Treatment for diabetes

Management of hyperglycaemia and maintaining glycemic levels to the non-diabetic state has been the top priority in the treatment of diabetes. Studies have shown that maintaining glycemic levels close to normal has beneficial effects in delaying the onset of secondary complications in type 1 and type 2 diabetes. Advances in metabolic testing and understanding of immune markers for type I diabetes have made the diagnosis of the disease easy in this decade. Discovery of insulin in 1921 was a major advancement in the treatment of diabetes type 1 which was associated with 100% mortality due to diabetic ketoacidosis before insulin. However, diabetic ketoacidosis and hypoglycaemia remain a major reason for

mortality in patients treated with insulin(Podar et al., 2000)(Skrivarhaug et al., 2005). The Diabetes Control and Complications Trial (DCCT), a multicenter clinical trial showed that intensive insulin therapy with three or more daily insulin injection using an insulin pump to keep glycemic level as close to normal was more effective in preventing metabolic complications and progression to long term secondary complications such as retinopathy, nephropathy when compared to normal insulin therapy (Control and Group, 1993).

Therapeutic innovations such as insulin analogues (Hirsch, 2005), continuous glucose monitoring systems, drugs for prevention of specific complications, improved immunosuppressant such as teplizumab (anti-CD-3) (Skyler, 2013) and alefacept (anti-CD-2) (Rigby et al., 2013), pancreatic transplantation (Robertson, 1999) and islet transplantation(Shapiro et al., 2000) has improved treatment of type 1 diabetes. However, lack of donor organ for allogeneic transplantation, the requirement of more than one donor organ for islet transplantation and the lifelong requirement for immunosuppressant has remained as obstacles for transplantation.

Orally administrable drugs are used to regulate hyperglycaemia in the early stages of type 2 diabetes. These drugs are aimed at either decreasing insulin requirement in insulin-resistant patients or at increasing the insulin availability to meet body requirements. Antihyperglycemic drugs such as metformin decrease insulin resistance, alpha-glucosidase inhibitors decrease insulin requirement, insulin secretagogues as sulfonylureas increase insulin secretion(Inzucchi et al., 2015).

1. 4 Tissue engineering

At the first scientific meeting on the subject tissue engineering in 1988, it was defined as "Tissue engineering is the application of the principles and methods of

engineering and the life sciences toward the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions"(Nerem and Sambanis, 1995) (Figure 1.1). The concept of tissue engineering for the treatment of diabetes involves the combined use of cells, such as mature islets or beta cells derived from stem cells, with a biomaterial scaffold that provides a suitable environment as the native extracellular matrix (ECM) for cell survival and function. Three main components for islet tissue engineering are 1) Viable cells that maintain the cluster morphology of native islets and secrete insulin in response to glucose concentration 2) Extracellular matrix components that enhance the viability and function of the islets 3) Biomaterial scaffold that provides mechanical support and helps maintain the three-dimensional architecture of the islets or encapsulation system that supports it function *in vivo* (Amer et al., 2014).

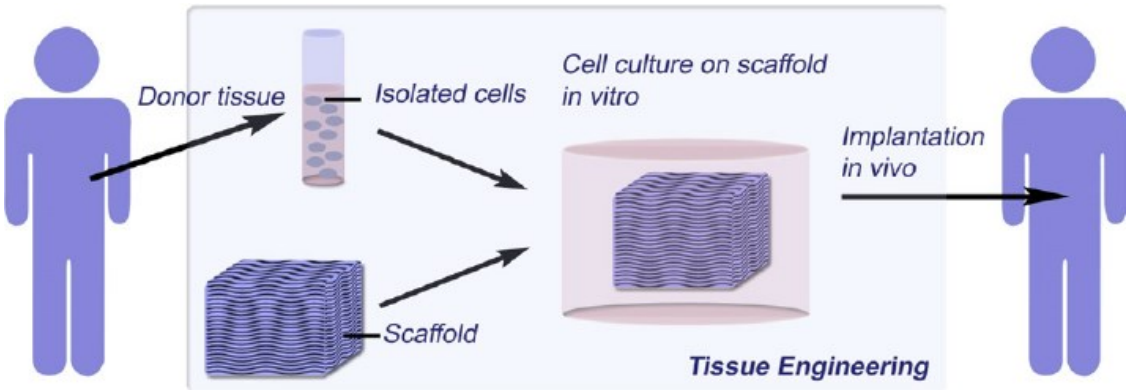

Figure1.1: The image explains the basic principle of tissue engineering wherein cell from a renewable source is isolated and cultured in scaffold *invitro* and transplanted to repair or restore a tissue function in patients (Stamatialis et al., 2008).

In normal physiology, insulin hormone is produced by beta cells in the islets of langerhans. Due to the shortage in the availability of islets for transplantation, any

cells that produce insulin in response to glucose level without adverse effect qualify for transplantation. Research in alternative cell sources for the replenishment of beta cells is categorised within one of the three 'R's. They are 1) Replacement with beta cells derived from stem cells or xenoislets, 2) Regeneration of beta cells from pancreatic progenitor cells and 3) Reprogramming from other nonendocrine adult cells (Orlando et al., 2014).

Islet function owes to the numerous cell-cell interaction among the different endocrine cells with the islet cluster and cell-matrix interaction with its extracellular matrix, a three-dimensional scaffold helps in maintaining the physiological structure of the islets and hence its function. Porous material scaffolds are shown to increase the viability and function of islet cells cultured in these scaffolds when compared to two-dimensional conditions (Chun et al., 2008)(Aloysious and Nair, 2013). Tissue engineering strategy for islet transplantation, in addition to biomaterial scaffolds, involves the use of materials for immunoisolation to enable the use of allergenic or xenogenic cell source and protection from autoimmunity characteristic of type I diabetes thus avoiding the detrimental effects of immunosuppression drugs(Nair and Aloysious, 2011).

Pancreatic islets are in high contact with the basement membrane around the islets in the pancreas. The extracellular matrix serves several functions such as structural support, acting as a reservoir of growth factors and signaling cells through integrin receptors(Hynes, 2009). Laminins, Collagen I, IV, V, Vitronectin, fibronectin are ECM proteins identified in the basement membrane of the pancreas. These proteins play specific roles in islet development and function as viability, attachment, gene expression and insulin release (Virtanen et al., 2008). ECM proteins

are utilised in the development of functional, biomimetic scaffolds to maintain the function of islets in tissue-engineered constructs.

1.5 Hypothesis

It was hypothesized that three-dimensional biomimetic nanofibrous scaffolds incorporated with specific ECM proteins could enhance stem cell differentiation to islet-like clusters and maintains its survival and functionality when transplanted *in vivo*

1.6 Objectives

- Develop a 3D Nanofibrous polymer scaffold for tissue engineering of the pancreas
- Immobilise ECM components on the nanofibrous polymer scaffold to make it biomimetic
- Isolate and characterize mesenchymal stem cells from adipose tissue
- Tissue engineer a pancreatic construct from the adipose-derived MSC on the three-dimensional biomimetic nanofibrous scaffold
- Evaluate the potential of the tissue-engineered construct to reverse hyperglycaemia in the experimental diabetic rat model

1.7 Significance

In the present study, a novel three dimensional porous nanofibrous patterned and the highly hydrophilic scaffold was fabricated using biodegradable synthetic polymers. The scaffold was shown to support the survival, architecture, function of islets and differentiation of islet-like clusters from adult stem cells. *Invivo* evaluation in diabetic rat models showed a decrease in hyperglycemic values. The scaffolds

were made biomimetic with covalent modification of extracellular matrix proteins to support the better function of the tissue-engineered construct. The studies demonstrate the feasibility of this tissue engineering approach for preparing transplantable islets and its further potential implications in islet transplantation.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes mellitus, Brief History

Diabetes has been described as early as 1500BCE in ancient Egyptian, Indian, Greek and Chinese texts with the same ideograph meaning " sweet urine" disease (Poretsky, 2010). Important aspects of the disease were elucidated during the nineteenth century. Oscar Minkowski and Joseph Von Mering proved that the secretions of the pancreas other than exocrine secretion were cause for the pathogenesis of diabetes mellitus(Medvei, 1993). Gustave Edward Laguesse in 1893 attributed the function of glucose regulation to islets of langerhans described by Paul Langerhans(Medvei, 1993). In 1921, Federick Banting and Charles Best identified and purified insulin whose deficiency causes diabetes(Medvei, 1993).

Diabetes development is triggered by various pathogenic processes from autoimmunity against beta cells of islets to defect in insulin action. Carbohydrate, fat and protein metabolism is adversely affected due to deficient secretion/insulin action(Ashcroft and Rorsman, 2012). Discovery of autoimmunity and other developments in molecular biology has helped to establish the heterogeneity of the disease and subdivide it to mainly type I and type II according to its etiology (Gale, 2001). Type I diabetes is characterised by little or no insulin secretion and the presence of autoantibodies whereas type II is characterised by insulin resistance.

2.2 Islets Of Langerhans

The islets of langerhans (Figure2.1) constituting the endocrine pancreas are unique micro organs responsible for maintaining homeostasis in the body. They are cell clusters of different types with endocrine function distributed throughout the pancreas and form 1-2% of the total pancreas volume(Ionescu-Tirgoviste et al., 2015)

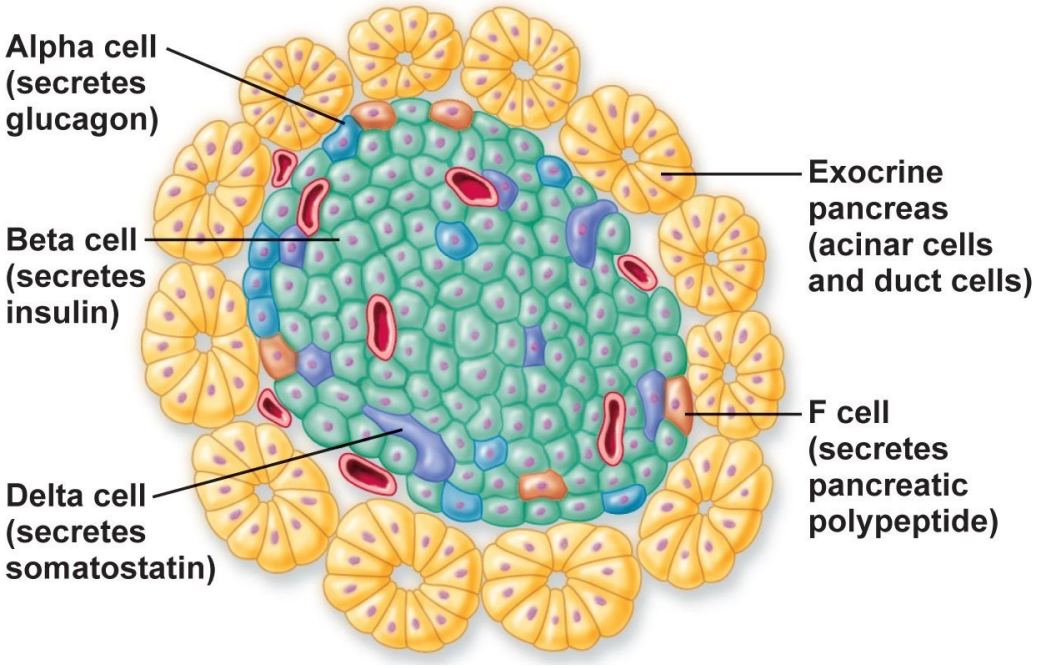

Figure 2.1 Schematic representation of the Islet of Langerhans. It consists of alpha cells, beta cells, delta cells and PP cells(The Pancreas, n.d.)

The size of islets in the pancreas varies in the range of 50 -280μm and occurs more densely in the tail region of the pancreas(Saito et al., 1978). Islets are composed of five types of cells that secrete five different hormones into the blood circulation. The ratio and arrangement of these cells vary among species. In rat islets, alpha cells secreting glucagon constitute 20% of islet volume, beta cells synthesising insulin constitute 70%, delta cells secreting somatostatin forms less than 10%, gamma cells secreting pancreatic polypeptide forms less than 5% and ghrelin producing epsilon

cells forms less than 1% of the total islet volume(Elayat et al., 1995). However, a single human islet is constituted by 57% beta cells, 32% alpha cells, 10%delta cells thus a human pancreas consists of 1.15cm^3 of beta cells, 0.66cm^3 of alpha cells and 0.21cm^3 of delta cells of the total islet volume of 2.02cm^3(Ionescu-Tirgoviste et al., 2015). The cytoarchitecture of the islets differs between species. Beta cells form the core of the islet cluster with alpha and delta cells occupying the periphery in rat islets whereas in human islets all the cells are intermingled with each other and a core of beta cells is not observed(Cabrera et al., 2006). Islets are highly vascularised favouring nutrient sensing and hormone release to maintain homeostasis in the body. Islet cells are fenestrated with capillaries that facilitate secreted hormones into the blood circulation(Jansson et al., 2016). Islets are innervated by nerve cells as neuro insular complex. These nerves affect hormone secretion and blood flow in the islets(Rodriguez-Diaz et al., 2011).

2.3 Insulin

Insulin, a dimeric peptide hormone consists of 51amino acids in two peptide chains, A chain, and B chain, linked by disulphide bonds(Wiley: Biochemistry, 4th Edition - Donald Voet, Judith G. Voet, n.d.). It is the main anabolic hormone that increases the uptake of glucose, increases glycogen synthesis, decreases gluconeogenesis, increases lipogenesis, reduces proteolysis by its action on liver, muscle and fat tissues (Dimitriadis et al., 2011).

Insulin is synthesised by beta cells of the pancreatic islets as preproinsulin which is cleaved in the rough endoplasmic reticulum to proinsulin and folded to structural conformation with three disulphide bonds(Steiner and Oyer, 1967). Proinsulin then matures in golgi body where a fragment is cleaved and released as c-

peptide forming the final dimeric insulin with two disulphide bonds(Steiner and Oyer, 1967). Insulin is secreted by beta cells in two phases, the first phase is the rapid release of insulin in response to increases in blood glucose, the second phase is the slow release of vesicles containing newly synthesised insulin(Hellman, 2009). Reduction in the first phase of rapid insulin release is an indicator of the onset of type II diabetes. Islets of langerhans release insulin in a rhythmic pulsatile manner with peak release sustaining for 5-15 minutes increasing the concentration of insulin in blood circulation(Pørksen et al., 2002). The oscillatory release of insulin is important to prevent the downregulation of insulin receptors maintaining the insulin sensitivity of target cells(Schofield and Sutherland, 2012). Downregulation of insulin receptors mainly attributes to insulin resistance, characteristic of type II diabetes(Schofield and Sutherland, 2012). Hence the pulsatile release of insulin than single insulin injections is ideal for controlling diabetes. This makes islet transplantation an ideal therapeutic option for diabetic patients(Hellman, 2009)

2.4 Current Treatment of Diabetes

Current treatment for diabetes aims at strictly controlling the blood glucose concentration, HbA1c levels to the normal range to delay progression to secondary diabetic complications.

2.4.1 Oral hypoglycemic drugs

Type II diabetes is characterised by insulin resistance and reduced beta-cell function. Physical inactivity and obesity contribute to insulin resistance, hence, exercise and controlled diet become the basis of the treatment in type 2 diabetes(Inzucchi et al., 2015: 2). Oral hypoglycemic drugs are used to control blood glucose concentration. The main drugs used for type 2 diabetes treatment are

classified into insulin sensitizers, insulin secretagogues, GLP-1 agonists and alpha-glucosidase inhibitors(Akram, 2013).

2.4.1.1 *Metformin*

Metformin is a biguanide or insulin sensitiser that suppresses gluconeogenesis from the liver and increases glucose absorption and glycogen synthesis in muscle(Rena et al., 2013). It enhances insulin action at the receptor level in tissues. American diabetes association recommends metformin as the initial oral drug for type 2 diabetes(Marathe et al., 2017).

2.4.1.2 *Thiazolidinediones(TZ*D)

Thiazolidinediones, mainly pioglitazone and rosiglitazone introduced in 1999 activates peroxisome proliferator-activated receptors (PPARs) that lead to an increase in fatty acid storage in adipocytes thus reducing circulating fatty acids which further promotes glucose oxidation for energy by the cells(Hauner, 2002). Significant weight gain is a side effect of these drugs(Rizos et al., 2009). In addition to weight gain, pioglitazone is reported to increase the risk of bladder cancer(Ferwana et al., 2013) and reduce bone density as it promotes differentiation of bone marrow stem cells to adipocytes thus reducing the formation of osteoblasts(Mannucci and Dicembrini, 2015).

2.4.1.3 *Sulphonylureas (SU)*

Sulphonylureas are secretagogues that bind to SU receptors causing the closure of adenosine triphosphate (ATP) dependent potassium channel leading to membrane depolarisation.(Proks et al., 2002) This, in turn, leads to the opening of voltage-gated calcium ion channels causing an influx of calcium ions(Proks et al., 2002). An increased concentration of calcium ions stimulates exocytosis of insulin-

containing vesicles. The main side effects of SU s are weight gain and hypoglycaemia.

2.4.1.4 *α Glucosidase (AG) Inhibitors*

Acarbose and miglitol inhibit the alpha-glucosidase enzyme in the intestine thus delaying carbohydrate absorption through the intestine(Chiasson et al., 1994). They are free from the common disadvantage of weight gain and hypoglycaemia associated with other glycemic drugs, however, flatulence, diarrhea and abdominal discomfort are caused in patients(Catalan et al., 2001).

2.4.2 Insulin therapy

The therapeutic effect of insulin was first discovered by Banting and Best in 1922 and since then it has been the foundation of treatment of type I diabetes and significantly reduced the fatality of the disease (Heller et al., 2007). Nowadays, recombinant human insulin is used to treat most diabetic patients especially diabetes I patients in the world. Insulin has been altered to form analogues with better pharmacokinetics such as rapid-acting, short-acting, intermediate-acting and long-acting(Vajo and Duckworth, 2000). Insulin analogues have the advantage of rapid absorption into the blood circulation after subcutaneous injection and uniform insulin activity(Garg et al., 1999). To mimic the physiological concentration of insulin in the body, intensive insulin therapy was developed where a combination of basal long-acting insulin analogue with premeal rapid-acting insulin was used(Marathe et al., 2017). This intensive therapy helped improve HbA1c levels in patients, however, it increased the frequency of blood glucose monitoring and patients were administered 4-6 injections per day or insulin pumps were used to achieve the physiological level of glucose(Schütt et al., 2006). Insulin therapy, in addition, caused side effects

mainly severe hypoglycaemia, insulin resistance, allergy to insulin, lipo hypertrophy and insulin edema(Control and Group, 1993).

2.4.3 Pancreas Transplantation

Pancreas transplantation opts as a treatment in patients with severe diabetes and end-stage renal disease or brittle diabetes(Association, 2006). 90% of pancreas transplantation is performed along with a kidney transplant, otherwise, the pancreas is transplanted after kidney transplantation or as single pancreas transplantation alone(Meloche, 2007). Complications of the transplantation include thrombosis, pancreatitis, bleeding and graft rejection(Meloche, 2007). Recipients of the transplant have to be protected lifelong with immunosuppressive drugs which in turn increases the chance of infections(Fishman and Rubin, 1998) and cancer(Dreno, 2003).

2.4.4 Islet Transplantation

Islet transplantation is alleged as a treatment option as it is a relatively simple procedure compared to whole pancreas transplantation, exclusive of major surgery and a high dose of immunosuppressive drugs. The concept of transplanting pancreatic islets was first reported in 1894(Robertson, 2004), however, it was not successful clinically until 1980 when islet autograft and allograft were successfully transplanted(Largiadèr et al., 1980). The islet isolation process was standardised to obtain high-quality islets via perfusion of collagenase enzyme in pancreatic ducts followed by digestion in Ricordi chamber and density gradient purification to remove exocrine cells from isolated islets(Markmann et al., 2003). Minilaparotomy method was adopted to lodge these isolated islets in the hepatic portal vein of the patients. The rate of success in allogeneic islet transplantation was less than 10% until 2000 when Shapiro et. al reported a 100% success in seven patients for one year with a

modified procedure widely known as Edmonton protocol (Figure 2.2)(Shapiro et al., 2000). This success was achieved with restrictions on patient body weight, the use of high-quality islets immediately after harvest from cadaveric donors and the use of sirolimus, tacrolimus, daclizumab while avoiding corticosteroids for immunosuppression(Shapiro et al., 2000).

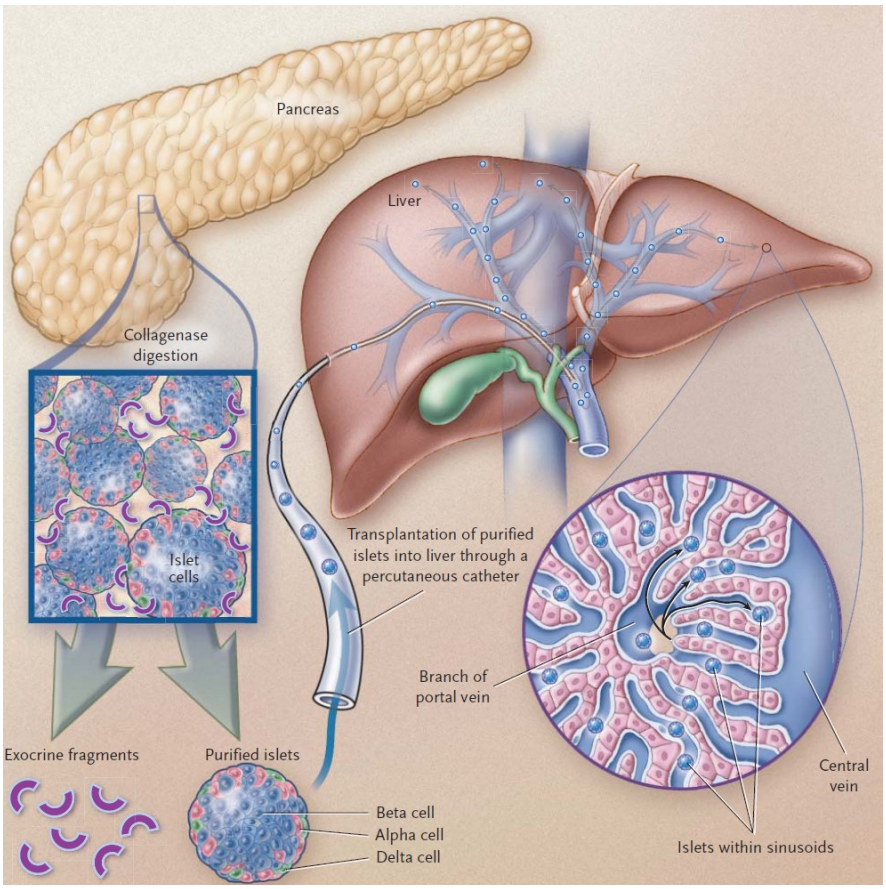

Figure 2.2 Process of islet transplantation: Pancreas from the cadaveric donor is digested with collagenase, purified with density gradient centrifugation and infused into the portal vein through percutaneous hepatic portal vein catheterisation (Robertson, 2015).

The liver was used as the site of implantation because early autologous islet transplantation was successful when implanted to the hepatic portal vein; however,

islet infusion to the site is complicated by thrombosis of the portal vein and portal vein hypertension. The use of anticoagulants to prevent clotting leads to hepatic bleeding at the site of puncture. In addition, the islets transplanted to the portal vein is exposed to environmental toxins and toxic immunosuppressant drugs absorbed through the intestine and delivered to the portal vein(Oetjen et al., 2003). Immunosuppressant drugs are toxic to beta cells and sirolimus used in the Edmonton protocol has an anti-proliferative effect that adversely affects the angiogenesis of transplanted islets (Carlsson et al., 2002). Furthermore, the islets transplanted to the portal vein is unable to release glucagon during hypoglycaemia (Gupta et al., 1997). Hence, the hepatic portal vein is not the ideal site for islet transplantation. Peritoneal cavity and omentum, successfully used in animal models, appeals as a potential site for islet transplantation clinically(Kin et al., 2003).

In recent transplantations, many refinements have been made to the original Edmonton protocol mainly in the immunosuppression drugs where sirolimus was eliminated for lymphodepletion antibodies, a combination of tacrolimus and mycophenolate mofetil (Senior et al., 2012). The hepatic bleeding has been reduced by the use of microfibrillar collagen flour in the catheter tract. This, in turn, has allowed the use of stronger doses of anticoagulant heparin to prevent portal vein thrombosis(Senior et al., 2012). With the changes in the transplantation protocol, insulin independence rate of more than 60% could be achieved over a period of four years. In addition, the positive impact on the prevention of progression of microvascular diseases as retinopathy, neuropathy, nephropathy was observed after islet transplantation with modified methods(Senior et al., 2012). Since the Edmonton protocol was first published, more than 500 diabetic patients have received islet

transplantation at various centers across the world, however, the procedure is performed only on a selected group of diabetic patients and their positive effect is limited by the use of immunosuppressants(Pellegrini et al., 2016). Additionally, the requirement of more than one cadaveric donor and scarcity of donors have restricted the availability of the treatment method for the majority of diabetic patients. Hence, alternative methods for beta cell replacement are of utmost significance.

2.5 Cell Sources for islet transplantation

Alternative cell sources for beta-cell implantation can be obtained from xenografts from pig islets, replication of existing beta cells, neogenesis of islet cells from pancreatic progenitor cells and islets derived from stem cell differentiation. Aggravated immune response due to the presence of α-galactosyl antigen and possible transmission of disease from animal to human patients has limited its further development to clinical settings. Differentiated islet cells from pancreatic progenitor cells or stem cells have grown in favor as an alternative source among research groups.

Cells are defined as stem cells when they possess the capability of self-renewal without differentiation and can differentiate to form specialized cell types in the presence of appropriate growth factors. Stem cells are mainly classified into two groups -1) embryonic stem cells and 2) adult stem cells.

2.5.1 Embryonic stem cells (ESC)

ESC are pluripotent cells capable of differentiating to ectoderm, mesoderm and endoderm, have high telomerase activity and isolated from the blastocyst stage of fertilised embryo(Thomson et al., 1998). Most embryonic stem cell studies have been performed with murine cells where studies have proved that embryonic stem

cells could be differentiated to endocrine pancreatic cells even though in a low percentage of 1-3%(Mfopou et al., 2010). Human embryonic stem cells were differentiated at a higher percentage of cells to the pancreatic progenitor stages by Brole'n et al., these cells expressed Pdx-1 marker characteristic of pancreatic progenitor cells, however, they failed to express insulin hormone *in vitro*(Brolén et al., 2005). A protocol that mimics the pancreatic islets development steps *in vivo* was developed to differentiate pancreatic progenitor from embryonic stem cells to insulin expressing cells(D'Amour et al., 2006). The protocol though successful in developing cells with insulin content lacked the insulin release response to changes in glucose concentration(D'Amour et al., 2006). In further studies, when differentiated embryonic stem cells were implanted *in vivo*, the cells matured to produce c-peptide(Kroon et al., 2008a). Though positive efforts have been made to differentiate embryonic stem cells to pancreatic islets, concerns have been raised in the use of embryonic stem cells because of teratoma formation in immunodeficient mice implanted with these cells and the ethical issues surrounding the use of embryos to derive these cells. Type I diabetic patients require immunosuppression to prevent autoimmunity destroying implanted cells, thus teratoma formation would limit the use of these cells in the treatment of humans(McCall et al., 2009).

2.5.2 Induced pluripotent stem cells

Induced pluripotent cell technology was developed by Shinya Yamanaka, who proved that mature adult cells could be reprogrammed into pluripotent cells by introducing genes for four specific transcription factors Oct4, Sox2, cMyc, and Klf4 (Takahashi and Yamanaka, 2006). Alipio et al used induced pluripotent cells from skin fibroblasts of the mouse to prove the concept that these cells could be

differentiated into pancreatic islet-like cells using specific differentiation protocol *in vitro* (Alipio et al., 2010). Recent studies have shown that human induced pluripotent stem cells could be successfully differentiated into islet-like cells that secrete insulin to glucose response *in vitro* and *in vivo* (Kim et al., 2016). However, the clinical application of these cells has been restricted because of the risk of tumor formation by reactivation of viral transgenes used to produce induced pluripotent stem cells(Godfrey et al., 2012). Studies on safer alternative methods such as using adenoviral vectors are being developed for producing induced pluripotent stem cells(Stadtfeld et al., 2008).

2.5.3 Adult Stem Cells

Adult stem cells are multipotent cells found in organs of an adult organism capable of self-renewal and regeneration of organs(Young and Black, 2004). Adult stem cells, unlike embryonic stem cells, can differentiate to cells of lineages they are committed to regenerate(Young and Black, 2004). However, in clinical application, adult stem cells are preferred to embryonic stem cells as they are free from the risk of teratoma development and ethical issues involved in the destruction of embryos (Young and Black, 2004). Different types of adult cells have been explored for their potential to differentiate into pancreatic islet cells.

2.5.3.1 Pancreatic Stem Cells

Pancreas tissue as well as islet increase in mass during development, after birth to adulthood and during pregnancy (Bonner-Weir, 2000). Researchers are divided over the mechanism of pancreatic tissue growth as cells grow by the proliferation of existing cells and islet neogenesis from stem cells. Neogenecists claim that the increase in cell mass is due to islet neogenesis from ductal progenitor

cells and hypertrophy of cells(Bonner-Weir and Sharma, 2002). Pancreatic stem cells lack characteristic markers for identification however, cells that express factors such as beta-galactosidase, PDX-1, neurogenin -3, tyrosine hydroxylase and GLUT-2 (glucose transporter) are considered as pancreatic progenitor cells (Jensen et al., 2000)(Teitelman et al., 1993)(Pang et al., 1994). Lineage tracing studies conducted using cre-lox-P to mark islets when mouse was 6-8 weeks and allowed to grow up to a year shows that the labeling index has not declined as would have been if the new islets were formed from stem cells, instead the labeling index remained same give a strong proof of growth by cell division theory(Dor et al., 2004). However, Studies on duct ligation of the pancreas where the main duct of the splenic lobe is ligated which leads to the death of distal acinar cells(Wang et al., 1995). In this study, it was observed that beta cells in the ligated portion doubled while the unligated portion remains unchanged(Wang et al., 1995). This model did not involve loss of preexisting beta cells and hyperglycaemia was not formed in the animal models as in streptozotocin-induced damage or near whole pancreatectomy(Wang et al., 1995)(Xu et al., 2008). Studies showed that neurog 3 expressions were activated at the ductal region and were expressed in neo islets formed in the damaged region(Xu et al., 2008). They also proved that neurog inactivation in these ductal cells dropped the beta-cell formation in the injured region. This study provides evidence of neogenesis in the case of ductal ligation and lineage tracing experiments could affirm the pancreatic stem cell concept.

2.5.3.2 Mesenchymal Stem cells

Mesenchymal stem cells (MSC) are non-heamatopoietic cells first identified in bonemarrow by the work of Friedenstein et.al (Friedenstein et al., 1970). The most

common source of mesenchymal stem cells have been bone marrow however marrow aspiration has been a painful invasive procedure and hence alternative and easy sources for MSCs have been studied. MSC s have been isolated from synovium, periosteum, adipose, and muscle (Yoshimura et al., 2007). Pittenger et al showed that these cells could differentiate into multiple lineages of mesenchymal origin such as adipocytes, chondrocytes and osteocytes (Pittenger et al., 1999). Several studies have shown that the mesenchymal stem cells can differentiate into multiple lineages that include myoblasts(Wakitani et al., 1995), early neuronal precursor cells(Woodbury et al., 2000) osteoblasts (Zaminy et al., 2008) (Jaiswal et al., 1997), chondrocytes (Mohan et al., 2015) and islet-like cells (Chandra et al., 2009)(Aloysious and Nair, 2013).

Adipose tissue as a source for mesenchymal stem cells has gained attention from researches due to the easy procedure to obtain adipose tissue, ease in isolating mesenchymal stem cells from the tissue and ability to bank the cells from patients(Mosna et al., 2010). Different protocols have been developed using various growth factors to differentiate mesenchymal stem cells to islets. Growth factors such as nicotinamide, exendin-4, betacellulin have been used together to achieve differentiation, however, those cells were not responsive to glucose stimulation(Timper et al., 2006). Growth factors activin, sodium butyrate,beta-mercaptoethanol, nicotinamide, betacellulin or GLP-1 have been used in a stepwise protocol to differentiate mesenchymal stem cells to endodermal lineage progressing to pancreatic endodermal lineage and finally to insulin expressing pancreatic islet-like cells. (Aloysious and Nair, 2013)(Chandra et al., 2009)(Muthyala et al., 2011). These islets like cells secreted insulin in response to glucose stimulation and helped

reduce hyperglycaemia when transplanted into streptozotocin-induced diabetic animal models. Clinical trials with insulin expressing mesenchymal stem cells from adipose have shown a 30% reduction in the requirement of exogenous insulin in transplanted patients(Trivedi et al., 2008).

MSCs are reported to have immunomodulatory through inhibition of effector function and hence safe from immunorejection in case of allotransplantation(Domínguez-Bendala et al., 2012). However, it is reported that MSC loses their immune-modulatory nature after the differentiation process and can be rejected in allotransplants(Huang et al., 2010) (Eliopoulos et al., 2005). Hence the use of mesenchymal stem cells differentiated islets requires the use of immunosuppression or immunoisolation.

2.6. Tissue engineering of Islet construct

Pancreatic islet transplantation has been limited by the shortage of available donors and hence research has been focused on producing islet cells from alternative cell sources in particular from stem cells. Protocols have been developed to direct the differentiation of adult and embryonic stem cells to islet-like clusters, however, the islet clusters formed often lack the function of mature islets (Bhat et al., 2019)(Takahashi et al., 2016). This could be attributed to the lack of interaction with the extracellular matrix as it has been proved that intricate cell - ECM interactions play important role in migration, proliferation and differentiation of islets in foetus during development(Stendahl et al., 2009a). Tissue engineering concept promises to overcome the limitations faced in islet transplantation by incorporating of cells, matrix and growth factors to develop functional tissue recapitulating *in vivo* process(Amer et al., 2014). Three-dimensional culture on scaffolds provides the

advantage of spatiotemporal and matrix interaction for cell differentiation leading to spherical architecture, maturation, improved function and survival of islet-like clusters(Takahashi et al., 2016).

2.6.1. ECM of Islets

Islet transplantation is deemed to be a potential cure for the diabetic state and provides an alternative to whole pancreatic transplantation. However, intrahepatic islet transplantation is limited by lower engraftment efficiency than whole pancreas transplantation. The process of enzymatic digestion and isolation of islets from its native environment disrupts its interaction with the extracellular molecules(Cheng et al., 2011). The pancreatic islets are surrounded by basement membrane made up of glycoproteins, the collagen that provides strength, attachment to cells and molecular sieves that entrap growth factors and environmental signals for the cells. The major components of the islet basement membrane are collagen, laminin, vitronectin, fibronectin, nidogen/ entactin (Stendahl et al., 2009b).

2.6.1.1 *Collagen*

Collagen molecules found in islet ECM membrane are collagen I, collagen III, collagen V collagen IV where collagens I, III and V form fibrillar structures when collagen iv forms hexagonal networks. The $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$ integrins are expressed in islets and they bind to GFOGER sequence in Coll-IV(McCall-Culbreath and Zutter, 2008). Kaido et al. reported that the expression of $\alpha1\beta1$ integrin in human fetal b-cell and they bind to collagen IV to attach and migrate. This interaction with collagen IV enabled insulin secretion(Kaido, Yebra, et al., 2004). However, Other studies could not prove the expression of a1 integrin

chain in islets of canine, porcine, and human pancreas(Wang et al., 1999). Thus, integrin -collagen IV interaction in islets could not be conclusively proved and non-integrin interaction of islets with collagen through discoidin domain may regulate cell adhesion, migration and differentiation(Chin et al., 2001).

2.6.1.2 *Laminin*

Laminins(LM) are glycoproteins composed of three polypeptide chains connected by disulfide bonds (Tunggal et al., 2000). The expression of LM -111 has been shown as the main laminin isoform in the islet basement membrane of foetal rodent pancreas(Jiang et al., 1999). Upon maturation, LM-111 is replaced by LM-511 in rodent pancreas(Jiang et al., 2002). The presence of LM-332 in human and rat islet matrix has been shown and it interacts with the alpha cells of the islets(Parnaud et al., 2006). Presence of other isoforms such as LM-411 and LM521 in the human islet matrix have been reported(Virtanen et al., 2008). α6 integrin and alpha-dystroglycan interacts with laminin -111 and induce differentiation in foetal rodent pancreas(Wang et al., 2005)(Jiang et al., 2001).

2.6.1.3 *Fibronectin*

Fibronectin is a high molecular weight dimeric glycoprotein that exists in the fibrillar form in the basement membrane (Kreis and Vale, 1999). Islet cells bind to the RGD sequence of fibronectin especially in developing pancreas islets interact with fibronectin with α3 and α5 integrins(Wang et al., 2005). It has been shown that islet interaction with RGD of fibronectin inhibits apoptosis in human islets(Pinkse et al., 2006). When fibronectin is added to islet culture *in vitro*, it has improved islet survival(Wang and Rosenberg, 1999).

2.6.1.4 *Vitronectin*

Vitronectin is a low molecular weight proteoglycan seen exclusively in the matrix of developing pancreas in foetus(Cirulli et al., 2000). Vitronectin receptor αvβ1 is seen upregulated in foetal islets and is downregulated in mature beta cells(Kaido, Perez, et al., 2004). The interaction of developing islets with vitronectin is important in the migration of beta cells to form islets, Hence, vitronectin plays an important role in migration and islet morphogenesis(Cheng et al., 2011).

2.6.2 Three dimensional scaffolds

Tissue engineering concept uses a three-dimensional environment for cell culture. Three-dimensional scaffolds improve the outcome of cell culture by providing architecture similar to the extracellular matrix of the tissues. It also promotes the differentiation of cells to form histologically similar tissues as *in vivo*. (Takahashi et al., 2016) The porosity and pore structure of scaffolds help in nutrient access to the cells in 3D structure and it promotes cell survival and function(Aloysious and Nair, 2013). Different synthetic and natural polymers have been used to make scaffolds for the purpose of islet culture and transplantation.

The positive effect of transplanting mature islets on PLGA scaffolds in diabetic animal models were demonstrated by Blomeier et al where 125 islets in PLGA scaffold could control blood glucose concentration when the same number of islets without scaffold failed to do so after transplantation in epididymal fat pad of diabetic mice(Blomeier et al., 2006). Consistent results have been obtained when human islets were transplanted in PLGA scaffolds and when coated with ECM components such as collagen I, Collagen IV, fibronectin showed higher survival rate for islets compared to two-dimensional culture(Daoud et al., 2011) and reverse

hyperglycaemia in diabetic animal models(Salvay et al., 2008). Mouse islets encapsulated in collagen and laminin along with MSCs in silk hydrogel showed increased insulin secretion and up-regulation of insulin and PDX1 gene expression compared to non encapsulated cells(Davis et al., 2012). Other biomaterials used for successful islet survival and engraftment are collagen (Nagata et al., 2002), self-assembled peptide nano gels (Lim et al., 2011), PEG hydrogels (Kizilel et al., 2010).

Stem cells have been differentiated in three-dimensional scaffolds to form islet-like clusters. These differentiated islet-like clusters showed greater viability, insulin secretion and spherical architecture compared to islets differentiated in 2D culture. Gelatin dextran dialdehyde scaffold has been shown to support the differentiation of mesenchymal stem cells to islet-like clusters and increase the survival rate of the differentiated islets(Aloysious and Nair, 2013) and gelatin polyvinyl pyrrolidone semi IPN scaffold is supports the growth and differentiation of pancreatic progenitor cells to islet-like cells which effectively reduced hyperglycaemia after implantation in diabetic rats (Muthyala et al., 2011). Human embryonic stem cell differentiated pancreatic islets were also functional and when transplanted in gelatin foam scaffold reduced hyperglycaemia in the diabetic mice model(Kroon et al., 2008a).

2.6.3 Immunoisolation

Immune rejection and autoimmunity in type I diabetes pose a significant challenge for the success of transplantation strategies. Cells including embryonic stem cells, adult stem cells and differentiated mesenchymal stem cells can elicit an immune response in recipients. Immuno isolation is one strategy to overcome the challenge of immune rejection, where semi-permeable polymer membranes form the

barrier between innate immunity and transplanted cells. Materials such as alginate(Schneider et al., 2005), PEG(Weber et al., 2007), silica(Pope et al., 1997), PU-PVP IPN (Polyurethane-polyvinyl pyrrolidone interpenetrating network)(Chandra et al., 2011)(Muthyala et al., 2011) have been used for micro and macro encapsulation devices to counteract immune rejection in animal models. Central necrosis of islets due to hypoxic conditions and device design issues for macro encapsulation are major limitations of immunoisolation strategy.

2.7 Effect of *In vivo* environment on Differentiated Islets

Protocols and three-dimensional culture systems have been developed to differentiate stem cells to islet-like clusters *in vitro*. Studies have reported insulin secretion in response to glucose stimulation and reversal of hyperglycaemia when transplanted to streptozotocin-induced diabetic models(Chandra et al., 2009)(Muthyala et al., 2011)(Pagliuca et al., 2014). However, an *in vivo* microenvironment would be necessary to promote further maturation of differentiated islets(Abdelalim and Emara, 2015). Several reports have shown *in vivo* maturation of differentiated islet cells or embryonic stem cell-derived pancreatic progenitors after implantation *in vivo* under kidney capsule or fat pad(Rezania et al., 2012)(Tang et al., 2004). Bruin et al showed that maturation of pancreatic progenitor cells occurs *in vivo* even when implanted with encapsulation devices (Bruin et al., 2013). This was in agreement with studies showing that hyperglycaemia in streptozotocin-induced diabetic animal models increases insulin content in porcine neonatal islets (Kin and Korbutt, 2007). Effect of hyperglycaemia in differentiated islets in humans have not been proved, however, it is demonstrated that newborns of diabetic mothers have increased beta-cell mass compared to newborns of non-

diabetic mothers(Steinke and Driscoll, 1965). Other studies have shown that maturation of pancreatic progenitor cells derived from stem cells occurs through chromatin remodeling(Xie et al., 2013). It has been reported that urocortin 3, a corticotropin-releasing factor that influences insulin secretion , expression is stimulated after *invivo* differentiation(Blum et al., 2012). Thus, these studies show that hyperglycaemia and other unknown factors *invivo* is essential for maturation of stem cell differentiated islet like clusters.

CHAPTER 3

MATERIALS AND METHODS

3.1 Isolation and Characterisation of Cells

The islet of Langerhans, adipose-derived mesenchymal stem cells were isolated from the pancreas and the retroperitoneal fat pad of Wistar rats. The cells were duly characterized after isolation.

3.1.1 Isolation and Characterisation of Rat Islets

3.1.1.1 *Isolation of rat islets*

Mature islets were isolated from the adult Wistar rat pancreas with approval from the Institutional animal ethics committee. The pancreatic tissue was collected aseptically and digested with collagenase V enzyme at 10mg/ml concentration for 10-15 minutes with continuous shaking at 37˚C (O'Dowd, 2009). Islet clusters were purified by filtering the digested tissue through a prewetted 40μm cell strainer (Li et al., 2009). The purity of the isolated islets was assessed by dithizone staining.

3.1.1.2 *Dithizone staining of Islets*

Diphenylthiocarbazone (Dithizone, DTZ) stock solution was prepared at 39mM concentration in DMSO (Dimethyl sulphoxide). The working solution was prepared by mixing 100μl of stock solution with 10ml Phosphate Buffered Saline, filtered with 0.2micron filter and used freshly to stain rat islets for 20 minutes at 37˚C. The islets are then observed and imaged under a phase-contrast microscope.

3.1.1.3 *Immunocytochemistry of Rat islets*

Endocrine cell composition in rat islets was characterised using immunofluorescence staining. The islets were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde solution for 20 minutes. The fixed islets were washed and permeabilised with 0.1% Triton X-100. The islets were blocked with bovine serum albumin (BSA) (10mg/ml PBS) and incubated with specific primary antibodies such as anti-insulin(Abcam), anti glucagon and anti-somatostatin (Dakocytomation, Denmark) overnight at 4˚C. The islets were then washed and stained with secondary antibodies tagged with FITC or Phycoerythrin (Abcam, USA and Santa Cruz Biotechnology, USA). The nucleus was counterstained with propidium iodide (Sigma, USA) and imaged with the confocal microscope.

3.1.2 Isolation and characterisation of rat adipose mesenchymal stem cells

3.1.2.1 *Isolation of rat adipose mesenchymal stem cells*

Wistar Rats weighing 250-300gms were chosen for the isolation of adipose tissue. The adipose samples from retroperitoneal fat pad were aseptically collected in PBS containing 10X antibiotic-antimycotic (Gibco, USA). The adipose tissue was washed and digested with 0.1% collagenase type I (Gibco, USA) for one hour at 37˚C. The digested samples were centrifuged at 2500 rpm for 10 minutes at 4˚C. The cell pellet was suspended in Dulbecco's Modified Eagle Medium-high glucose (DMEM HG) (Gibco, USA) medium containing 10%FBS and 1% antibiotic-antimycotic (Ab/Am). The isolated primary cells were seeded and cultured in a $25cm^2$ cell culture flask (Thermo Scientific™ Nunc™, India) in a humidified incubator with 5% CO_2 at 37˚C. The morphology of the cells was observed under phase-contrast microscopy (Olympus IX 71). After reaching confluence the cells

were subcultured with 0.25% Trypsin -EDTA (Gibco, USA) and seeded at a density of $2x10^4$ cells /cm^2 for cell expansion.

3.1.2.2 *Cytoskeletal Organisation*

F-Actin of mesenchymal stem cells (MSC) cultured on tissue culture plates were stained with phalloidin tagged with alexafluor488 dye (Invitrogen, USA). MSC s was fixed with 4%paraformaldehyde for 20 minutes. After multiple washes with PBS, the cells were permeabilised with 0.1% Triton X 100 for a minute. The cells were then stained with Alexa fluor 488 phalloidin for 30 minutes in the dark. Nucleus was counterstained with propidium iodide. The stained cells were imaged with a fluorescence microscope (Olympus IX 71, USA).

3.1.2.3 *Expression of Stem cell markers*

The expression of mesenchymal stem cell markers in the isolated cells was analysed with immunofluorescence staining. Monolayers of adipose-derived mesenchymal stem cells were cultured on coverslips and were fixed with 4% paraformaldehyde and stained for mesenchymal stem cell markers such as CD105, CD90, CD45-PE/CD34-FITC (BD Biosciences) and Vimentin (Santa Cruz, Biotechnology). For intracellular antigen Vimentin, the cells were permeabilised with 0.1% Triton X-100. The cells were blocked with bovine serum albumin (10mg/ml) for 20 minutes at 37˚C. The cells were incubated with specific primary antibodies for 60 minutes at 37˚C. Cells incubated with CD90, CD105 and Vimentin primary antibodies were stained with FITC labeled anti-mouse secondary antibody (Invitrogen, USA) for 60 minutes in the dark. Propidium iodide and Hoescht were used to counterstain the nucleus. The stained cells were imaged with a fluorescence microscope (Olympus IX 71).

Flow cytometric analyses of the isolated MSC s were performed using BD FACS Aria Cell Sorter (Becton and Dickinson, USA). The cells were trypsinized to form single-cell suspension with 0.25% trypsin-EDTA, washed and stained with CD105, CD44 and CD45-PE/CD34-FITC antibodies. 10^4 cells were counted and recorded for each measurement and analysed using gating Diva software.

3.1.2.4 ***Multilineage differentiation***

Adipogenic, osteogenic and chondrogenic differentiation potential of rat adipose-derived mesenchymal stem cells were analysed using appropriate differentiation media. Adipogenic medium constituted of 10^{-7} M dexamethasone (Sigma, USA), 0.5mM isobutylxanthine(Sigma, USA), 50µM indomethacin (Sigma, USA) in DMEM HG with 10%FBS. Mesenchymal stem cells conditioned for 14 days with the adipogenic medium were fixed with 4% paraformaldehyde and stained with oil red O, a stain for lipid vesicles in the cells. Mesenchymal stem cells treated with osteogenic medium which consists of 10^{-7} M dexamethasone(Sigma, USA), 20mM β glycerolphosphate (Sigma, USA), 50 µg/ml ascorbate 2 phosphate (Sigma, USA) in DMEM HG with 10%FBS, for 28 days were fixed and stained with alizarin red which stains calcium deposition in the matrix. Chondrogenic differentiation in MSC s were induced by culturing cells in DMEM HG medium supplemented with 10%FBS, 2mM L-Glutamine (Gibco, USA), 1X Non-essential amino acids (Gibco, USA), 50µg/ml ascorbic acid (Sigma, USA), 40µg/ml proline(Sigma, USA), 10^{-7}M dexamethasone for 14 days. The cells were fixed and stained with 1% alcian blue that binds to proteoglycans synthesised by the cells.

3.2 Fabrication and characterisation of scaffold

3.2.1 Fabrication of scaffold

The electrospinning method was used for making scaffolds. The procedure for electrospinning was followed from our earlier published work with slight modifications(Vaikkath et al., 2016). Briefly, the method is described as follows, a two-step method was adopted to fabricate the three-dimensional fibrous scaffold by electrospinning on lattice patterned metallic collectors followed by layering the lattice patterned membranes to form three dimensional porous scaffold. Polycaprolactone (Sigma, USA) (Mw=70,000 - 90,000) solution at 20% w/v was prepared in chloroform: methanol mixture (7:3) concentration. To this solution, premelted polycaprolactone- polytetrahydrofuran-polycaprolactone triblock polymer (Mn= 2000) was added to make the final ratio of PCL: PCL-PTHF-PCL at 90:10 and blended by continuous stirring. The solution was then subjected to electrospinning using Holmarc electrospinning equipment (Holmarc Opto-Mechatronics, India). Two lattice patterned metallic collectors with metallic wires arranged at 90˚ to each other at a distance of 3.5 mm and 1.5 mm to each other were used. A 10 ml syringe with a 21G needle was used to eject the solution at a rate of 2ml/h and the fibers formed by applying a voltage of 10kV were collected on the patterned collectors attached to an XY stage kept at a distance of 15 cm. The relative humidity and temperature were maintained at 50% and 25˚C respectively. The XY stage moved at a uniform speed of 1500 steps/S and was controlled by a computer program (Software: Electrospinner, Holmarc Opto-mechanotronic, India). The membranes obtained from 3.5mm and 1.5mm collectors were designated as large lattice and small lattice membranes which were layered to form the three-dimensional porous large lattice and small lattice

scaffolds of size 1cm x 1cm and thickness of 1 mm respectively. The scaffolds were sterilised with UV irradiation for 2 hours and stored aseptically for cell culture studies.

The three-dimensional scaffolds were compared to normal electrospun scaffold which was fabricated by electrospinning the solution onto a steel mandrel rotating at a speed of 500 rpm for 2hrs. The other electrospinning conditions were kept the same.

3.2.2. Fourier Transform Infrared (FTIR) Analysis

PCL/ PCL-PTHF-PCL scaffold was characterised by Fourier Transform Infrared (FTIR) spectroscopy analysis via the Attenuated total reflectance (ATR) method using Thermo Nicolet 5700 FT-IR with Diamond ATR accessory, USA. The spectra in the range of 4000 - 400cm^{-1} were compared to that of PCL.

3.2.3 Differential scanning calorimeter Analysis

Calorimetric behaviour of PCL and PCL-PTHF-PCL was measured. Ten-milligram samples were weighed and kept in an aluminum pan. The experiment was conducted in a nitrogen environment in order to prevent oxidative degradation.

3.2.4Membrane Thickness measurement

The thickness of the electrospun patterned large lattice, small lattice, and normal electrospun membranes was measured with Taylsurf CLI 1000 (Taylor Hobson precision, UK) profilometer. Step height measurement of the membrane thickness was done by scanning a focused white light from point A to B across the attached electrospun membrane on a glass slide with 1000 data points/mm. Each membrane (n=3) were measured at 5 randomly selected areas. The mean thickness of the membranes was quantified with Taylsurf CLI 1000 software.

3.2.5 Scaffold Morphology

Morphology of the three-dimensional scaffolds was analysed using environmental scanning electron microscopy (ESEM), Quanta FEI, Hillsboro, USA. Fiber diameter and inter-fiber distance were measured from ESEM micrographs using image analysis software (Image J, National Institutes of Health, USA).

3.2.6 Pore size and porosity measurement:

Pore size distribution and porosity of the 3D electrospun scaffolds were analysed using Micro CT (Micro CT 40, Scanco Medicals, Switzerland). The large lattice and small lattice scaffolds were irradiated with 45keV at 177μA intensity and 350 slices with a scanning resolution of 8μm were obtained. The two-dimensional slices were compiled to obtain the three-dimensional images of the scaffolds from which the pore size was analysed. Micro CT tomography V5.5 and Micro CT Evaluation Programme V6.0 was used as image processing software and evaluation software respectively.

3.2.7 Contact angle measurement

Surface wettability of PCL/PCL-PTHF-PCL membrane was analysed by static contact angle measurements using a contact angle equipment (Dataphysics, contact angle OCA, Germany). Membranes of PCL were used as control. Distilled water and glycerol (ACS grade 99.5%) were used as liquids in measuring the contact angle. All measurements were carried out by the motor-driven drop method and analyzed using Dataphysics software. At least 5 measurements were carried out on each sample by maintaining the drop time as constant, at room temperature.

3.2.8 Swelling

Swelling ratios of the large lattice and small lattice scaffolds were studied in PBS at 37^{o}C and compared to the normal electrospun scaffold. The samples were cut into 1cmx1 cm with 1 mm thickness of known dry weight were put into PBS for different time periods. The wet weight was measured at the end of each time period and the swelling ratio was calculated using the equation: Swelling ratio = (Wet Weight – Dry Weight)/Dry Weight (**Equation 1**)

3.2.9 Mechanical Testing

The static tensile properties of the large lattice and small lattice scaffolds compared to the normal electrospun scaffolds were analysed with a universal testing machine (INSTRON 3345, UK). Scaffold samples used were rectangular strips of 40 mm length and 10mm width and tested for its tensile strength at 10N load with crosshead speed 25mm/min at room temperature and 50% humidity. Five samples for each electrospun scaffold were tested.

3.2.10 Cytotoxicity tests

3.2.10 .1 *Direct contact method*

Cytotoxicity of the scaffold on contact with the cells was evaluated by the direct contact method. The UV sterilised samples of large lattice, small lattice and normal electrospun scaffolds in triplicate were placed on confluent rat adipose mesenchymal stem cells and incubated at 37˚C with 5% CO_2 for 48 hours. The cells were examined under phase contrast optical microscope for change in morphology of the cells in the test samples. Confluent cells without scaffold were kept as positive control.

3.2.10 .2 *MTT assay*

Cell viability after exposure to scaffold extract was evaluated by MTT assay (3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, USA). The scaffold extract was prepared by incubating PCL/PCL-PTHF-PCL scaffold ($6cm^2$/ml) in DMEM HG medium with 10%FBS and 1%Ab/AM at 37˚C for 24 hours. L929 cells were seeded at a density of 10^4 cells per well in 96 well culture plates. The scaffold extract was diluted to 100%, 50% and 25% concentration with culture medium. An equal volume of the scaffold extract at each concentration was added to the cells and incubated at 37˚C with 5% CO_2 for 24 hours. Cells cultured in the normal medium were considered as control and cells with added Phenol extract (13mg/ml phenol in culture medium) was considered positive control for the experiment. After 24 hours incubation, the test extracts were replaced with 20 µl of 5mg/ml MTT solution and incubated at 37˚C with 5% CO_2 for 3 hours. MTT solution was discarded and formazan crystals formed in cells were dissolved using dimethyl sulphoxide. The optical density of the resulting purple solution was measured at 550nm wavelength using ASYS UVM 340 plate reader. Percentage viability of the cells was calculated using the formula:

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

Equation 2.

3.2.11 Cell Penetration

To analyse the depth of cell penetration in the large lattice, small lattice scaffolds compared to the normal electrospun scaffold, cryosections of cell-seeded

scaffolds were taken. The scaffolds (n=3) seeded with rat adipose-derived mesenchymal stem cells were cultured for 7 days in DMEM HG medium with 10%FBS and 1%Ab/AM at 37˚C with 5% CO_2. The scaffolds were embedded in tissue freezing medium (Leica Biosystems, Germany) and frozen at -80 °C. The sections were cut longitudinally at 8-micron thickness. The sections were then fixed with 4% paraformaldehyde for 20 minutes, permeabilised with 0.1% triton x-100 and stained with DAPI for 20 minutes and fluorescent images were captured using an Olympus IX71 microscope. The depth of cell penetration was measured using Olympus cell sense software.

3.2.12 Cell Proliferation

Cell attachment and proliferation on the small lattice, large lattice and normal electrospun scaffold were monitored at different time points (1 day, 3 days, and 7 days) using metabolic activity based MTT assay. The UV sterilised scaffolds conditioned with the culture medium were seeded with 10^5 rat adipose mesenchymal stem cells. After initial attachment for 2 hours, cell-seeded scaffolds were transferred to fresh culture plates supplemented with culture medium and incubated at 37˚C with 5% CO_2. Medium change was given every 2 days. Cells cultured on cell culture treated surface were considered as control. Three samples of each scaffold per time period were studied.

3.3 Rat islet on scaffold

3.3.1 Islet culture on scaffolds

The 3D electrospun scaffolds and tissue culture plates were pre-wetted with culture medium and 200 mature rat islets were seeded on 3D scaffolds and cell culture plate. The groups are cultured in DMEMHG medium supplemented with

10%FBS and 1%Ab/AM at 37˚C with 5% CO_2 for 7 days. Fresh medium was replenished every 2 days.

3.3.2 Islet cell morphology

The morphology of the rat islets cultured in the scaffold was analysed with environmental scanning electron microscopy after 7 days in culture. The islets on tissue culture plates were observed under a phase-contrast optical microscope.

3.3.3 Islet viability

Viability of the islets on the scaffolds and tissue culture plates were compared after 7 days of culture. The islet seeded scaffolds and islets on tissue culture plates were stained with fluorescein diacetate and propidium iodide (Invitrogen, USA). The islets were then imaged using a confocal microscope (Nikon A1R si laser scanning confocal microscope, USA). The percentage viability was calculated from image analysis using Image J software (Image J, USA)

3.3.4 Immunocytochemistry of islets

Expression of islet-specific hormones insulin, glucagon and somatostatin in islets seeded in scaffolds and tissue culture plates were analysed by immunofluorescence staining. The nucleus was counterstained with Hoechst stain (Sigma, USA) and imaged with a confocal microscope (Nikon A1R si laser scanning confocal microscope, USA).

3.3.5 Glucose stimulated Insulin release assay

Islet seeded scaffold and islets on tissue culture plates were washed and preincubated in with KREBH(Krebs ringer bicarbonate -HEPES) (Appendix A-1) buffer for 3 hours to remove residual insulin from the cells. The groups were then washed and incubated in KREBH buffer containing 5mM glucose, 25mM glucose,

30mM KCL for 30 minutes respectively and the supernatant was collected. The supernatant samples containing released insulin were analysed using rat insulin ELISA (Mercodia, Sweden) according to the manufacturer's protocol.

3.4 Rat adipose mesenchymal stem cell on scaffolds

3.4.1 Differentiation of rat adipose mesenchymal stem cells

Adipose-derived mesenchymal stem cells were seeded on pre-wetted scaffold and tissue culture plates at a concentration of 10^6 cells/cm^3 and 10^6 cells/cm^2 respectively. Differentiation of mesenchymal stem cells to islet cells with the endocrine function was accomplished by a three-stage protocol. For initiation of differentiation, the cells were supplied with serum-free media A (SFM A) containing 50μM/L β-mercaptoethanol and 4nM activin A in DMEM HG. After 72 hours of culture, the next stage of differentiation was initiated by changing media to serum-free media B (SFM B). SFM B comprises of 1% (v/v) non essential amino acid, 2mM/L L-glutamine, 1X B27, 20ng/ml basic fibroblast growth factor (bFGF) and 20ng/ml epidermal growth factor (EGF). The cells were cultured in SFM B for 7 days at 37˚C with 5% CO_2 with fresh medium replenished every 2 days. From day 11, the cells are exposed to serum-free media C (SFM C) comprising of 10ng/ml betacellulin, 10ng/ml activin A, and 1X B27 and 10mM/L nicotinamide for the following 10 days.

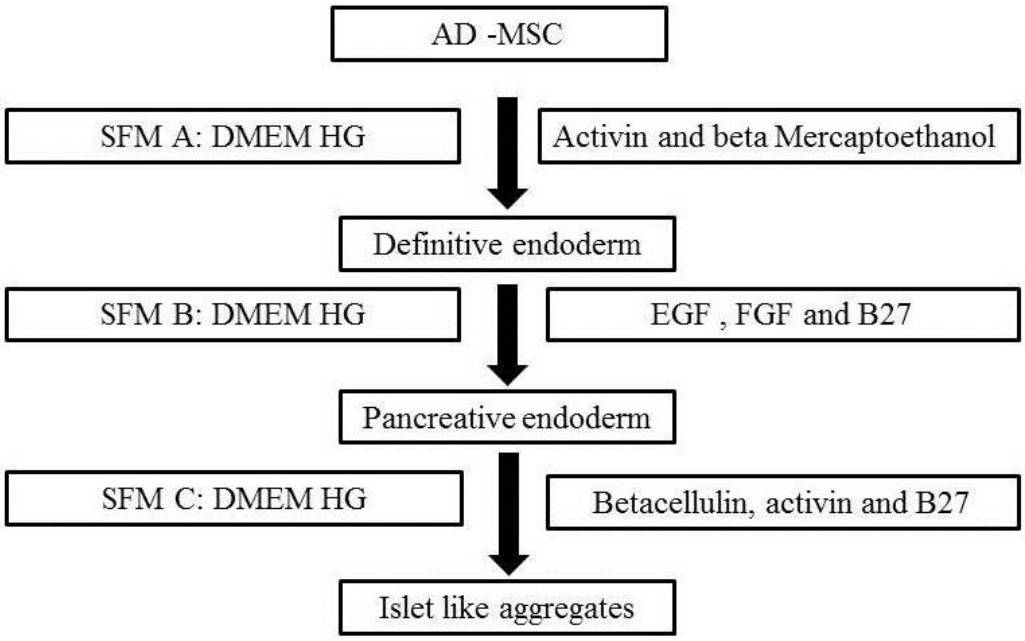

Figure 3.1: Flow Chart of the differentiation protocol

3.4.2 Immunofluorescence analysis

The islet-like clusters (ILC) differentiated from mesenchymal stem cells on 2D tissue culture plates and 3D electrospun scaffolds (Large lattice, small lattice and normal electrospun scaffold) were analysed for the expression of islet-specific hormone insulin (Abcam) by immunofluorescence staining. The islets were washed three times with PBS and fixed with 4% paraformaldehyde solution for 20 minutes. The ILC s formed on scaffolds and 2D plates were washed and permeabilised with 0.1% Triton X-100. The ILCs were blocked with BSA (10mg/ml PBS) and incubated with specific primary antibodies such as anti-C-peptide (Abcam) overnight at 4˚C. The ILCs were then washed and stained with secondary antibodies tagged with FITC or Phycoerythrin (Abcam, USA and Santa Cruz Biotechnology, USA). The nucleus

was counterstained with propidium iodide (Sigma, USA) and imaged with a confocal microscope (Nikon A1R si laser scanning confocal microscope, USA).

3.4.3 Morphology of ILCs on scaffolds

The morphology of mesenchymal stem cells and Islet like cluster formation when differentiated on the large lattice, small lattice and normal electrospun scaffolds on days 2, 4 and 20 were examined using environmental scanning electron micrographs(Quanta FEI, Hillsboro, USA). Change in the mesenchymal stem cell cytoskeletal morphology and clustering during differentiation was analysed by F-actin staining with phalloidin tagged with alexafluor488 dye (Invitrogen, USA). The size of ILCs formed on the large lattice, small lattice and normal electrospun scaffolds compared to 2D culture was calculated using image analysis software Image J (National Institutes of Health, USA).

3.4.4 Viability Assay

Viability of ILC s formed on the small lattice, large lattice and normal electrospun membrane and 2D culture were analysed by staining with fluorescein diacetate and propidium iodide. The samples were incubated in DMEM medium without FBS containing 10mg/ml of fluorescein diacetate and propidium iodide for 10 minutes at 37˚C. The stained samples were washed with PBS thrice and observed under a confocal microscope (Nikon A1R si laser scanning confocal microscope, USA). The percentage viability of the ILCs was calculated using Image J software (Image J, USA) from the fields of interest and calculating the corrected cell fluorescence in the red and green fluorescence(MacGregor et al., 2006). The ratio of corrected fluorescence of green hue divided by the total corrected cell fluorescence was calculated as the percent of live cells.

3.4.5 Glucose stimulated insulin release assay of ILC

ILCs differentiated on scaffolds (large lattice, small lattice, normal electrospun scaffolds) and ILCs on tissue culture plates were washed and preincubated in with KREBH buffer for 3 hours to remove residual insulin from the cells. The groups were then washed and incubated in KREBH buffer containing 5mM glucose, 25mM glucose, 30mM KCL for 30 minutes respectively and the supernatant was collected. The supernatant samples containing released insulin were analysed using rat insulin ELISA (Mercodia, Sweden) according to the manufacturer's protocol.

3.5 *Invivo* studies

3.5.1 Diabetic animal model

Animal studies were performed after approval from the Institutional animal ethics committee, SCTIMST. Wistar rats (male) about 250-300gms were used for the study as an islet, adipose tissue donors and recipients of the tissue-engineered construct. The rats were housed in individually ventilated cages with access to sterile water and food.

3.5.2 Diabetes Induction and Metabolic Monitoring

Diabetes was induced in Wistar rats by injection of streptozotocin (Sigma) at a concentration of 40mg/kg. The rats were kept on fasting overnight before the injection of streptozotocin. Streptozotocin was freshly prepared in sodium citrate buffer (pH 4.5) and injected intraperitoneally. Food was given 30 minutes after streptozotocin administration, water was provided ad libitum. Blood glucose levels of the rats were monitored using glucometers (One Touch Select glucometers, Life Scan

Inc.) for 7 days and rats with blood glucose levels above 300 mg/dl were chosen for implantation studies.

3.5.3 Implantation of Tissue-engineered construct.

For implantation in diabetic animal models, they were grouped into three- test group, control and diabetic control. The test group was implanted with differentiated islet-like clusters in the scaffold, the control group received scaffolds seeded with mature rat islets and the diabetic control group did not receive an implant. A total of four scaffolds were used for each group. For the test group, $0.5x10^6$ mesenchymal stem cells were seeded in each scaffold and differentiated to islet-like clusters following the standardised protocol. For the control group, each scaffold was seeded with 250 mature rat islets and incubated at 37˚C with 5% CO_2 in a humidified atmosphere. The scaffolds were encapsulated in 2% sodium alginate crosslinked with 1.5M calcium chloride solution before implantation. The constructs were implanted in the omental pouch of the diabetic rats (Figure1). The diabetic recipients were anesthetized by intraperitoneal administration of ketamine (7mg/kg) and xylazine(50mg/kg). The abdomen was shaved and prepared for implantation. A midline incision was made and the omentum was exposed, scaffolds were wrapped with the omentum and sutured to form the omental pouch and returned to the peritoneal cavity, the abdominal muscle and skin were sutured in two layers using 3-0 silk suture (Mersilk, Johnson&Johnson, USA). Tetracycline and insulin were administered and betadine was applied on the wound daily for 7 postoperative days. Food and water were given ad libitum post-transplantation and the animals were monitored for 60 days, fasting blood glucose levels were recorded every 5 days until the end of the study period.

Intraperitoneal glucose tolerance tests were performed at 30 days post-transplantation. After overnight fasting 50%D-glucose (Invitrogen, USA) solution at 2g/kg was administered intraperitoneally. Blood glucose levels were measured before and at fixed time intervals (15, 30, 60 and 120 minutes) after D-glucose injection.

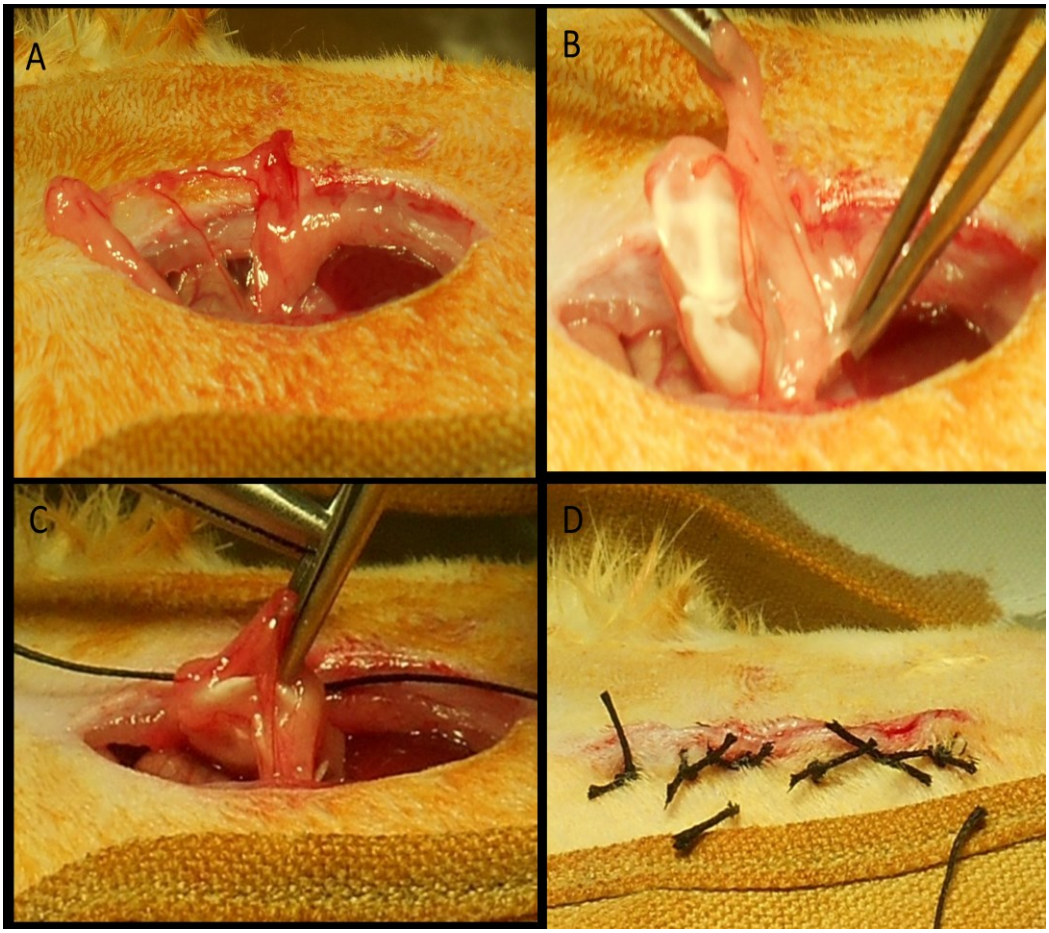

Figure 3.2: Site of implantation: A) Omentum of rat, B) &C) Omental Pouch with the implant, D) Sutured wound

3.5.4 Serum collection and Insulin assay

Blood was collected by orbital venous plexus bleeding and allowed to clot at room temperature for 5 to 10 minutes. The blood samples were centrifuged at 4000 rpm for 10 minutes. The serum was collected into a fresh sterile vial and stored at -

80˚C until analysis. The serum insulin was measured using a rat insulin ELISA kit according to the manufacture's protocol (Mercodia, Sweden).

3.5.5 Histology

Pancreas collected from the animal models during implant retrieval were fixed in 10% neutral buffered formalin (NBF) and stored at RT until histological evaluation. The formalin-fixed samples were dehydrated in alcohol and paraffin-embedded and sections were taken at 5 μm thickness using a microtome (Leica, Germany). The pancreas sections were stained with Mayers hematoxylin (Sigma, India) and counterstained with eosin (Sigma, India). Pancreas sections were immunostained for expression of insulin.

The implanted constructs were frozen in the cryofreezing medium (Leica, India). Cryosections at 8 μm thickness were taken for hematoxylin and eosin staining and immunostaining of insulin hormone.

3.6 Surface modification of scaffold

3.6.1 Modification of 3D large lattice scaffold

The electrospun PCL/PCL-PTHF-PCL large lattice scaffolds were hydrolyzed by immersing in 0.5M NaOH (SD Fine, India) for 1 hr each at room temperature followed by 0.1M HCL (SD Fine, India) for protonation and formation of carboxylic groups. The scaffolds were washed with PBS and immersed in 50mM 2-(N-morpholino)ethanesulfonic acid buffer (MES buffer) for 1 hr at room temperature. After removal of excess MES buffer, a mixture of 2mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma Aldrich) and 1mg/ml N-hydroxysulfosuccinimide (NHS, Sigma Aldrich) were added to the

scaffolds to which ECM components such as collagen IV, vitronectin, fibronectin (50 µL at 1 mg/mL) (Sigma) and a mixture of all three ECM components and crosslinked for 3hrs at 37 ºC. The scaffolds were washed with PBS thrice to remove unbound proteins. The presence of protein on the scaffold was identified by staining with eosin and picosirius red and photographs were taken.

3.6.2 FTIR analysis

PCL/ PCL-PTHF-PCL large lattice scaffold coated with collagen IV, vitronectin, fibronectin was analysed with Fourier Transform Infrared (FTIR) spectroscopy via ATR method (Thermo Nicolet 5700 FT-IR). The spectra in the frequency range of 4000-400cm^{-1} were recorded and the resultant peaks obtained were analysed.

3.6.3 XPS Analysis

X-ray photoelectron spectroscopies (XPS) of the coated large lattice scaffold were measured using Kratos Axis ultra XPS, Manchester, UK with Al Kα radiation as an X-ray source for excitation. Casa XPS software was used for further analysis of elemental peaks.

3.6.4 Viability of Mesenchymal Stem cells on modified scaffolds

Viability of mesenchymal stem cells on large lattice scaffolds coated with collagen IV, vitronectin, fibronectin and combination of all three ECM proteins was analysed by staining with fluorescein diacetate and propidium iodide. The samples were incubated in DMEM medium without FBS containing 10mg/ml of fluorescein diacetate and propidium iodide for 10 minutes at 37˚C. The stained samples were washed with PBS thrice and observed under a confocal microscope (Nikon A1R si laser scanning confocal microscope, USA).

3.6.5 Differentiation of rat adipose mesenchymal stem cells on ECM modified large lattice scaffold

Adipose-derived mesenchymal stem cells were seeded on the prewetted scaffold and tissue culture plates at a concentration of 10^6 cells/cm^3 and 10^6 cells/cm^2 respectively. Differentiation of mesenchymal stem cells to islet cells with the endocrine function was accomplished by a three-stage protocol. For initiation of differentiation, the cells were supplied with serum-free media A (SFM A) containing 50μM/L β-mercaptoethanol and 4nM activin A in DMEM HG. After 72 hours of culture the next stage of differentiation was initiated by changing media to serum-free media B (SFM B). SFM B comprises of 1% (v/v) non essential amino acid, 2mM/L L-glutamine, 1X B27, 20ng/ml basic fibroblast growth factor (bFGF) and 20ng/ml epidermal growth factor (EGF). The cells were cultured in SFM B for 7 days at 37˚C with 5% CO_2 with fresh medium replenished every 2 days. From day 11, the cells are exposed to serum-free media C (SFM C) comprising of 10ng/ml betacellulin, 10ng/ml activin A, and 1X B27 and 10mM/L nicotinamide for the following 10 days.

3.6.6 Immunofluorescence analysis of rat ILC

The islet-like clusters (ILC) differentiated from mesenchymal stem cells on 2D tissue culture plates and 3D electrospun scaffolds (Large lattice, small lattice and normal electrospun scaffold) were analysed for the expression of islet-specific c-peptide (Abcam) by immunofluorescence staining. The islets were washed three times with PBS and fixed with 4% paraformaldehyde solution for 20 minutes. The ILC s formed on scaffolds and 2D plates were washed and permeabilised with 0.1% Triton X-100. The ILCs were blocked with BSA (10mg/ml PBS) and incubated with

specific primary antibodies such as anti-C-peptide (Abcam), overnight at 4˚C. The ILCs were then washed and stained with secondary antibodies tagged with FITC or Phycoerythrin (Abcam, USA and Santa Cruz Biotechnology, USA). The nucleus was counterstained with propidium iodide (Sigma, USA) and imaged with a confocal microscope (Nikon A1R si laser scanning confocal microscope).

3.6.7 Glucose stimulated insulin release assay of ILC

ILCs differentiated on scaffolds (large lattice, small lattice, normal electrospun scaffolds) and ILCs on tissue culture plates were washed and preincubated in with KREBH buffer for 3 hours to remove residual insulin from the cells. The groups were then washed and incubated in KREBH buffer containing 5mM glucose, 25mM glucose, 30mM KCL for 30 minutes respectively and the supernatant was collected. The supernatant samples containing released insulin were analysed using rat insulin ELISA (Mercodia, Sweden) according to the manufacturer's protocol.

3.7 Statistical Analysis

Statistical analysis was performed using Graph pad prism 5 software. The results are represented as mean ± standard deviation. One way ANOVA (Analysis of variance) followed by post hoc Tukey test was used to analyse the results. The P-value ≤ 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

The results obtained in the current study are explained in this chapter. The results are divided into five subsections. The first subsection details the results of isolation and characterisation of rat islets and mesenchymal stem cells. The results for the fabrication and characterisation of the polymeric scaffold are detailed in the second subsection. The effect of scaffold architecture and porosity on rat islets, mesenchymal stem cells and its differentiation to islet-like clusters are detailed in the third subsection. The fourth subsection details the results obtained from the *in vivo* evaluation of the tissue-engineered construct in the diabetic rat model. The fifth subsection details the results of the conjugation of ECM proteins on the 3D nanofiber scaffolds and its effect on the differentiation of mesenchymal stem cells to islet-like clusters.

4.1 Isolation and Characterisation of Cells

4.1.1 Isolation and Characterisation of Rat islets

Mature islets were isolated from rat pancreas after collagenase V digestion. Under light microscopy, the isolated islets showed spherical cluster morphology with size ranging from 50-250 μm (Figure 4.1a). The isolated islets stained reddish-orange after incubation with dithizone which binds to zinc ions in beta cells of islets (Figure 4.1b)**.** The viability of the islets after isolation was analysed by fluorescence staining of islets with fluorescein diacetate, propidium iodide and observed under confocal microscopy (Figure 4.1C). The islets showed greater than 95% viability and the live

cells stained green and dead cells stained red. The islets were positive for insulin, glucagon and somatostatin expression after immunofluorescence staining (Figure 4.2)..

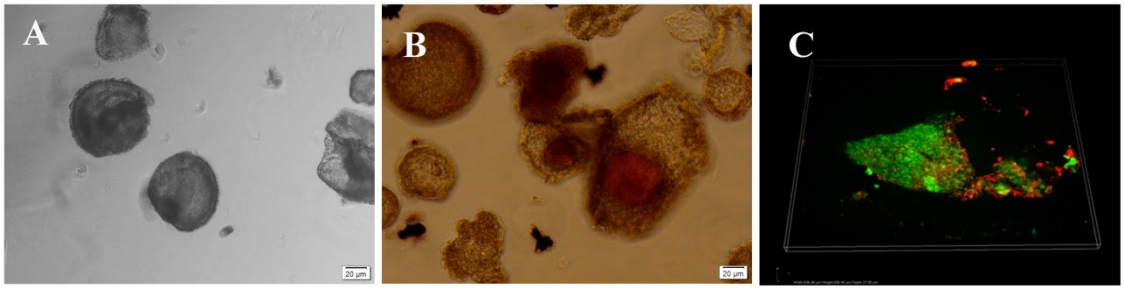

Figure 4.1: A) light micrograph of isolated islets shows their spherical morphology B) Dithiazone stained islets show reddish-orange staining of beta cells C) Live- dead staining of isolated rat islets, live cells are stained green and dead cells are stained red.

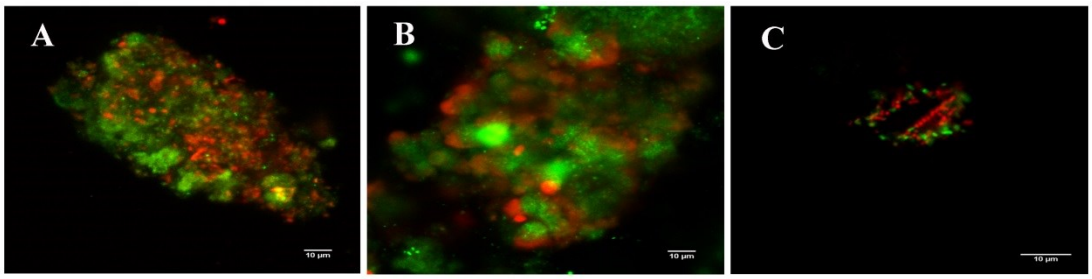

Figure 4.2: Isolated rat islets are stained for expression of islet hormones A) insulin B) Glucagon C) somatostatin. The nucleus is stained red with propidium iodide

4.1.2 Isolation and Characterisation of rat mesenchymal stem cells

Isolation of rat mesenchymal stem cells

Adipose tissue isolated from the retroperitoneal fat pad of Wistar rats were digested with collagenase I and centrifuged to separate mesenchymal cells from mature adipocytes. After an initial 24 hours in culture, cells with fibroblastic morphology adhered to the surface of tissue culture treated dish, which grew to confluency in approximately 7 days (Figure 4.3).

4.1.3 Characterisation of adipose-derived mesenchymal stem cells

International society for cellular therapy (ISCT) (Dominici et al., 2006)has proposed adhesion to the plastic surface, presence of specific surface markers and multipotent differentiation potential as three main criteria for characterising mesenchymal stem cells. Hence, morphology, positivity and negativity for specific surface markers and trilineage differentiation potential of rat adipose-derived mesenchymal stem cells were analysed.

4.1.4 Morphology

Actin and vimentin staining (Figure 4.4) of the isolated cells revealed the characteristic long spindle morphology of the mesenchymal stem cells when attached to tissue culture-treated surface.

4.1.5 Surface marker analysis

Immunocytochemical staining (figure 4.5) of the isolated cells showed positive staining of surface antigens CD105, CD90, and negative staining for haematopoietic marker CD45 and CD34. When quantified using flow cytometry

(Figure 4.6), the cells showed 77.5% positivity for CD105, 78.2% for CD44 and 0.2% for CD34/45.

4.1.6 Multilineage differentiation of MSC

The capacity of the isolated cells for trilineage differentiation was analysed (Figure 4.7). The cells differentiated to osteogenic, chondrogenic and adipogenic lineage when cultured in specific differentiation medium. Oil droplets formed during adipogenic differentiation of MSC was confirmed by oil red o staining (Figure 4.7 a). Osteogenic differentiation was confirmed using alizarin red stain for calcium deposition, which appeared red (Figure4.7 b). Alcian blue staining was performed to confirm the chondrogenic differentiation of mesenchymal stem cells. Alcian blue stains the sulphated proteoglycans deposited during differentiation (figure 4.7 c)

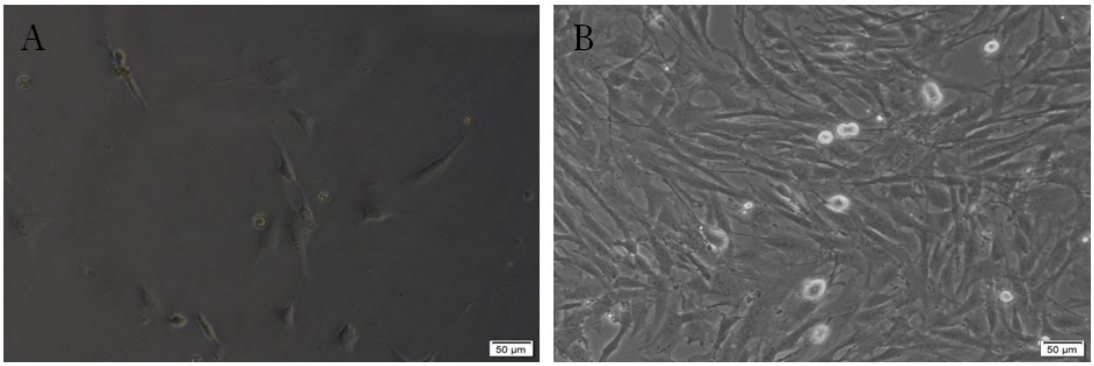

Figure 4.3: Phase contrast image of isolated rat adipose mesenchymal stem cells A) cells 24 hours after isolation B) cells confluent after 7 days in culture

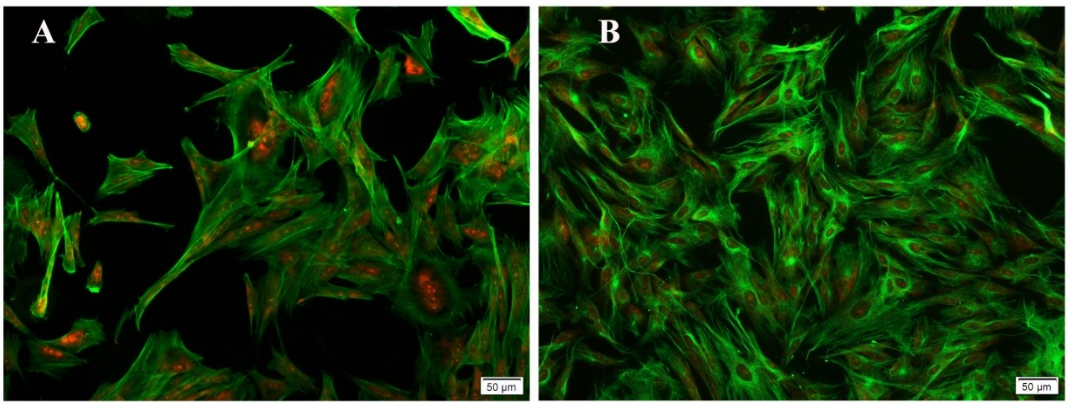

Figure 4.4: Fluorescent staining of mesenchymal stem cells for cytoskeleton A) actin B) vimentin is seen in green. The nucleus is stained red with propidium iodide.

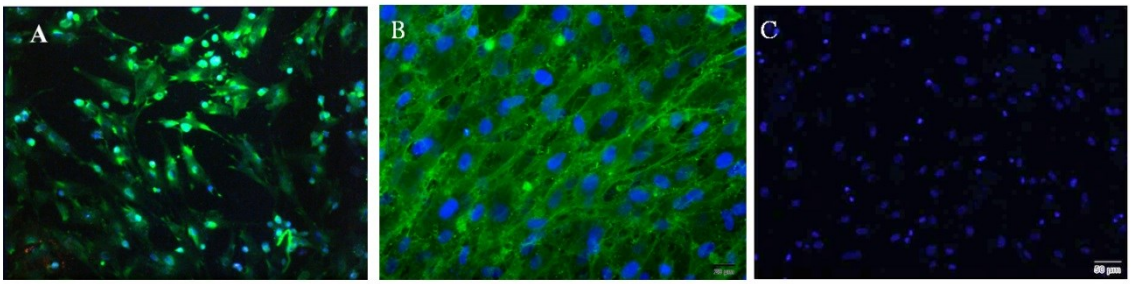

Figure 4.5: Fluorescent micrograph of mesenchymal stem cells stained for surface markers. MSCs were positive for A) CD105 B) CD 90 and C) negative for CD34/45

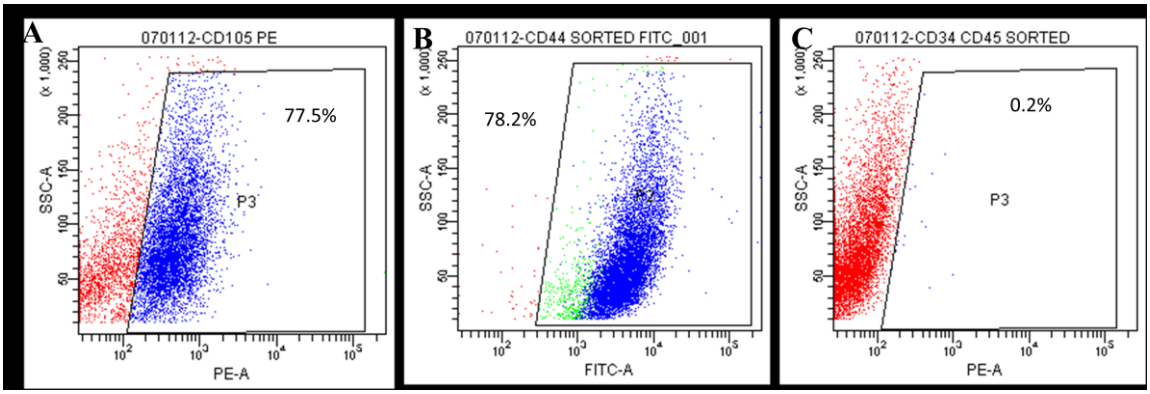

Figure 4.6: Flow cytometry analysis of rat adipose-derived cells shows the percentage of cell population positive for mesenchymal stem cell markers a) 77.5% positive for CD105 b) 78.2% positive for CD44 c) 0.2% negative for CD34/45

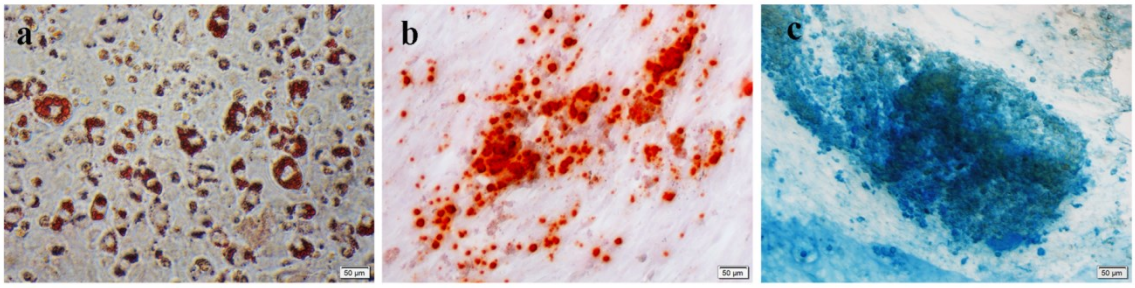

Figure 4.7: Multilineage differentiation of rat adipose mesenchymal stem cells a) Oil droplets formed during adipogenesis stained by oil red O b) Calcium deposition during osteogenesis stained by alizarin red stain c) Sulphated proteoglycans deposited during chondrogenic differentiation stained with alcian blue.

4.2. Fabrication and characterisation of scaffolds

4.2.1 Polycaprolactone/Polycaprolactone-polytetrahydrofuran-polycaprolactone Scaffold

The first objective of the study was to fabricate a three dimensional hydrophilic fibrous and porous scaffold. The technique of electrospinning was employed as it can successfully develop nanofiber polymeric scaffolds with fibres ranging from nanometer to micrometer so as to mimic the ECM fibrils. The electrospinning process is influenced by environmental and processing factors such as temperature, humidity, solution concentration, flow rate, the distance of needle tip from the collector, rate of movement of collector and time. One major disadvantage of the electrospinning process is the difficulty in developing scaffolds that are three dimensional in the thickness direction with uniform porosity and pore connectivity. Macroporous scaffolds are necessary to maintain the spherical morphology, function and viability of islets of Langerhans, which range in size from 50-200μm. Hence, a two-step method was developed to fabricate three dimensional macroporous,

nanofibrous scaffolds. It consists of electrospinning lattice structured membranes on metallic patterned collectors and layering them to form three dimensional macroporous scaffolds. Two types of scaffolds with different porosity range was developed utilising patterned metallic collectors of different sizes. The two metallic patterned collectors used were made of stainless steel wires at 90^{o} and at a distance of 1.5mm (referred to as small lattice) and 3.5mm (referred to as large lattice) to each other. The collectors were attached to a stage with XY directional movement controlled by a computer program (Software: Electrospinner, Holmarc Opto-mechanotronic, India). The schematic representation of the electrospinning process is shown in figure 4.8. The fibres were electrospun at 2ml/h and 10KV, 15cm distance, the XY stage moved at a speed of 1500 steps/S. The humidity and temperature were maintained at 25 º C and 50% relative humidity in the fume hood. The timing of electrospinning each membrane was crucial in later forming the porosity and was standardised to 15 minutes for large lattice membranes (Figure 4.9 a) and 5 minutes for small lattice membrane (Figure 4.9 b). Twenty electrospun membranes of each lattice sizes obtained were layered to form three dimensional scaffolds with two different pore size ranges. The superiority of these scaffolds was compared to normal electrospun membrane scaffold (Figure 4.9c) formed by normal spinning on a mandrel rotating at 500rpm and without a program-controlled XY stage. The other electrospinning conditions were kept constant.

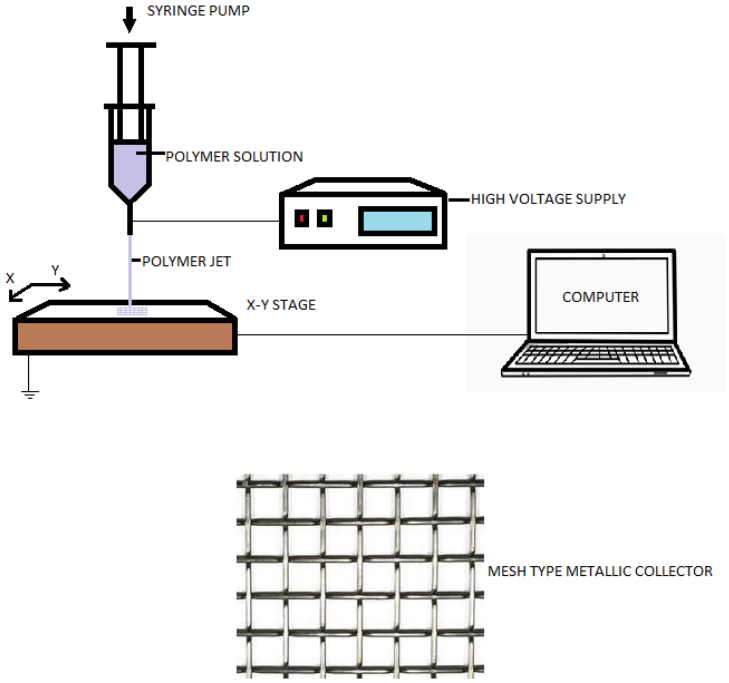

Figure 4.8 Schematic representation of electrospinning process

Polycaprolactone (PCL) and hygroscopic triblock copolymer, Polycaprolactone-polytetrahydrofuran-polycaprolactone (PCL-PTHF-PCL) were selected to fabricate the three dimensional macroporous, nanofibrous scaffolds by the above-mentioned method. Polycaprolactone is synthetic, aliphatic linear polyester that is biodegradable and FDA approved for biomedical applications in the human body. PCL can be electrospun with different solvent systems, alone or in combination with other polymeric components. The advantages of the PCL scaffold in tissue engineering scaffold is however limited by its hydrophobic nature. A co spinning system involving a hygroscopic triblock copolymer, Polycaprolactone-polytetrahydrofuran-polycaprolactone (PCL-PTHF-PCL), can change the hydrophobicity of electrospun PCL scaffolds. When blended and electrospun together, a highly hydrophilic scaffold is obtained (Vaikkath et al., 2016). Blending

with a hydrophilic material is an easy method to make PCL hydrophilic. PCL/PCL-PTHF-PCL solution at the final blend ratio of 90:10 was electrospun to form the desired lattice structured membranes which were layered to form the final three-dimensional lattice structured macroporous scaffolds. The scaffolds were sterilised with UV irradiation for 2 hours and stored aseptically for cell culture studies.

4.2.2 Step height measurement of membrane thickness

Step height measurement with surface profilometer was used to measure the thickness of the membranes formed by electrospinning. In step height measurement, the mean height of the electrospun membrane from glass determines the thickness of the membrane (Affandi et al., 2009). The average thickness (Figure 4.10) of individual small lattice, large lattice and normal membrane were 90.85 ± 2.91, 78.33 ± 10.03 and 82.12 ± 5.96 μm respectively. Twenty membranes of small lattice and large lattice membranes were layered and edges were sealed by applying pressure to form the three-dimensional scaffold of approximately 1.5 to 2 mm thickness (Figure 4.11).

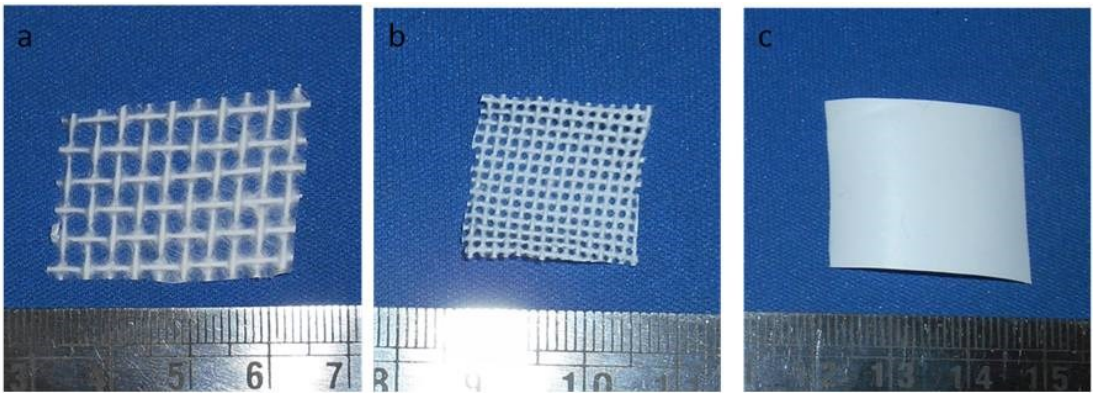

Figure 4.9: a-c shows the large lattice, small lattice membrane and normal electrospun membrane

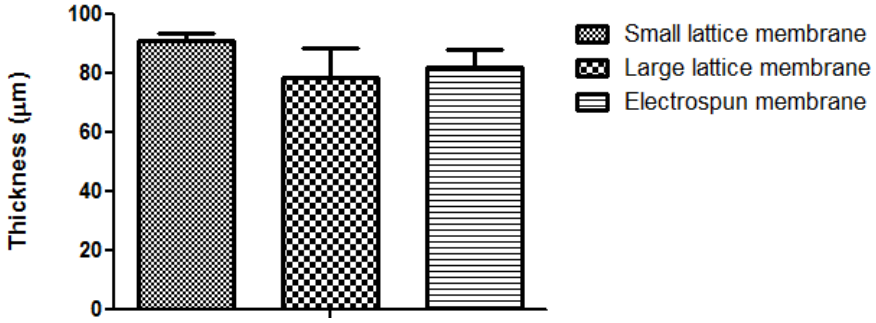

Figure 4.10: Thickness of the electrospun large lattice, small lattice and normal electrospun membranes. The data represented is mean thickness ± Standard Deviation

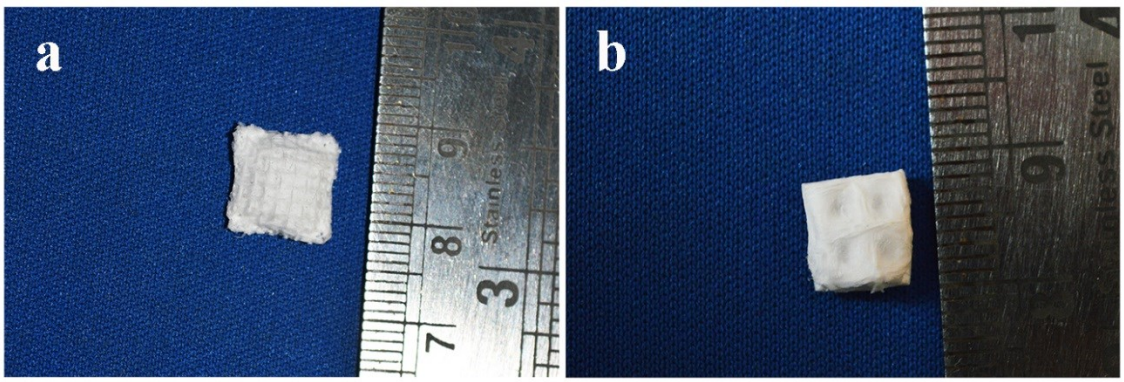

Figure 4.11: Photographic image of (a) small lattice and (b) large lattice scaffold

4.2.3 Morphology:

The morphology of porous three-dimensional scaffolds was analysed using an environmental scanning electron microscope (Quanta FEI, USA). The small and large lattice scaffolds had regions of distinct fiber densities (Figure 4.12 and 4.13). The lattice structure is formed by dense bands of fibers that surround the network of randomly arranged fibers that form the highly porous regions of the scaffold. Such a structure is formed as a result of more fibers depositing on the metallic wire region compared to the spaces in between the wires. More fibers are seen on the void region of small lattice scaffold compared to large lattice since the spacing between the wires was less for these scaffolds. The normal electrospun membrane is uniformly made of randomly arranged fibers (Figure 4.14). The fiber diameters of normal electrospun membranes ranged from 400nm to 5μm with more fibers below 1μm (Figure 4.12d). The diameter of fibers on the porous region of the small lattice scaffold ranged from 0.5 to 3.6 μm (Figure 4.13d) and that of the large lattice scaffolds ranged from 0.2 to 5μm(Figure 4.14b).

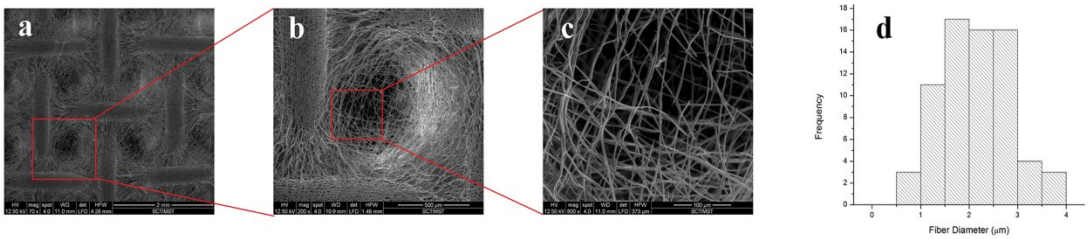

Figure 4.12: a) Overview of the small lattice scaffold. b) Magnified image of single lattice c) fibers formed in the interspacing d) fiber diameter of fibers formed in the interspacing

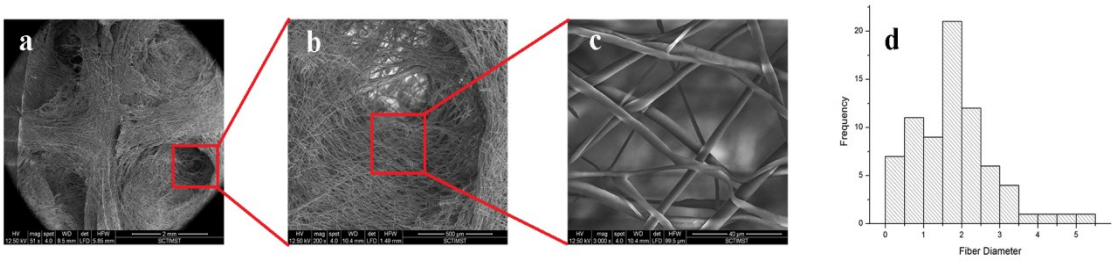

Figure 4.13: a) Overview of the large lattice scaffold. b) Magnified image of single lattice c) fibers formed in the interspacing d) fiber diameter of fibers formed in the interspacing

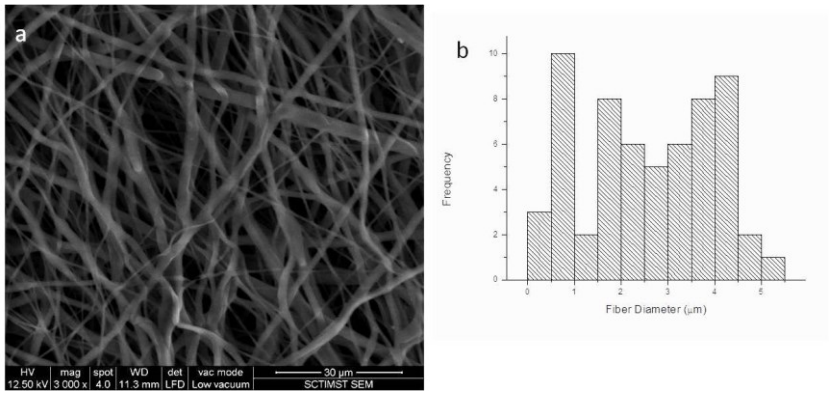

Figure 4.14: a) Environmental scanning electron micrograph of normal electrospun scaffold b) Fiber diameter histogram

4.2.4 Porosity

Micro CT scan data was used to measure the porosity and pore size of the scaffolds. The porosity of large lattice and small lattice scaffolds was 89.05 and 90.82% respectively. The pore size ranged from 24μm to 250 μm for large lattice scaffold and 24μm to 190μm in small lattice scaffold (Figure 4.15).55.5% of the total pore volume is constituted by pores greater than 100μm in large lattice scaffolds whereas 45% of total pore volume of small lattice scaffold is constituted by pores greater than 100μm(Figure 4.16). The pore size was found to increase with the increase in space between the metallic wires of the collector; hence the large lattice scaffold has more pores in the range of 100-250 μm compared to small lattice scaffold.

4.2.5 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra were used to identify the chemical composition of the polymer scaffold. FTIR spectra (Figure 4.17) of PCL/PCL-PTHF-PCL scaffold exhibited asymmetric CH_2 stretching at 2919 cm^{-1}, symmetric CH_2 stretching at 2851 cm^{-1}, carbonyl stretching at 1724 cm^{-1} and asymmetric C-O-C stretching at 1162 cm^{-1} and these peaks were similar for PCL scaffold.

4.2.6 Differential scanning calorimetry

Differential scanning calorimetry shows two separate peaks for the co-electrospun scaffold at 15.24 ˚C and 56.3 ˚C, the same as the individual peaks obtained for PCL-PTHF-PCL and PCL respectively. Hence it can be concluded that

PCL and PCL-PTHF-PCL remain as a physical blend in the electrospun scaffold (Figure 4.18).

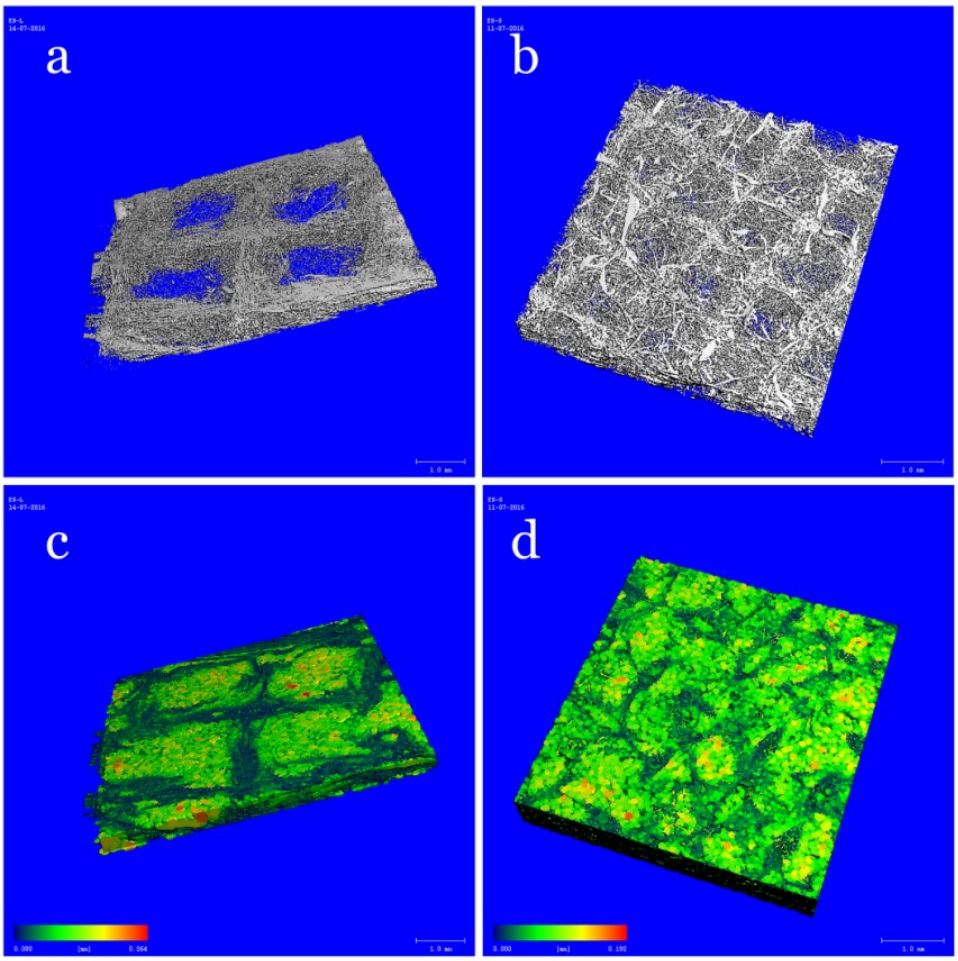

Figure 4.15: MicroCT image of a) large and b) small lattice scaffold. C and d represents the spacing profile of the large and small lattice scaffold

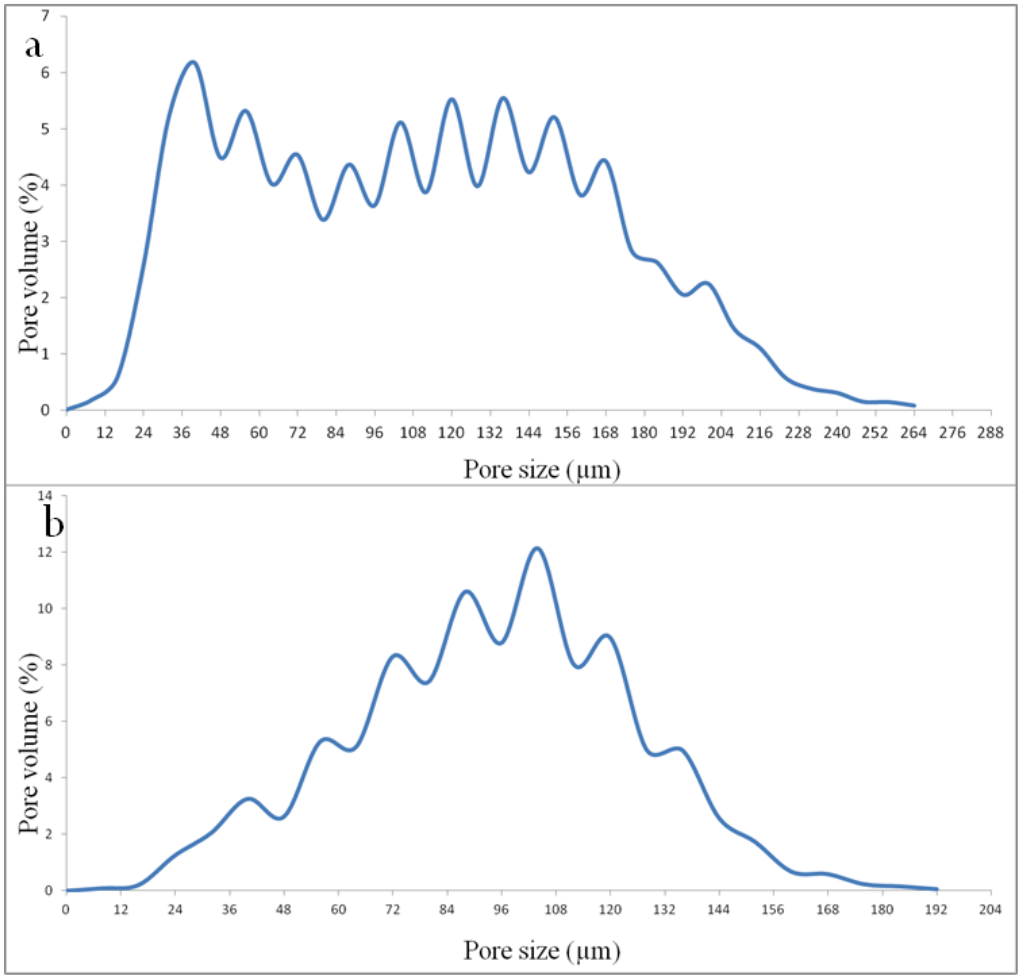

Figure 4.16: Histogram showing pore size range of a) large lattice b) small lattice membrane

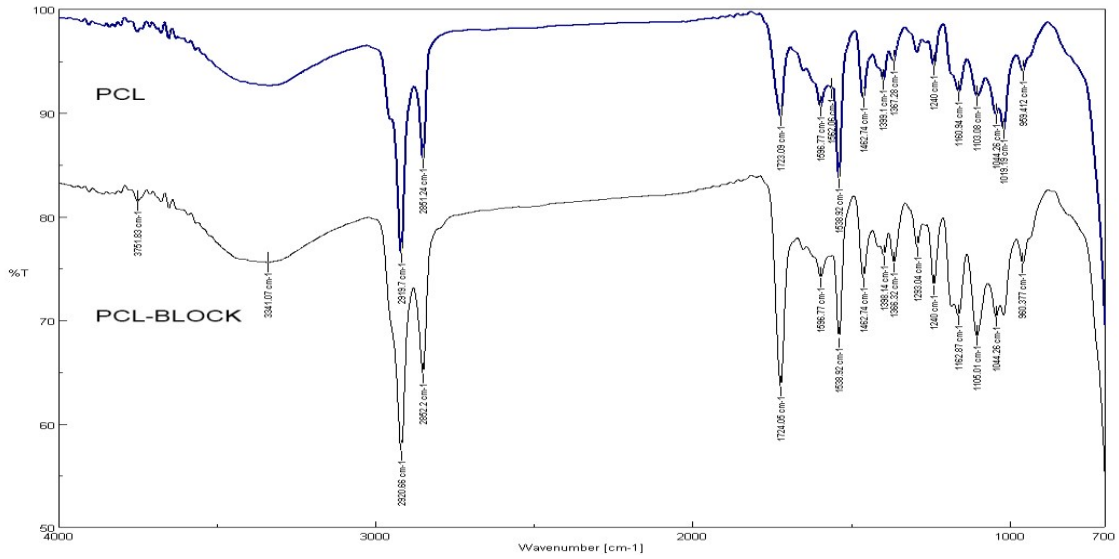

Figure 4.17: FTIR Spectra of PCL/PCL-PTHF-PCL (PCL-Block) and PCL scaffold. Groups: C= O : 1740-1690 cm^{-1}, Alkyl C-H stretch:2950-2850 cm^{-1}, C-O-C Stretching: 1167cm^{-1}

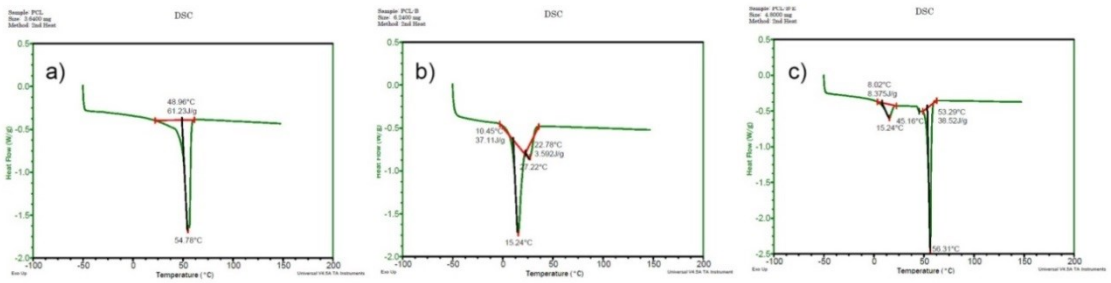

Figure 4.18: Differential scanning calorimetry of a) PCL (54.78˚C) b) PCL-PTHF-PCL (15.24˚ C) and c) PCL /PCL-PTHF-PCL blend (15.24˚C and 56.31˚C)

4.2.7 Hydrophilicity of the scaffold:

To measure the hydrophilicity of the scaffold material, the static water contact angle test was performed. The high rate of absorption of water droplets by the material prevented obtaining a measurable value of contact angle for the PCL/PCL-

PTHF-PCL material. Glycerol, a highly viscous polar solvent was chosen to overcome the high absorption experienced with the use of water. Contact angle formed by glycerol (Figure 4.19) at the time of contact was measured and a contact angle of 59.3˚ was obtained whereas glycerol contact angle of PCL membrane was 129.1˚(Vaikkath et al., 2016). Co spinning of the triblock polymer with PCL has significantly ($p \leq 0.001$) increased the hydrophilicity of the material.

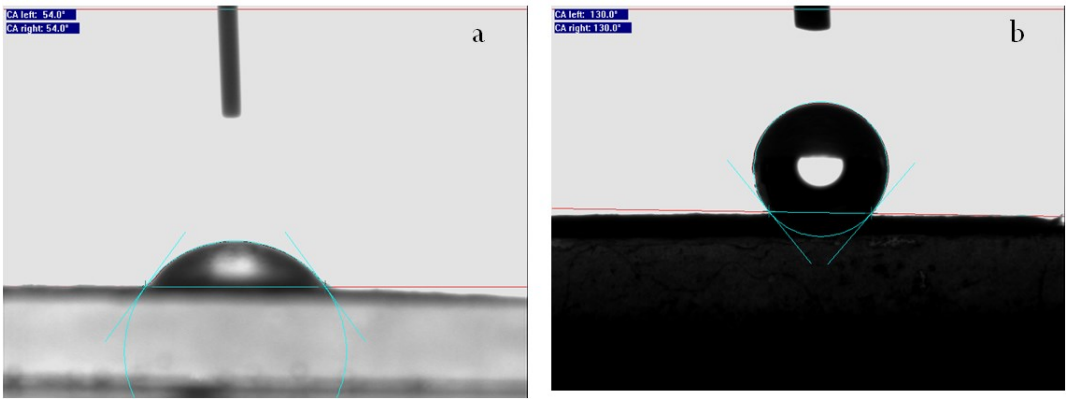

Figure 4.19: Air -glycerol contact angle of (a) PCL/PCL-PTHF-PCL membrane (b) PCL membrane.

4.2.8 Swelling Ratio

The swelling ratio of the three-dimensional scaffolds and electrospun membrane as a function of time was studied (Figure4.20). The scaffolds as well as the electrospun membrane absorbed PBS and reached the peak swelling ratio within 5 minutes and stayed constant henceforth. The small lattice scaffold showed the highest swelling ratio of 10 compared to the large lattice scaffold (swelling ratio of 8) and the normal electrospun scaffold. The higher swelling ratio of the small lattice scaffold may be due to a higher density of fibers compared to the large lattice

scaffolds. The high swelling capacity of the three-dimensional scaffolds aides in better nutrient exchange for the cells.

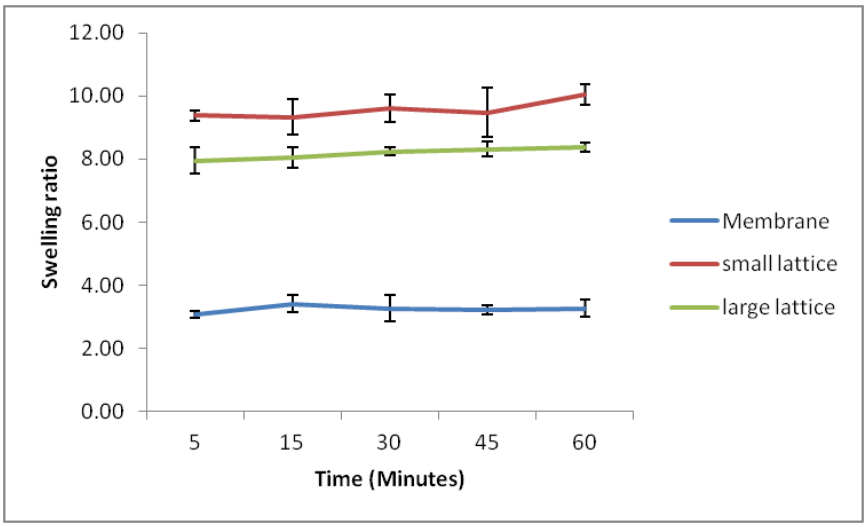

Figure 4.20: represents the swelling ratios of small lattice and large lattice scaffolds compared to the electrospun membrane.

4.2.9 Mechanical testing

Mechanical properties of the small lattice, large lattice, and normal electrospun scaffolds were compared and are shown in table 4.1. The stress at maximum load decreased for the large lattice membrane compared to the small lattice and the normal electrospun scaffold. Modulus and stress at maximum load decrease with the increasing lattice size of the electrospun scaffold. Thus large lattice scaffolds were softer compared to small lattice and normal electrospun scaffold.

Table 4.1: Mechanical performance of scaffolds

Sample code	Stress at maximum load(MPa)	Modulus (MPa)
Small lattice	1.4±0.19	4.4±0.6
Large lattice	0.90±0.07	3.0±0.4
Con.electrospun scaffold	3.3±0.8	12.2±3.5

4.2.10 *In vitro* cytotoxicity of scaffold

Direct contact test (Figure 4.21) of PCL/PCL-PTHF-PCL scaffold with adipose-derived mesenchymal stem cells showed no change in the cell morphology and proliferated to form a confluent layer of cells in the presence of the scaffolds. Test on extract (MTT assay) of the scaffolds showed 100% cell viability with 25% extract of the scaffold and more than 80% viability with 50 and 100% extracts (figure 4.22). These tests showed that PCL/PCL-PTHF-PCL scaffold was non-cytotoxic to cells.

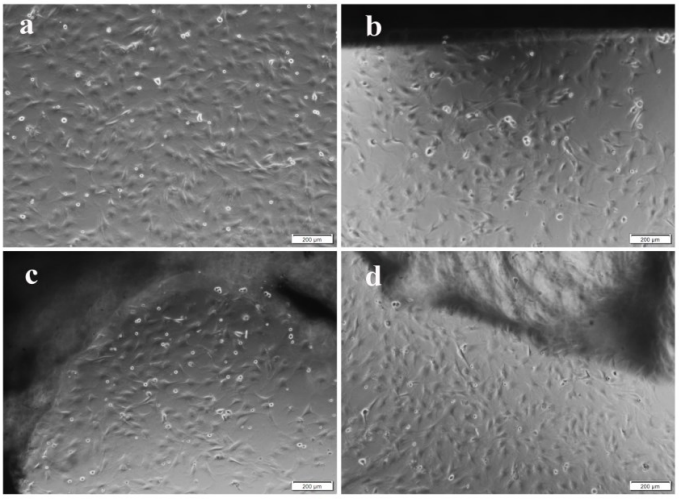

Figure 4.21 shows Direct contact test: a) Control b) electrospun membrane c) large lattice membrane, d) small lattice membrane

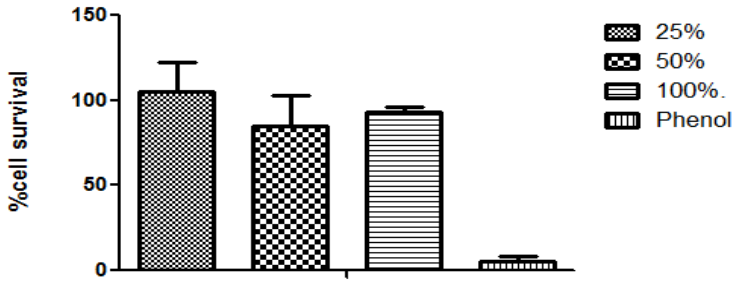

Figure 4.22: MTT assay of PCL/PCL-PTHF-PCL electrospun membrane for percentage cell viability with L929 cell lines

4.2.11 Cell Penetration

The effect of increased pore size on the penetration and colonisation of the scaffolds by cells was evaluated. Figure 4.23 shows the representative images of large lattice, small lattice and normal electrospun scaffold sections with cell nuclei stained with Hoechst. Rat adipose-derived mesenchymal stem cells that were seeded on one side of the scaffolds have infiltrated into the depths of the small lattice and

large lattice scaffolds after 7 days of culture when compared to the electrospun scaffold where the cells have formed a sheet on its surface. The depth of infiltration was measured to be 641.54±62.08 μm in large lattice scaffold and 367.81 ±86.80 μm in small lattice scaffold compared to 53.548±8.35 μm in the normal electrospun scaffold. This shows that the porous nature of these scaffolds have helped the cells to colonize the depths of the scaffolds when compared to the normal electrospun scaffold, where the absence of large pores limited cell penetration and resulted in cell sheet formation on its surface.

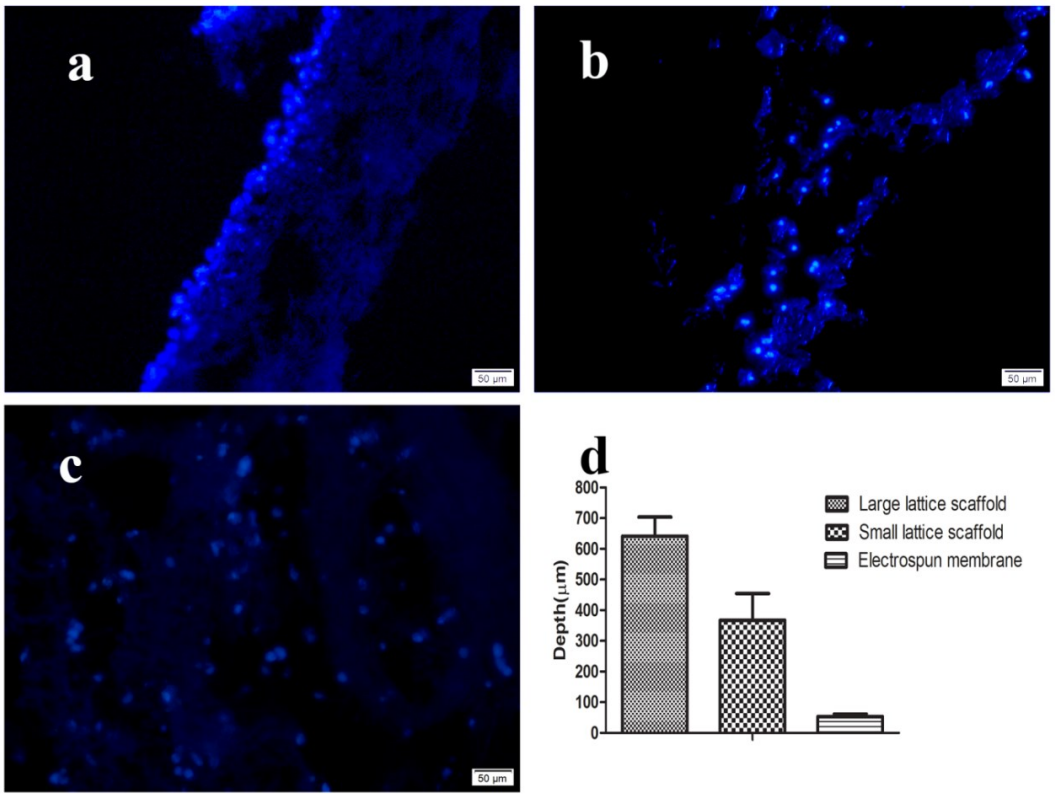

Figure 4.23: a-c shows cell penetration in the electrospun membrane, small lattice scaffold and large lattice scaffold respectively (d) shows the depth of penetration in the scaffolds. The result is expressed as the mean ± standard deviation.

4.2.12 Cell Proliferation

Metabolic activity-based MTT assay was used to measure the activity and viability of cells in the different pore size scaffolds at different time points in culture. Mesenchymal stem cells seeded on the large lattice, small lattice and normal electrospun scaffolds were (Figure 16) evaluated for viability and activity on day 1, 3 and 7 of culture and was compared with cells growing on tissue culture-treated surface. It was observed that the optical density increased over the culture period for all groups indicating cell proliferation. On day 7 metabolic activity on a large lattice scaffold was significantly higher ($p < 0.05$) than the small lattice scaffold.

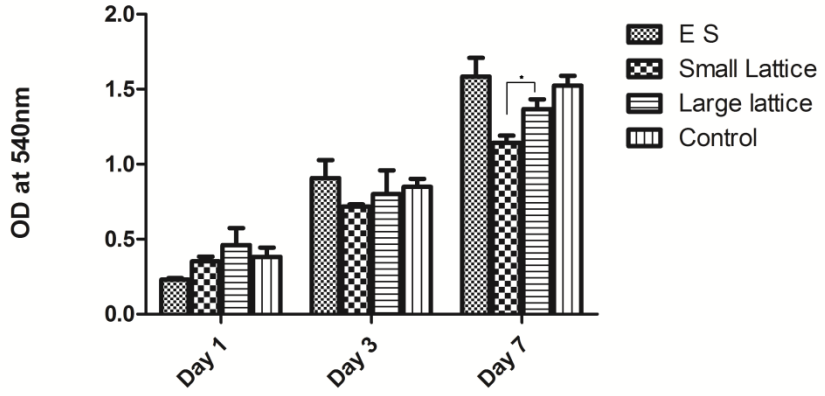

Figure 4.24 shows cell activity measured by MTT assay. The activity of the cells increased with culture time.

4.3 Evaluation of rat islets on three-dimensional nanofibrous scaffolds

4.3.1 Islet seeding in three-dimensional scaffolds

The islets were seeded on the large lattice, small lattice and normal electrospun scaffold. The seeded islets were cultured for 7 days with medium change on alternate days. At the end of 7 days culture, the morphology of islet in scaffolds

was analysed with ESEM (Environmental scanning electron microscopy). The micrographs (Figure 4.25) showed that islets seeded in large lattice scaffolds retained their spherical morphology and were seen embedded in the random fibers of the scaffold. Islets could not be observed in the small lattice and normal electrospun scaffolds. The islets could not embed in the small lattice and normal electrospun scaffold due to the small pore size of these scaffolds. ESEM analysis showed that the large lattice scaffold provided the favourable pore size and structure among the three selected scaffolds that can support mature rat islets to lodge and grow in culture. Since the order of pore size of the small lattice and normal electrospun scaffold were small to support mature rat islets, further studies on viability, insulin release function of the islets were carried out using large lattice scaffold and compared to the islets on 2D culture.

4.3.2 Immunostaining of islets cultured on the scaffold

The islets in large lattice scaffolds were stained for the expression of islet hormones after 7 days in culture and imaged using confocal microscopy (figure 4.26). The cells showed positive expression of hormones insulin, glucagon and somatostatin.

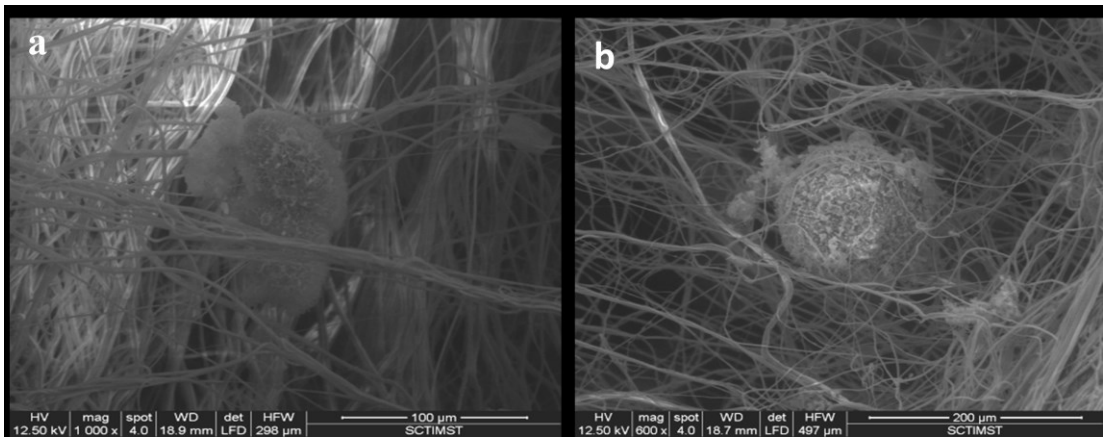

Figure 4.25 a, b- shows the mature rat islets in the large lattice scaffold could attach and maintain their spherical morphology in large lattice scaffold in *in vitro* culture

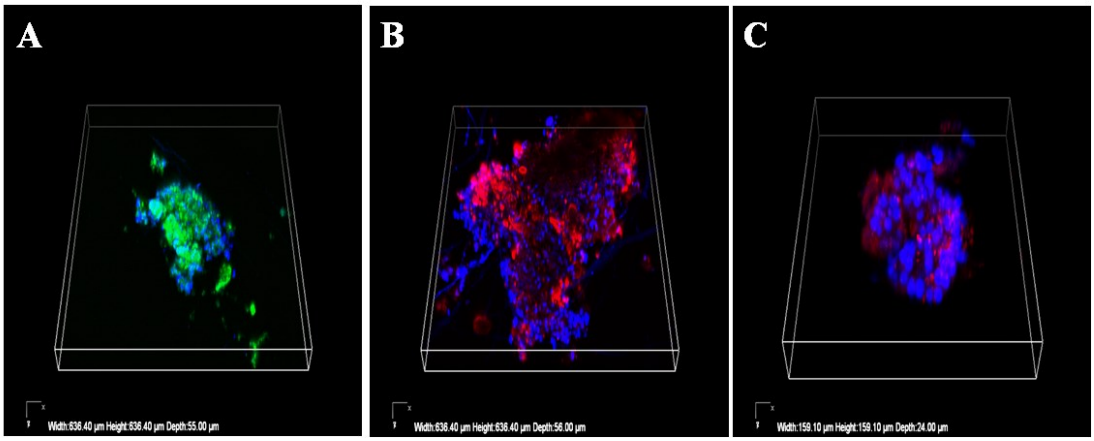

Figure 4.26 Immunostaining of rat islets on large lattice scaffold for expression of A) Insulin staining B) Glucagon staining C) somatostatin staining. The nucleus is stained blue.

4.3.3 Viability of Islets on the scaffold

After 7 days in culture, the viability of the islets on thelarge lattice scaffolds was analysed and compared with the islets on the cell culture plate (Figure 4.27). The percentage viability of islets on the scaffold was calculated using image analysis. It

showed that 56.82±8.2 % of islet cells on the scaffold were viable compared to 39.47±7.9% islet cells on the cell culture plate. The islets exhibited increased viability because of the three-dimensional support given by the large lattice scaffold.

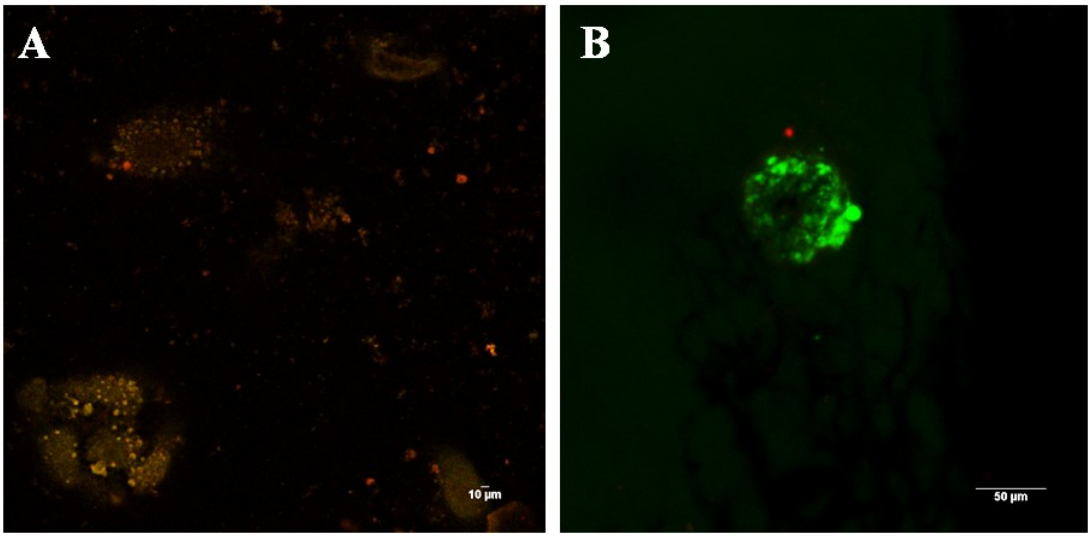

Figure 4.27 Fluorescent staining of viability with fluorescein diacetate and propidium iodide in islets A) cultured in 2D cell culture plate B) islet cultured in large lattice scaffold. Dead cells stain red and live cells stain green.

4.3.4 Islet function on the scaffold

When compared to islets on the cell culture plate, islets seeded on scaffold showed an improved increase in insulin secretion at 5mM glucose and 25mM glucose. Rat islets on large lattice scaffold showed 1.7 fold increased secretion at 5mM glucose and 1.3 fold increase in insulin secretion at 25mM glucose (Figure 4.28)

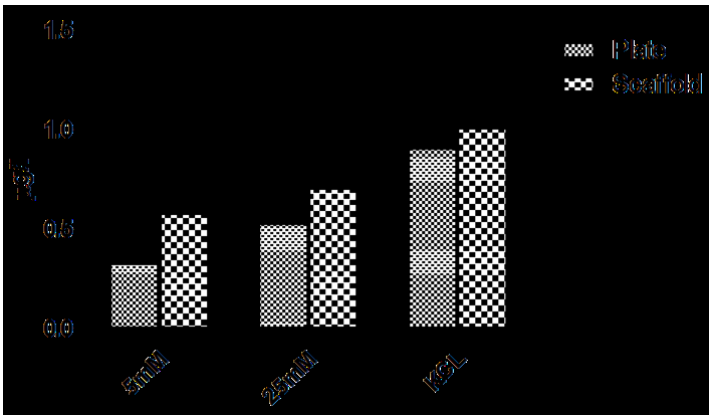

Figure 4.28 Islet cell functionality was assessed by glucose stimulation assay. Insulin cultured on large lattice scaffold showed higher insulin secretion compared to islets cultured on two-dimensional culture plates

4.4 Rat adipose-derived mesenchymal stem cells on 3D scaffolds

4.4.1 Cell adhesion on scaffolds

Rat adipose-derived mesenchymal stem cells were seeded on the small lattice, large lattice and normal electrospun scaffolds and the morphology of the cell were assessed with ESEM (Figure 4.29). The cells were attached on the surface of the fibers and were confluent after 7 days of culture. The actin cytoskeleton of the cells in scaffolds was stained with phalloidin conjugated with Alexa fluor 488(Figure 4.30A). The MSCs exhibited long parallel filaments of actin proving they maintained their spindle morphology in the scaffolds. The mesenchymal stem cells were 99% viable on these scaffolds (Figure 4.30 B).

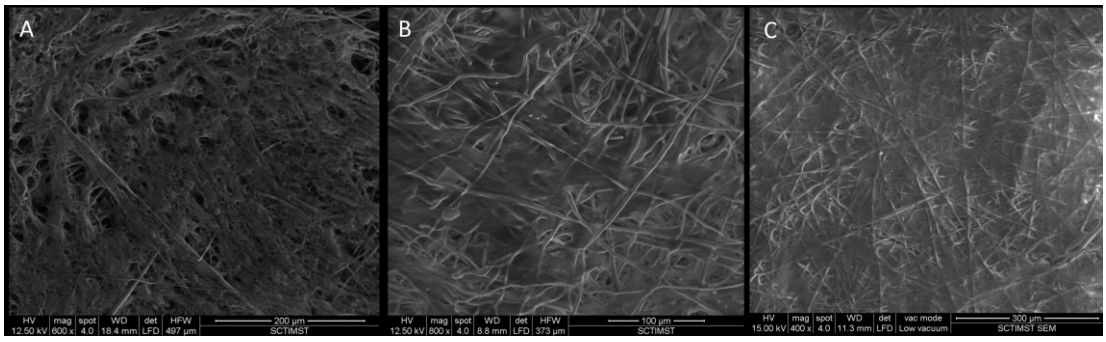

Figure 4.29: ESEM of confluent mesenchymal stem cells on A) small lattice, B) large lattice and C) normal electrospun scaffold

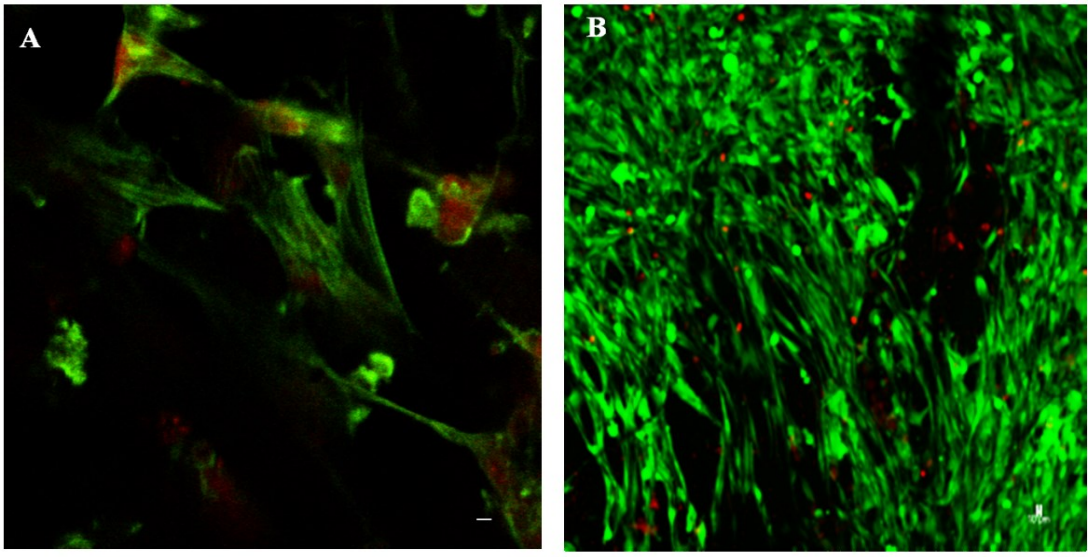

Figure 4.30: A) long parallel actin filaments of MSC s seeded on scaffold B) fluorescent live dead micrograph of MSC cultured on scaffolds. Scale bar 10μm

4.4.2 Differentiation of rat mesenchymal stem cells to islet-like clusters *in vitro*

Rat mesenchymal stem cells at a seeding density of 10^6 cells were suspended in SFM A medium and plated on tissue culture dishes. Figure 4.31A shows the cells attached on the surface of the culture plates and cellular aggregation occurred within 48 hours in culture. These aggregated cells were further incubated in SFM B medium, at day 4 cell aggregation was complete (Figure 4.31B) and clusters were

formed. These clusters were cultured in SFM C medium and by day 20 they acquired the spherical morphology (Figure 4.31C) similar to the pancreatic islets and hence termed as islet-like clusters (ILC s)

The MSC cells seeded on the small lattice, large lattice and normal electrospun scaffold were differentiated in the same pattern. ESEM micrographs show the differentiation pattern of MSCs in the small lattice (Figure 4.32), large lattice (Figure 4.33) and normal electrospun (Figure 4.34) scaffolds at 2, 4 and 20 days.

The cells are anchored to the fibers of the electrospun scaffolds. Three-Dimensional Cell aggregation and clustering were observed on day 2 and day 4 of differentiation assisted by the 3D morphology of the small lattice and large lattice scaffolds. In normal electrospun scaffold, the cells are anchored to the fibers on the surface of the scaffold and defined aggregation and 3D cluster formation were not observed. At day 20, ESEM micrographs show well defined and dense 3D spherical islet-like clusters within the small lattice and large lattice scaffold. However, in the normal electrospun scaffold, the cell aggregate lacked the spatial organization observed in the small and large lattice scaffolds and random nonspherical aggregates were formed

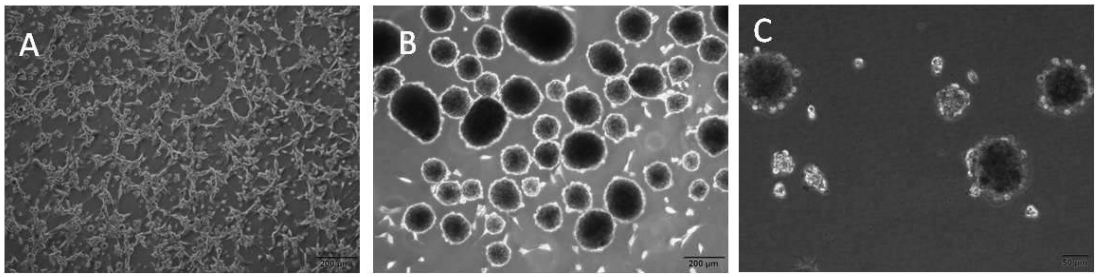

Figure 4.31: Differentiation of MSC on tissue culture treated surface on A) day 2 cell clustering begins b) Day 4 - clusters morphology is formed c) day 20- spherical morphology acquired

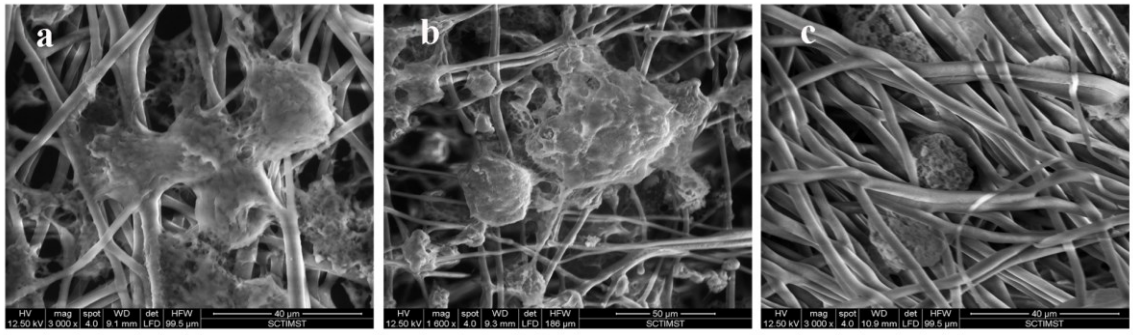

Figure 4.32: Differentiation of MSC on small lattice scaffold on A) day 2 cell aggregations is visible b) Day 4 cell aggregation and cluster formation is on progress c) at day 20 small spherical cell clusters are seen attached to the fibers on the small lattice scaffold

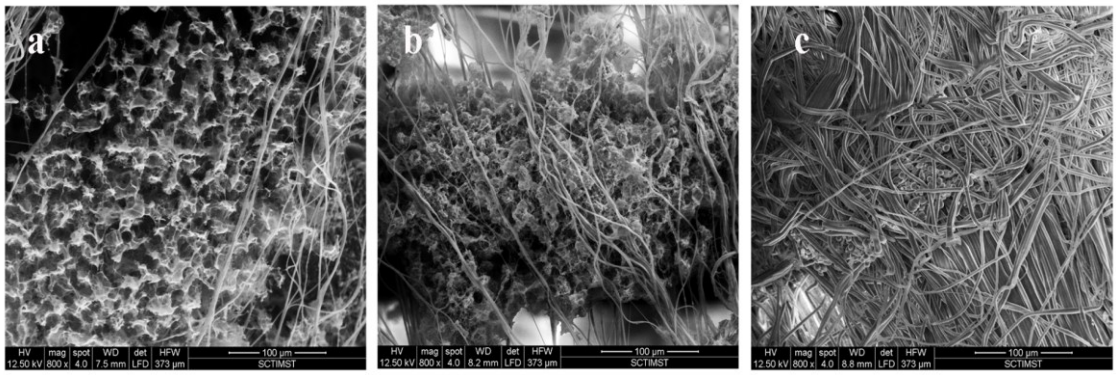

Figure 4.33: Differentiation of MSC on large lattice scaffold show visible change compared to small lattice and 2 d plates. A) On day 2 large number of cells are seen aggregating in the pores of large lattice scaffold b) On Day 4 cell aggregation is not complete and large number of cells are seen in the aggregate c) On day 20 cell aggregation and cluster formation is complete and islet clusters of medium sizes are seen attached to the fibers in the pores of the large lattice scaffold.

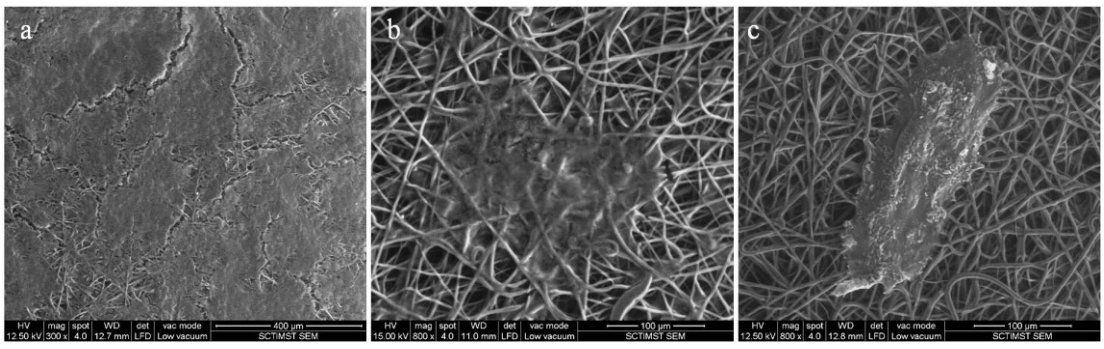

Figure 4.34: Differentiation of MSC on normal electrospun scaffold A) On day 2 the confluent MSC are seen covering the surface of the scaffold. Beginning of cell aggregation can be observed b) on Day 4 cell aggregation is progressing and the cells are spread on the surface of the scaffold c) on day 20 cell aggregation is complete forming islet like cluster irregular in morphology

4.4.3 Cytoskeletal Organisation

A change in the cytoskeletal organisation of the differentiated cells was observed from the fluorescent micrographs. The diameter of the ILCs formed (Figure 4.35) were measured from these fluorescent images using Image J software. The ILCs formed on the small lattice and large lattice scaffolds were small with an average diameter of 16.35± 3.86 µm and 51.95± 10.90µm compared to normal electrospun scaffold and tissue culture plates where the average diameter of ILCs formed were 145.87 ±18.39µm and 252.78± 27.18 µm respectively.

The fluorescent images (figure 4.36) of cytoskeleton actin of differentiated MSCs show peripheral actin condensation and change in cell morphology that is compatible with the epithelial phenotype of the islets of Langerhans. Cortical actin was also observed in cells differentiated in tissue culture-treated plates

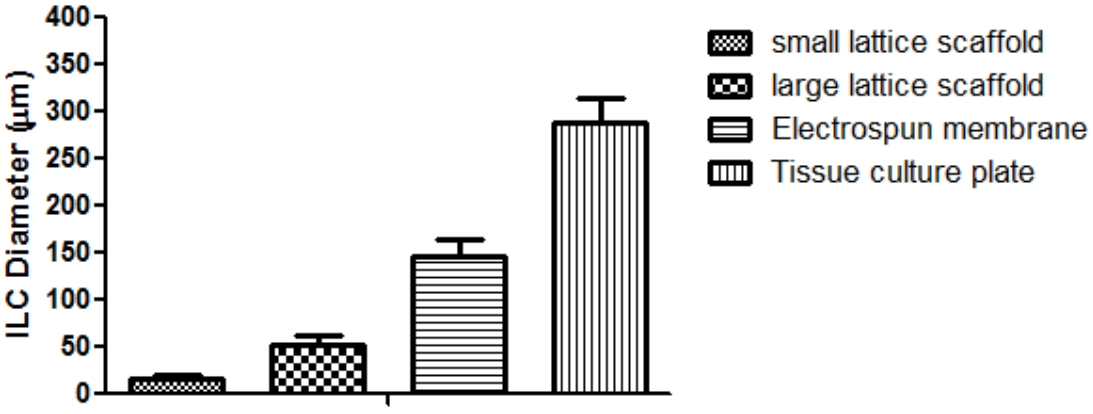

Figure 4.35: The average diameter of ILC s formed on 3D scaffolds and 2D tissue culture plates

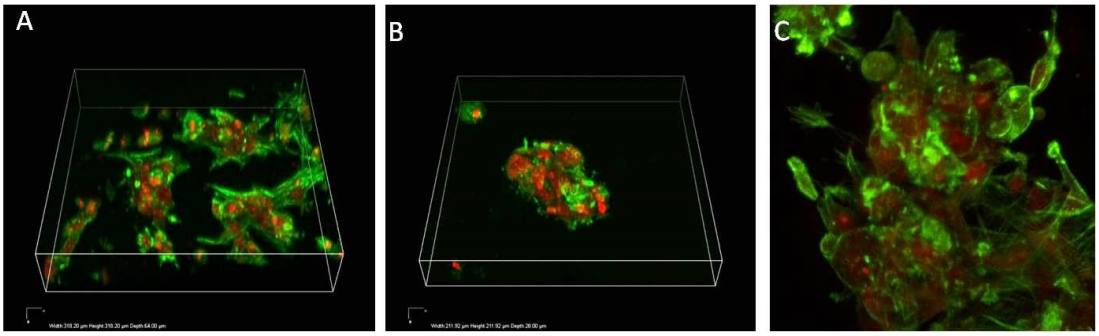

Figure 4.36: MSCs shows peripheral actin condensation and acquire epithelial phenotype after differentiation on A) small lattice B) large lattice C) normal electrospun scaffold

4.4.4 Viability

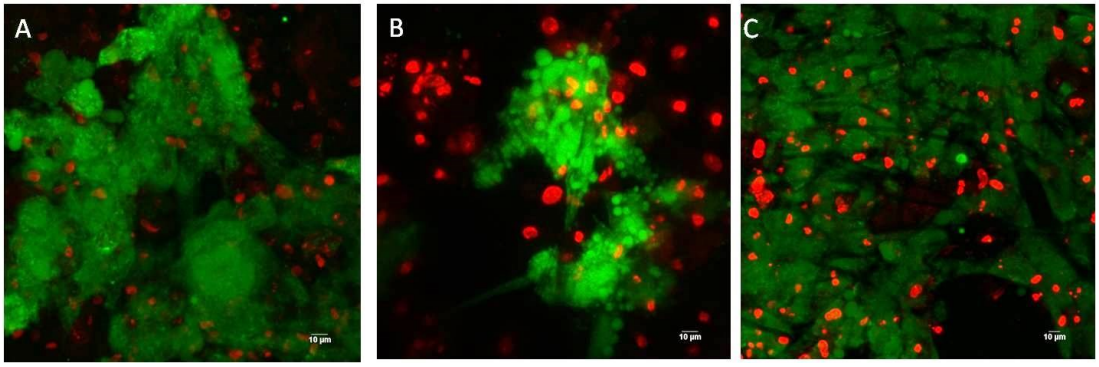

Figure 4.37: Fluorescent micrographs of viability staining of ILCs on A) small lattice B) large lattice C) normal electrospun scaffold. Fluorescein diacetate stains the live cells green and propidium iodide stains the dead cells red.

The percentage viability of ILC s formed was measured after 20 days of differentiation from fluorescent micrographs (Figure 4.37) with image J software using a procedure slightly variant from(MacGregor et al., 2006). The mean percentage viability of ILCs (Figure 4.38) on small lattice scaffolds were 94.32± 6.12% compared to 81.82 ±8.7% on large lattice scaffold and 68.95± 16.51% on

normal electrospun scaffold. The core death of the ILCs were observed more on ILCs formed on large lattice and normal electrospun scaffold compared to small lattice scaffold.

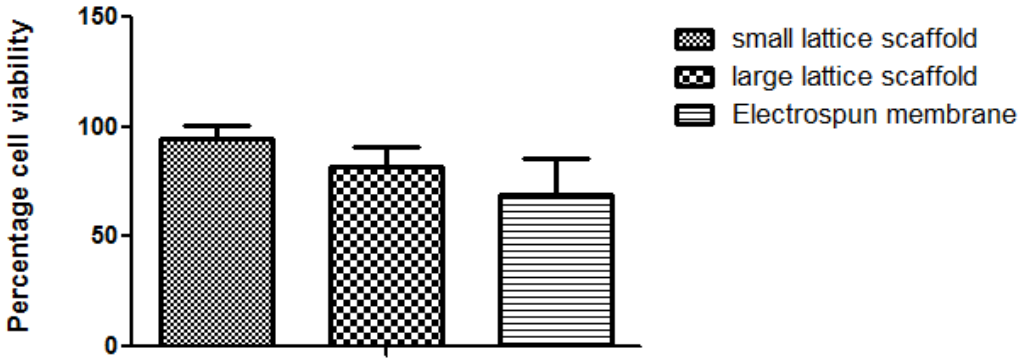

Figure 4.38: Mean percentage viability of ILCs on scaffolds

4.4.5 Hormone Expression in differentiated ILCs

After 20 days of differentiation immunofluorescence staining of differentiated ILCs on the large lattice, small lattice and normal electrospun scaffolds showed positive c-peptide expression (Figure 4.39). The c-peptide expression was seen in cells within the clusters formed in both scaffolds whereas c-peptide expression was faint and seen in a low number of cells in normal electrospun scaffolds. The ILCs formed on 2D culture plates showed positive expression of c-peptide.

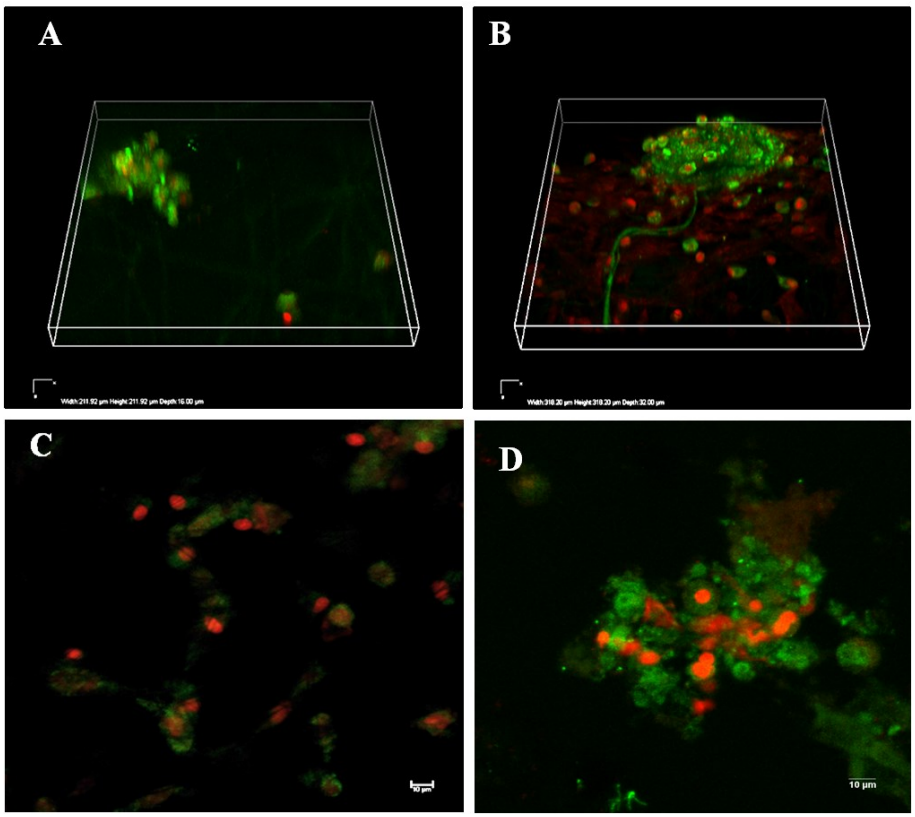

Figure 4.39: Immunofluorescence staining for C-peptide expression of ILCs on (A) small lattice scaffold (B) Large Lattice scaffold (C) Normal electrospun scaffold and 2D culture plate. The nucleus is stained red with propidium iodide.

4.4.6 Function of differentiated ILCs

The ILCs differentiated on the large lattice, small lattice, normal electrospun scaffolds and 2D culture plates were assessed for insulin secretion against glucose stimulation (Figure 4.40). The insulin release of ILCs differentiated on large lattice scaffolds was 1.4 fold higher compared to ILCs on 2D culture when stimulated with 25mM glucose. However, there was no statistical difference in insulin release among small lattice, normal electrospun and 2D culture plates. Application of 30mM KCL with 5mM glucose has improved the insulin secretion in large lattice scaffold. KCL

causes the depolarisation of membrane leading to increased calcium ions inside cell resulting in exocytosis of insulin-containing vesicles.

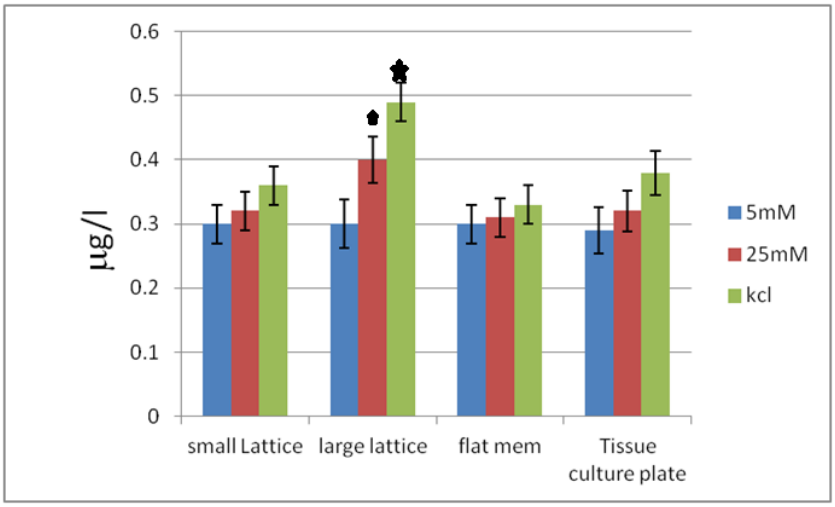

Figure 4.40: Insulin release in response to glucose stimulation. ILCs differentiated on the large lattice scaffold showed a higher release of insulin to 25mM glucose challenge compared to other groups.

4.5 *Invivo* assessment of differentiated ILCs

The physiological effect of tissue-engineered islet construct to reverse hyperglycemia in a diabetic rat model was evaluated. The islet-like clusters (ILC) differentiated from rat adipose mesenchymal stem cells ($2x10^6$ cells) on 3D nanofibrous scaffolds were encapsulated in sodium alginate as an immune response was seen in the preliminary study and implanted in an omental pouch of the diabetic rat models (n=6). Mature rat islets (1000-1200numbers) seeded on 3D nanofibrous scaffolds encapsulated in sodium alginate (n=6) and diabetic model (n=6) without implant was used as controls.

4.5.1 Blood Glucose concentration after transplantation

The scaffolds with mature islets on transplantation in the experimental diabetic rats, brought down the blood glucose concentration significantly by day 15 to levels 188.33±74.78mg/dl from 466.83±33.77mg/dl, whereas the tissue-engineered ILCs brought down the blood glucose concentration of diabetic rats by day 30 to levels 241.8± 53.518 mg/dl and maintained until the endpoint of the study (Figure 4.41). The diabetic rats without implant had remained hyperglycemic with blood glucose values ranging at 500-600mg/dl up to 30 days and died by the end of this period.

4.5.2 Response to the glucose challenge

Intraperitoneal glucose tolerance test (IPGTT) (Figure 4.42) was performed on the diabetic rats with and without transplanted constructs at 30 days post-transplantation. The blood glucose concentration decreased 30 minutes after intraperitoneal injection of glucose in groups transplanted with Islets in scaffolds and ILCs in scaffolds and reached basal level by 120minutes. The blood glucose level in diabetic rats increased to reach levels ranging from 500-600mg/dl at 120mins. The AUC (area under the curve) was significantly lower ($P<0.05$) for scaffolds transplanted with mature rat islets compared to ILCs on the scaffold. This test indicates that the differentiated ILCs on the scaffold are functional to reduce glucose concentration in blood, however, it is not as effective as mature islets seeded in scaffolds.

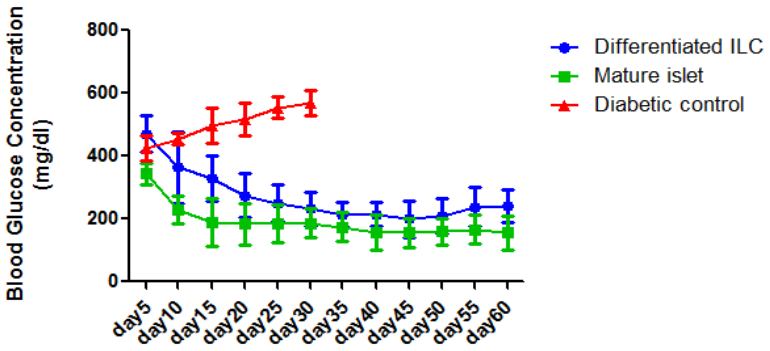

Figure 4.41: Blood glucose levels in diabetic mice transplanted with ILCs in scaffolds, mature islets in scaffold and control diabetic rats

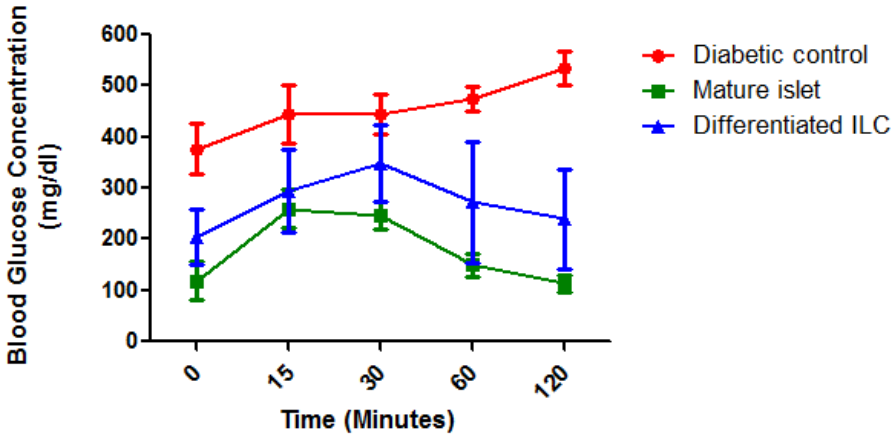

Figure 4.42: Blood Glucose measurements during the IPGT test in rats implanted with ILCs in the scaffold, mature islets in scaffolds and diabetic rat controls

To quantify the insulin secreted *in vivo*, serum was collected from the implanted groups and diabetic controls and quantified via ELISA**.** A higher level of insulin expression was observed in rats implanted with tissue-engineered constructs compared to diabetic control. The highest expression of serum insulin was observed in groups implanted with mature islets in scaffolds however less than half of this

insulin level was observed in groups implanted with differentiated ILCs on the scaffold (Figure 4.43).

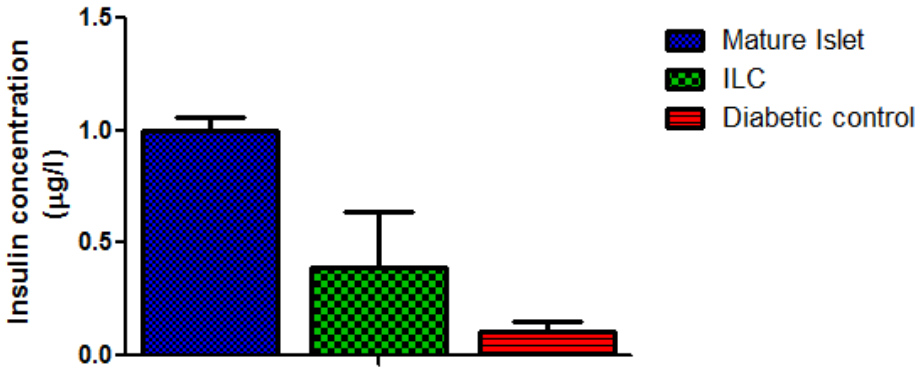

Figure 4.43: Insulin concentration in blood serum of rats. The highest expression was observed in rats implanted with mature islets in scaffolds. This correlates with the faster reduction in hyperglycemia of this group compared to that in the rats implanted with ILCs on the scaffold.

4.5.3 Architecture and viability of Implants

The viability and architecture of islets and ILCs in implants were assessed when explanted after 60 days. The viability staining with fluorescent diacetate and propidium iodide reveals that islets and ILCs were viable at the end period. Viable blood vessels were also observed in the explants (Figure4.44). H&E staining also showed that ILCs and Islets retained their spherical morphology and the presence of blood vessels (Figure 4.45). Immunohistochemical analysis showed insulin-positive cells in the retrieved implants (Figure 4.46).

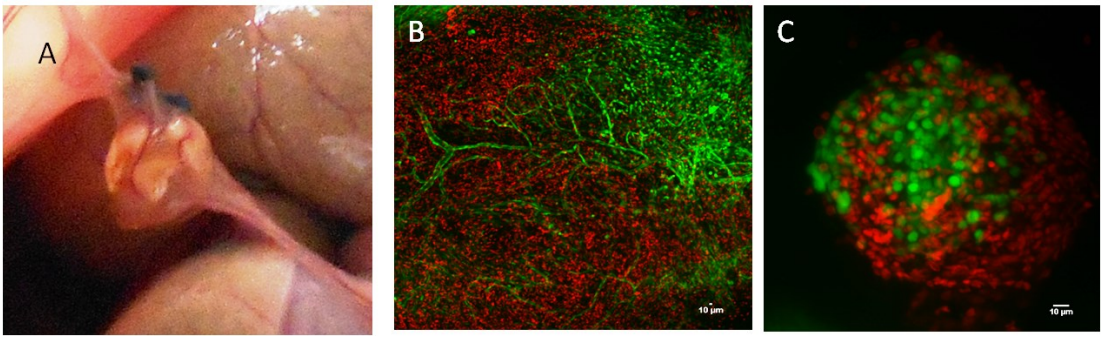

Figure 4.44 A- Photographic image of implant retrieved, B) Live dead staining of retrieved implant shows viable vascular structures in the implanted scaffolds C) shows the spherical ILCs which have 30% viable cells.

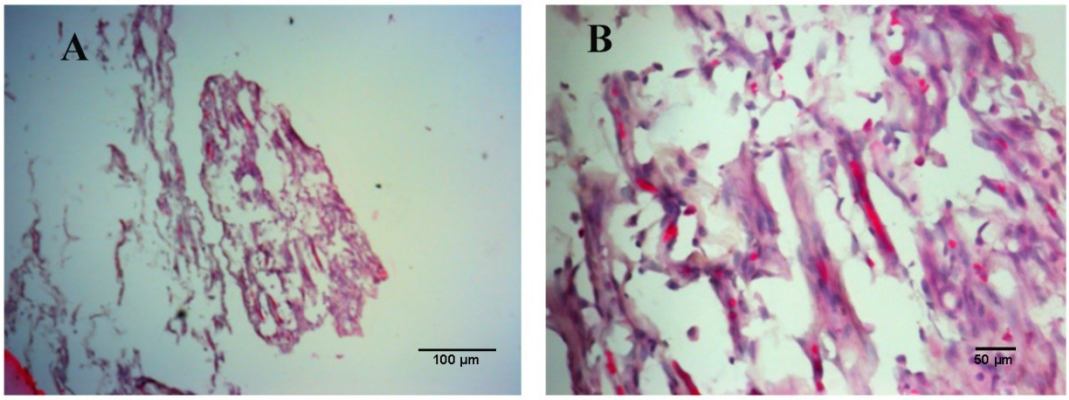

Figure 4.45: H &E staining of representative of retrieved scaffolds containing either ILC or islets (A -4X magnification, B-20X magnification) where blood vessels can be seen on the implanted scaffold

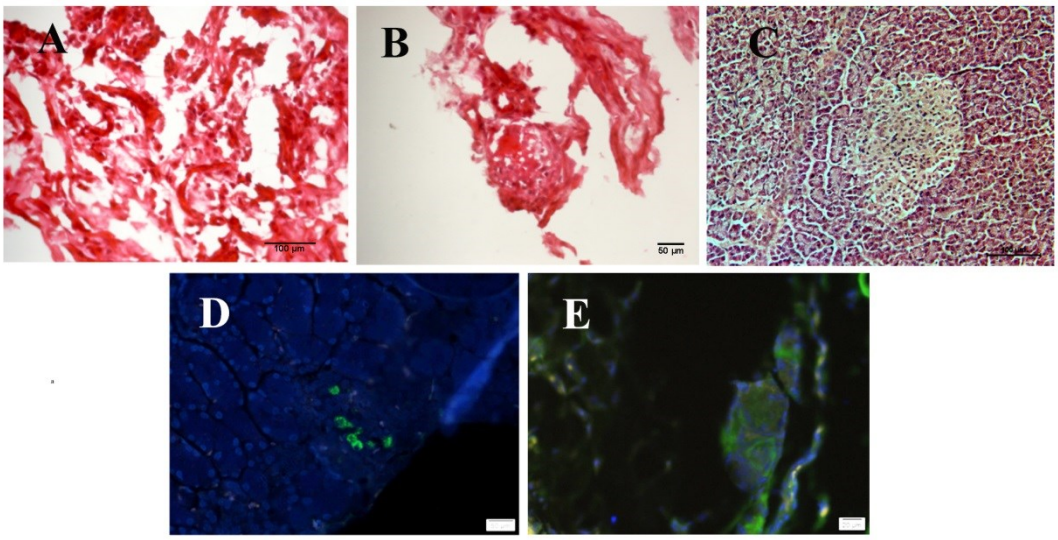

Figure 4.46: H&E staining of retrieved scaffolds (A)- ILC in the scaffold, (B)-Islet in scaffold shows the spherical morphology of islets (C): H&E staining of rat pancreas, (D) Insulin immunostaining of rat pancreas,(E) Insulin immunostaining of retrieved scaffold

4.6 Surface modification of scaffolds

The extracellular matrix, a complex network of proteins, performs important functions to maintain the integrity of a tissue. ECM is responsible for providing a structural framework to the tissue; in addition, ECM harbours growth factors and transmits chemical signals that control important cellular physiology such as differentiation, viability and proliferation. ECM interaction influences foetal islet tissue development and regulates the differentiation process of insulin-producing beta cells. In developing pancreas, collagen IV, vitronectin, fibronectin have been identified as the main ECM constituents. In order to improve insulin secretion in islet-like clusters differentiated from adipose mesenchymal stem cells, we attempted

to modify scaffold surface with ECM constituents of the foetal pancreas- collagen iv, vitronectin and fibronectin.

4.6.1 Surface Modification

The membranes were hydrolysed to introduce carboxylic acid groups. The ECM proteins collagen IV, fibronectin, vitronectin, and mixture of all three were coated on the surface of the scaffold by cross-linking with EDC and NHS. Homogenous covalent attachment of the proteins on the scaffolds was visualised using eosin staining(Figure 4.47). Eosin, an acidic dye that binds to basic components of the proteins to stain them pink, hence the stain was used to visualise protein coated on the scaffolds. The modified scaffolds showed protein attachment with higher staining intensity compared to unmodified scaffolds. Collagen IV coated scaffolds were, in addition, stained with picosirius red (figure4.48) to demonstrate the homogenous attachment of the protein.

4.6.2 FTIR Analysis

The coated scaffolds were examined using the FTIR ATR method. Figure 4.49 shows the spectra of protein-coated scaffolds compared to the uncoated PCL/PCL-PTHF-PCL scaffold. After covalent modification of the scaffold surface with proteins, –N-H Stretch at 3400cm^{-1} and N-H bend at 1625 cm^{-1} were observed indicating protein attachment.

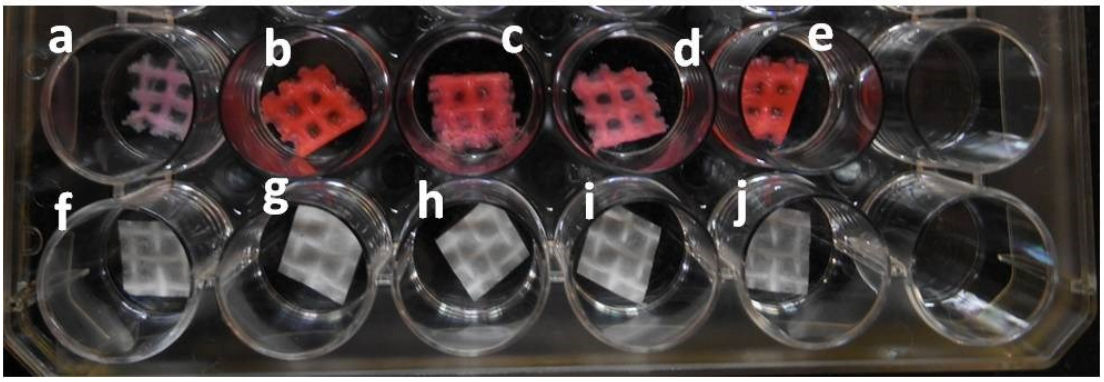

Figure 4.47: a) Uncoated b) Collagen IV C)Fibronectin d) Vitronectin e) All 3 ECM combined coated scaffold after eosin stain**,** f-j) unstained scaffold

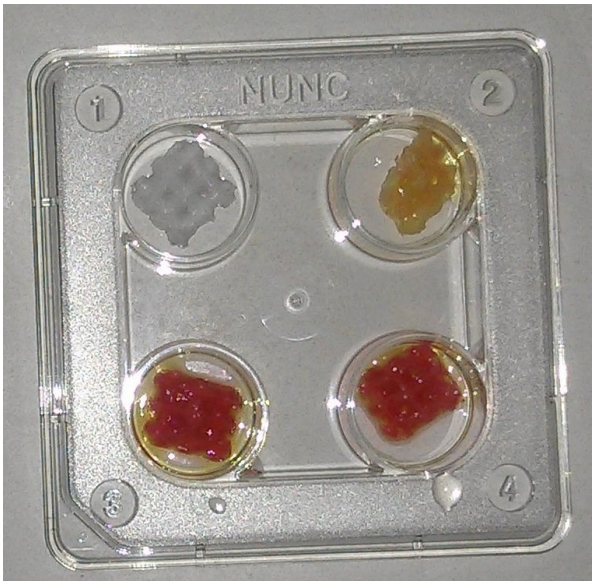

Figure 4.48 Picosirius red stains white coloured collagen-coated large lattice scaffold red. The collagen-coated scaffolds are stained red. Uncoated scaffold control is seen yellow after staining.

4.6.3 XPS Analysis

The surface composition of the scaffolds was examined with XPS. The atomic composition of the scaffolds showed the addition of nitrogen in the modified scaffold compared to the unmodified scaffold. The nitrogen peak was observed at 400ev (Figure 4.50) and a 12.4 atomic % nitrogen content was observed on the coated scaffold compared to the unmodified scaffolds.

4.6.4 Viability

The viability of mesenchymal stem cells on the scaffolds was verified using fluorescent live dead staining (Figure4.51). Fluorescein diacetate is converted to fluorescein, a green fluorescent compound by live cells whereas propidium iodide can only penetrate a membrane compromised cells to bind to its nucleus and fluoresce red indicating cell death. The cells were grown on modified and unmodified scaffolds for 7 days and imaged by confocal microscopy. The cells showed characteristic morphology and good adherence and growth on all scaffolds. The cells were viable on PCL/PCL-PTHF-PCL, collagen IV, fibronectin, vitronectin and all three ECM proteins combined coated scaffolds

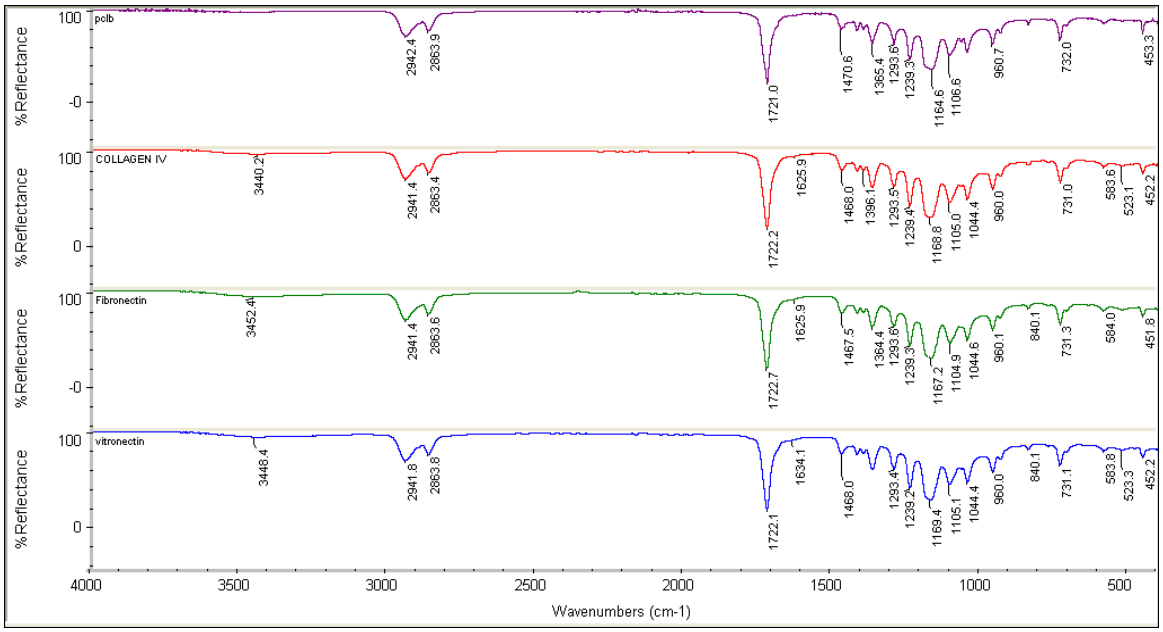

Figure 4.49 FTIR spectra of the large lattice scaffold after surface modification with ECM proteins. A small peak for –N-H Stretch at 3400cm^{-1} and N-H bend at 1625 cm^{-1} were observed

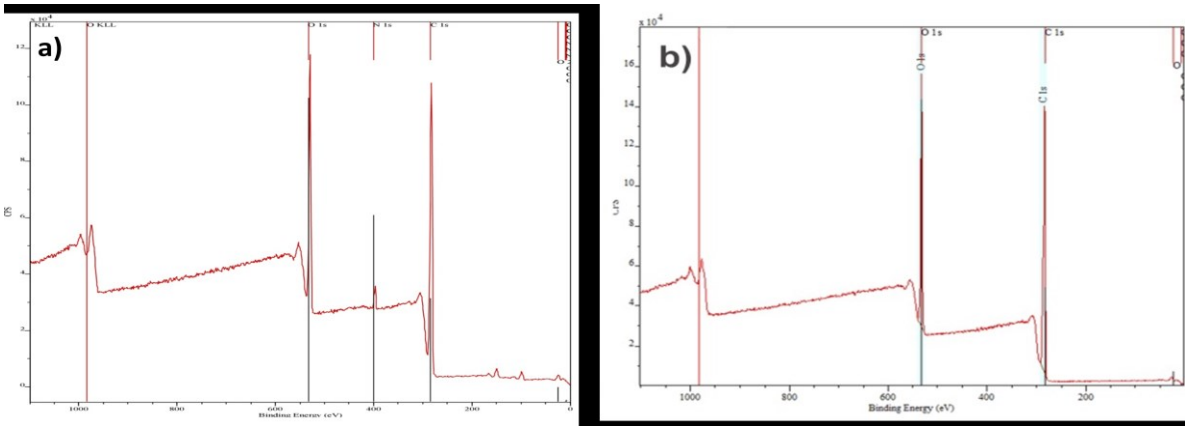

Figure 4.50 XPS of a) Coated scaffold showing a peak at 400ev for nitrogen b) uncoated PCL/PCL-PTHF-PCL electrospun scaffolds

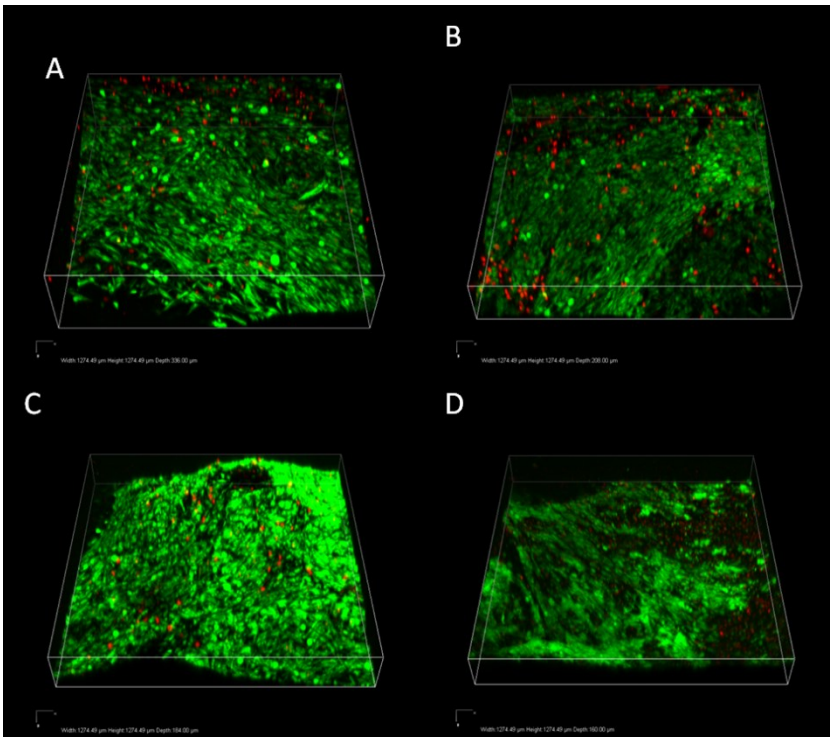

Figure 4.51 viable mesenchymal stem cells growing to confluency on A) Collagen IV B)Fibronectin C) Vitronectin D) All 3 ECM combined coated scaffold. Live cells are stains green and dead cells are stained red.

4.6.5 Differentiation and immunostaining

The mesenchymal stem cells were seeded on modified and unmodified scaffolds at the concentration of $1x10^6$ cells and differentiated according to the three-stage protocol. The ILC s formed on the scaffolds were stained for the expression of c-pep and visualised with confocal microscopy. Figure 4.52 represents strong positive c pep expression of ILCs on collagen IV, vitronectin, fibronectin-coated and all three proteins combined on scaffolds and the ILCs exhibited the 3D cluster morphology of the islets. The presence of insulin by-product c-pep indicates that the differentiated cells synthesises and release insulin.

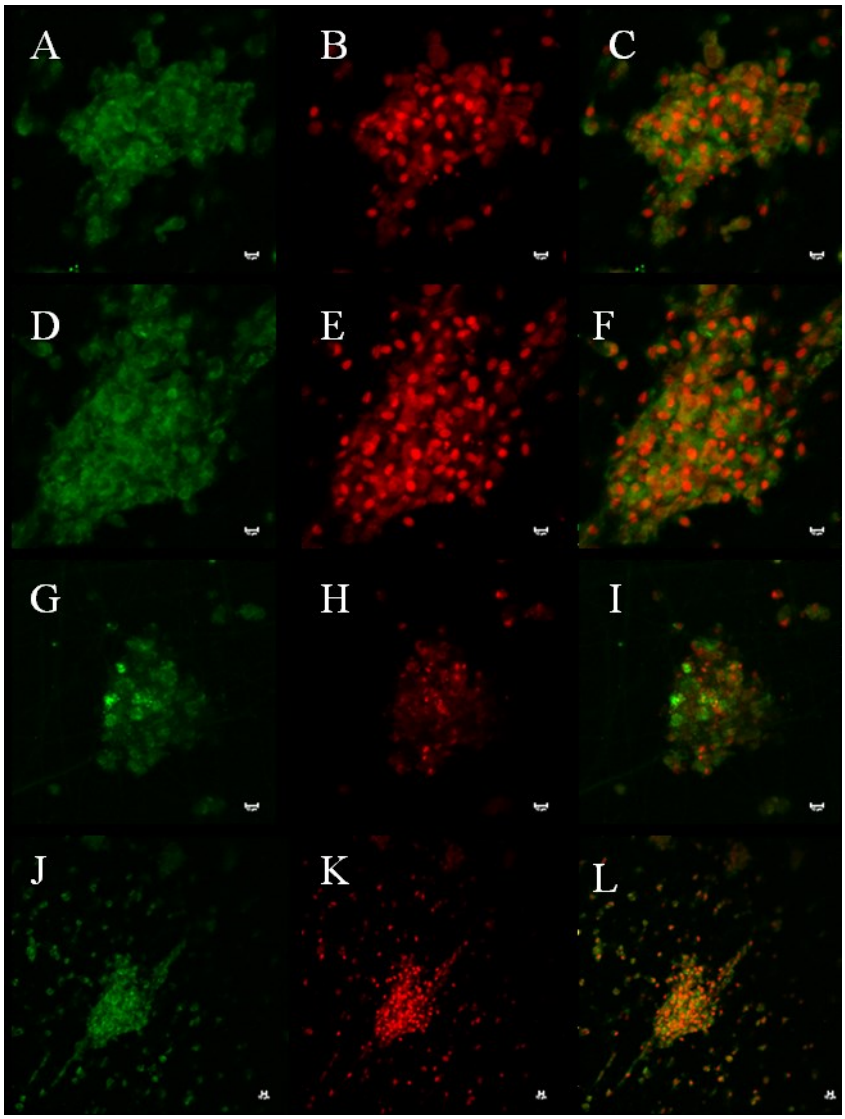

Figure 4.52: C-pep staining (Green Cpep, Red: nucleus) of ILC on (A-C) collagen IV coated scaffolds, (D-F) Vitronectin coated scaffold, (G-I) Fibronectin coated scaffold and (J-L) All three protein-coated scaffold.

4.6.6 Glucose Challenge Assay

The glucose challenge assay showed that ILCs differentiated on modified scaffolds had a higher release of insulin when challenged with 25mM glucose. The ILCs on scaffolds modified with all three ECM proteins showed a higher ($P \leq 0.05$)

insulin release at 25mM glucose challenge compared to the scaffolds modified by fibronectin and vitronectin proteins (Figure 4.53). Vitronectin modified scaffolds showed lesser insulin release compared to the other groups. KCL depolarisation did not show significant difference fibronectin, collagen IV and all three combined groups however release after depolarisation for the groups were better compared to the vitronectin group.

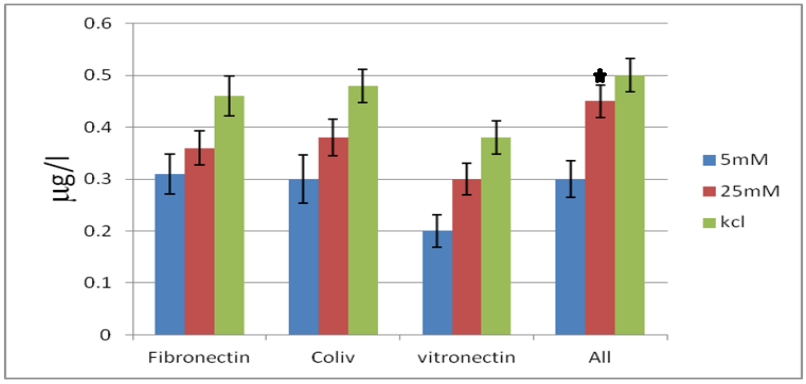

Figure 4.53: Insulin release profile- significantly higher ($P \leq 0.05$) at 25mM for large lattice scaffold coated with all 3 ECM molecules

Our result confirms that ECM molecules have a combinatorial effect on the cell processes and hence it is ideal to have the ECM together at the optimum concentration for a physiologically relevant outcome.

CHAPTER 5

DISCUSSION

The results of the study detailed in chapter 4 are discussed in this chapter. The results are correlated with the published literature.

5.1 Isolation and Characterisation of Cells

5.1.1 Islets of Langerhans

Mature rat islets were isolated through enzymatic digestion of the pancreas after excising the tissue from euthanised rats and cut to small pieces to increase the surface area for collagenase digestion of the tissue. Enzymatic digestion is influenced by various factors such as temperature, time, enzyme concentration and changes in these factors that influence the yield and viability of the isolated islets(Carter et al., 2009). Our laboratory standardised protocol(Aloysious and Nair, 2014) was strictly followed to have consistency in the quality of isolated islets. The isolated islets were spherical in the morphology of various sizes (50-250μm) and the average islet yields were 500±150 islets per tissue. This was consistent with the literature(de Groot et al., 2004). Studies have estimated the total number of islets per rat pancreas as 3000-5000 and isolation yield ranges from 10-30% of the total number of islets in a pancreas(de Groot et al., 2004). The isolated islets were tested positive for purity by dithizone staining. Dithiazone, a zinc chelating agent, binds with the intracellular zinc in the secretory vesicles of beta cells thus staining them red(Yuan, 2011). The islets were positive for insulin, glucagon and somatostatin expression in immunofluorescence staining. The isolated islets were 95% viable upon staining with

fluorescein diacetate and propidium iodide. Hence we could confirm that the isolated islets were viable and functional.

5.1.2 Mesenchymal stem cells

In accordance with the criteria proposed by The Society for Cellular Therapy (ICST) to define MSC (Dominici et al., 2006), mesenchymal stem cells isolated from rat adipose tissue were studied for its plastic adherence, positive and negative expression of surface markers and multipotent differentiation capacity. MSCs do not express a unique surface marker specific for itself similar to CD34 in hematopoeitic stem cells(Sethe et al., 2006), hence several markers that are positive (CD105 (SH2), CD73 (SH3/4), CD44, CD90) on MSC and antigenic negativity of hematopoietic marker CD45 are relied on to identify MSC(Chamberlain et al., 2007). The isolated cells displayed good plastic adherence after day 1 as evident from the actin and vimentin staining of the adherent cells. In the flow cytometry and immunostaining experiments, our cells expressed 77.5%, 78.2% positivity for CD105, CD44 respectively and negligible percentage (0.2%) of cells expressed CD34/45. Previous studies have reported a similar percentage of expression for hematopoietic markers CD34/45 which is consistent with our results however a lower percentage of 17.15% expression of CD44 was reported(Bayati et al., 2013). CD44 helps MSCs in adhering to matrix proteins such as fibronectin, hyaluronan and migrating towards wound sites for tissue regeneration(Zhu et al., 2006). Trilineage (chondrogenic, adipogenic and osteogenic) differentiation of the cells along with flow cytometry data was used to confirm the isolated cells as mesenchymal stem cells.

5.2 Fabrication of scaffold

Scaffolds form the three-dimensional framework in place of the extracellular membrane for developing tissue construct. Polymeric materials, natural and synthetic, have been explored to mimic the ECM in tissue engineering. Scaffolds are specifically defined to have high porosity, interconnected pores, high biocompatibility, and suitable surface chemistry for cell function, controlled degradation and appropriate mechanical properties. Polycaprolactone (PCL) has many advantages in its application as a scaffold in tissue engineering. It is an FDA approved synthetic, aliphatic linear polyester, a biodegradable polymer that has good compatibility with different polymeric components, several methods of fabrication of scaffold and is non-toxic to cells (Woodruff and Hutmacher, 2010). One limiting factor in its use as the scaffold is the inherent hydrophobicity. The hydrophobic nature of this polymer has led researchers to use surface modification methods to improve its wettability(Martins et al., 2009)(Liu et al., 2012). We adopted an easy method of blending a hydrophilic triblock copolymer, PCL-PTHF-PCL, with PCL and co-electrospinning to drastically reduce its hydrophobicity(Vaikkath et al., 2016). Electrospun scaffolds can be good for islet tissue engineering since they exhibit valuable features of extracellular matrix as nanofibrous architecture, high surface area to volume ratio, high cellular adhesion and mechanical support(Lee et al., 2013) The co-electrospinning of the two polymers has brought down the hydrophobicity of the resultant scaffold to 59.3˚ from 129.1º of PCL, as measured by static contact angle method with glycerol. The presence of terminal PCL chains in the triblock polymer allows good miscibility of both polymers in the blend solution.

However, the DSC thermogram of the blend scaffold shows two distinct melting peaks, indicating the absence of any covalent linkages between the PCL and the graft copolymer. Phase segregation of the low molecular weight component has been reported for PLA/PDMAEMAand PLA-b-PEG-b-PLA triblock copolymer(Kim et al., 2003)(Viswanathan et al., 2015). Some level of segregation of the low molecular weight PCL-PTHF-PCL to the surface in the electrospun form may be expected and be a reason for the enhancement of hydrophilicity(Vaikkath et al., 2016)

PCL is a recognised non-cytotoxic material; nevertheless, cytocompatibility tests were performed to determine the compatibility of the co- electrospun scaffold. Direct contact test, fluorescence staining for viability and test on extract showed no change in morphology of the cells with 98% viability confirming cytocompatibility of the co-electrospun scaffold.

The limiting factor in the use of the electrospinning method for scaffold fabrication is the difficulty in developing three-dimensional structure and high pore size in the range of 50-200 micrometers as required in islet tissue engineering. Pore size and porosity of scaffolds play an important role in tissue engineering as it influences cellular processes such as cell infiltration, adhesion, migration, morphology, and phenotype expression(Rnjak-Kovacina and Weiss, 2011). In the case of islet tissue engineering, cell aggregation and 3D cluster formation are crucial in maintaining its viability and function. Various methods such as salt leaching(Lee et al., 2005), sacrificial fibers(Baker et al., 2012), cryogenic electrospinning(Simonet et al., 2007), wet electrospinning(Yokoyama et al., 2009), direct-write electrospinning(Lee et al., 2012) has been studied to increase

pore size in electrospun scaffolds. The increase in pore sizes achieved by these methods ranged from 10μm to 500μm, however, no single method was successful to increase pore size with interconnected pores and result in changes in electrospun morphology, delamination of the scaffold, heterogenous pores resulting in poor cell infiltration(Rnjak-Kovacina and Weiss, 2011). Our two-step method of fabrication could successfully develop three-dimensional scaffolds up to 2 mm thickness with 55.5% and 45% of total pores greater than 100μm in the large lattice and small lattice scaffolds respectively.

This porosity was achieved in the scaffolds by using patterned collectors. The collectors were wire meshes with void spaces of two different radiuses. Li et.al has shown that the presence of the void space causes the electrostatic field to orient towards the opposite sides of the collector resulting in stretching and alignment of the electrospun fibers(Li et al., 2003). The fibers arranged itself on the metallic wires and loose random fibers were seen in the void spaces. Li et. al has suggested that coulombic interactions are responsible for the arrangement of fibers on the metallic part and it would further enhance the arrangement of fibers (Li et al., 2003). However, upon continuous electrospinning, the pattern formation was gradually lost and fibers get randomly arranged and lose the porosity. This observation was consistent with studies by Vaquette et.al (Vaquette and Cooper-White, 2011). Hence, the time of electrospinning was crucial in getting the desired pore size in the final three-dimensional scaffolds. This was optimised as 5 minutes and 15 minutes for each small lattice and large lattice membranes respectively. These membranes were then layered to form the 3D construct of 2mm thickness with porosity greater than 100 micrometers.

The large lattice, small lattice scaffolds were highly hydrophilic and absorbed water as indicated in the swelling studies. The swelling ratio of the small lattice scaffold was higher compared to the large lattice and normal electrospun scaffold as the numbers of fibers were higher in these scaffolds. The scaffolds achieved saturation within 5 minutes in PBS; this fast water absorption capability is attributed to the presence of hydrophilic PCL-PTHF-PCL in the scaffolds and concurrent wicking action of the nanofibers. The high swelling of the scaffolds is advantageous for fast oxygen and nutrient exchange for the cells(Aloysious and Nair, 2014).

Another important aspect of tissue engineering scaffold governing cell fate is its mechanical properties. The large lattice scaffold had the lowest modulus (3.0±0.4 MPa) compared to the small lattice (4.4±0.6 MPa) and normal electrospun scaffolds (12.2±3.5 MPa). As the void space of the patterned collector increases, the modulus of the resulting scaffold decreases. The tensile strength of the patterned porous scaffolds compared to the normal electrospun scaffold followed the same trend. A similar observation was reported by Vaquette et al and Neves et al (Vaquette and Cooper-White, 2011),(Neves et al., 2007). The porosity and fiber alignment have contributed to the reduction in modulus and tensile strength of the scaffold. Therefore, the mechanical properties of the scaffold could be tailored by our method of scaffold fabrication and a soft porous scaffold befitting for application in soft tissues such as the pancreas could be developed.

Colonisation of the scaffold by the cells of interest is important for tissue engineering strategy. In normal electrospun scaffolds, the cells grew to confluence on the surface of the scaffold. Strategies such as perfusion over static seeding were

studied to increase the depth of cell infiltration and colonisation in the electrospun scaffold(Pham et al., 2006). However, the increase in the thickness of the nanofiber layer resisted the cell infiltration of the scaffold. Kim et al used salt developed techniques to develop macropores, nevertheless, the small interconnected pathways between the macropores prevented cell infiltration into the depths of the scaffold (Kim et al., 2008). Hence large interconnected pores are necessary for cell movement and colonisation of the scaffold. Cell penetration up to 641.54±62.08μm in large lattice scaffold and 367.81 ±86.80μm in small lattice scaffold compared to 53.548±8.35μm in normal electrospun scaffold could be achieved after seeding on one side of the scaffold. Cell infiltration up to 100μm and 200-250μm has been reported earlier for porous electrospun scaffolds(Zhu et al., 2008) (Vaquette and Cooper-White, 2011).

5.3 Islets on the scaffold

The culture of islets on scaffold has been shown to improve their viability and function compared to two-dimensional culture(Aloysious and Nair, 2014)(Muthyala et al., 2011). In the current study, the efficiency of three-dimensional electrospun scaffolds for the *in vitro* culture of rat islets were evaluated. The isolated rat islets were seeded on the small lattice, large lattice and normal electrospun scaffolds and cultured for 7 days. ESEM analysis showed that the large lattice scaffold provided the favourable pore size and structure among the three selected scaffolds that can support mature rat islets to lodge and grow in culture. This shows the importance of pore dimensions of a three-dimensional scaffold in the culture of islets *in vitro* and maintaining the physiological architecture of islets(Aloysious and Nair, 2014). The

order of pore size of the small lattice and normal electrospun scaffold were small to support mature rat islets, hence further studies on viability, insulin release function of the islets were carried out using large lattice scaffold and compared to the islets on 2D culture. The viability of islets in culture plates was reduced compared to the large lattice scaffold consistent with the literature(Chun et al., 2008). Insulin release in response to high glucose stimulation was higher in large lattice group compared to two-dimensional cultures as the scaffold assist in maintaining the viability of the islets. The results are consistent with literature as it has been proved that three-dimensional scaffolds improve the function and viability of the islets in culture and *in vivo*(Aloysious and Nair, 2014)(Kin et al., 2008).

5.4 MSC differentiation on Scaffold

The beta cells of islets of Langerhans are glucose 'thermostats', efficiently sensing a rise in blood glucose level and releasing insulin to the bloodstream to strictly maintain physiologic glucose levels within a narrow range(Bluestone et al., 2010). Loss of function of beta cells results in uncontrolled glucose levels leading to diabetes-related acute conditions as ketoacidosis, severe hypoglycaemia and secondary complications including heart disease, blindness and kidney failure(*JAMA*, 2003)(Kaveeshwar and Cornwall, 2014). Insulin therapy is the core treatment for patients with diabetes, nonetheless, acute complications and progression to secondary complications are unavoidable.

Transplantation of cadaveric donor islets with immunosuppression or immunoisolation strategies is considered a logical cell replacement therapy for diabetic patients chiefly for type I diabetes. However, the use of this strategy has

been limited by the requirement of more than one donor and the shortage of available islets for transplantation. Replacement of beta cells from stem cells is considered ideal to overcome this obstacle (*Regenerative Medicine*, 2012). Adult stem cells are preferred alternative cell source for beta cell replacement considering the ethical and legal issues involved with the use of embryonic stem cells. Adult Hematopoietic stem cells(Couri and Voltarelli, 2008), pancreatic progenitor cells (Xu et al., 2008)(Muthyala et al., 2011), mesenchymal stem cells from bone marrow(Figliuzzi et al., 2009)(Phadnis et al., 2011) and induced pluripotent stem cells (Alipio et al., 2010) have been evaluated for cell transplantation. The invasive procedures required for isolation of these stem cells and the scarcity of their sources have restricted their clinical advancement. On the contrary, adipose tissue has the advantage of being easily accessible, simple isolation of tissue, stem cells and its high expandability with minimum patient discomfort(Schäffler and Büchler, 2007).

Our earlier reports have confirmed the effectiveness of differentiating adipose-derived mesenchymal stem cells to islet-like cells (Aloysious and Nair, 2013)(Chandra et al., 2009)(Chandra et al., 2011). In our current study, we explored the effect of scaffold architecture and porosity on the differentiation process and formation of ILCs. The adipose-derived mesenchymal stem cells were seeded and differentiated on the large lattice, small lattice and normal electrospun scaffolds following our established protocol and the results were compared with 2D cultures on tissue culture plates. The differentiation protocol follows a three-step strategy to convert mesodermic adipose-derived mesenchymal stem cells into pancreatic ILCs of endodermic lineage in a defined serum-free medium. In the differentiation protocol, as shown in the flow chart given in chapter 3, figure 3.1 in which the first step

involves the use of beta-mercaptoethanol and activin A in Serum-Free Medium A (SFM A), gradual cellular aggregation begins in 24 hours. Beta mercaptoethanol and activin induce cells into endodermal lineage by the upregulation of HNF-3 beta, TCF-2 and Sox-17 transcription factors(Chandra et al., 2011). After the initial two days, the cells were grown in SFM B supplemented with fibroblast growth factor and epidermal growth factor, glutamine and amino acids to proceed differentiation to pancreatic endoderm by upregulation pancreatic endoderm specific transcription factors such as PDX-1, Ngn3, NeuroD. FGF induces PDX^{+} cells in the definitive endoderm cells formed via ERK/MAPK and AKT activation and promotes its proliferation along with EGF by activating the Notch pathway(Mfopou et al., 2010). These cells are then exposed to nicotinamide, activin and betacellulin at the third stage of differentiation. Activin A induces differentiation to hormone-producing pancreatic endoderm cells and betacellulin acts as a mitogen and their combined activity support maturation and hormone synthesis by the differentiated cells(Demeterco et al., 2000). Nicotinamide helps preserve the viability and function of the differentiated cells via poly (ADP-ribose) polymerase (PARP) and improved glucose sensitivity(Chen et al., 2004)(Otonkoski et al., 1993). The islet-like clusters derived from mesenchymal stem cells at the end of differentiation resembled the cluster morphology of mature islets and was positive for c-peptide staining consistent with the literature(Aloysious and Nair, 2013)(Phadnis et al., 2011)(Chandra et al., 2009).

The mesenchymal stem cells seeded on the 3D porous large lattice, small lattice and normal electrospun scaffolds followed the same pattern on differentiation as in 2D cell culture plates. Initial gradual 3D cell clustering was more prominent on

the large lattice, small lattice scaffolds compared to the normal electrospun scaffold. The cells clustered to form 3D spherical islet-like clusters of defined diameters at the end of the differentiation process on the small lattice and large lattice scaffolds. However, the ILCs formed on normal electrospun scaffolds were irregular clumps attached on the surface of the scaffold and lacked 3D spherical geometry. On 2D culture plates, cells became less adherent to tissue culture surface after initial cell clustering and detached to form random cell clumps after 48 hours in culture, however, in the 3D scaffolds multilayered spherical islet-like clusters were formed by cells well anchored to the fibres in the scaffolds. Three-dimensional cell positioning and clustering without loss of cells during medium change is due to the efficient anchorage provided by the fibres of the electrospun scaffolds and the interconnected pathways between the fibres help in the cell movement(Gallego-Perez et al., 2010). Cell interactions with ECM or synthetic substrates have been shown to regulate survival, insulin secretion and preservation of spherical morphology (Aloysious and Nair, 2014)(Stendahl et al., 2009b)(Muthyala et al., 2011). Fluorescent micrographs of the clustering cells on scaffolds showed peripheral actin condensation characteristic of islet endocrine cells which has epithelial-like phenotype(Hardikar et al., 2003). Actin condensation to the cell periphery supports cadherin based cell-cell adhesion in 3D(Gallego-Perez et al., 2012). The morphology of the islets influences its secretory behaviour and viability. Maintaining the spherical morphology is crucial for higher hormone secretion capacity of the islets(Lucas-Clerc et al., 1993). It is reported that in the 3D cluster of islets cell-cell adhesion and communication are facilitated through a high expression of E-cadherins and surface adhesion proteins (Guo-Parke et al., 2012). Cell-Cell adhesion and

communication are crucial for the function of heterogeneous tissue as islets of Langerhans.

It is reported that the size and homogeneity of islet clusters influence its viability, function and hence the degree of success after transplantation. Size of the islets influence transplantation outcome mainly due to the hypoxic condition in the portal vein, small islets were shown to be more superior in survival rate in the hypoxic and normoxic state(Lehmann et al., 2007)(MacGregor et al., 2006). In our study, we could show that the diameter and size of differentiated islet-like clusters are determined by the pore size of the scaffolds. The islet-like cells formed in scaffolds had restricted diameter with ILCs on small lattice scaffolds being the smallest in comparison to clusters on the normal electrospun scaffold and 2D culture. The ILCs formed on large lattice scaffolds were 51.95 ± 10.90 μm in diameter, the size recommended by (50–100 μm) Lehmann et.al as the optimal size for islets survival in hypoxic conditions(Lehmann et al., 2007). The ILCs formed on normal electrospun scaffolds varied in size, shape and spheroid morphology. The ILCs formed on tissue culture plates were large in the size range of 282.78 ± 27.18 μm. They were free-floating without attachment to the matrix and tended to fuse in medium to form a large irregular mass of cells. This observation was consistent with earlier studies(Gallego-Perez et al., 2012).

The size of the islets also determines its viability and secretory function with small islets having greater viability percentage than larger islets. The ILCs formed on small lattice scaffolds were 94.32±6.12 % viable compared to large lattice scaffolds (81.82±8.79), normal electrospun (68.95±16.51) and 2D culture. This is consistent

with the earlier studies as isolated islets and ILCs differentiated from stem cells lack microvasculature hence the oxygen and nutrient supply to the core of the cluster depends on diffusion(Giuliani et al., 2005). In this scenario, the radius of the islet cluster (diffusion distance) determines the partial oxygen tension towards the center of the cluster. Higher the cluster size lower the oxygen tension and nutrient supply into the core of the islets thus resulting in necrosis and cell death(Komatsu et al., 2017). Hypoxic conditions, in addition, results in decreased insulin secretion. When pO2 levels fall below 7 mmHg, insulin secretion decreases, hence, low oxygen tension and low concentration of glucose available to the core of the islet cluster adversely affect its insulin secretory function(Garcia-Contreras et al., 2017)(Papas et al., 1996)(Muthyala et al., 2017). Consistent with the literature, insulin release was lower for ILCs in normal electrospun scaffold and 2D culture compared to ILCs on the large lattice scaffold. Reduced insulin secretion in the small lattice scaffold could be due to a low number of beta cells formed as the size of the clumps were a few cells thick (16.35 ± 3.86 μm).

5.5 In vivo transplantation of tissue-engineered islet construct

The transplantation of islets to diabetic patients offers a long term therapy, however, the requirement of two or more cadaveric donors and intensive immunosuppressive drug therapy have limited the option of islet transplantation to patients with brittle diabetes and have considerable complications. The Edmonton protocol used for intravascular (portal vein) islet transplantation had initial success because of the use of lesser toxic immunosuppressants and complete avoidance of corticosteroids, however, islet graft gradually lost function over few years post-

transplantation as hepatic intravascular site caused blood mediated inflammatory reaction(Ryan et al., 2005). Biomaterial scaffolds and immunoisolation materials have the advantage to improve islet viability and function by providing mechanical support, protection from immune reaction and maintain the islet implant at the site of transplantation(Wang et al., 2017). Immunoisolation strategy can enable allogenic/ xenogenic islet source for transplantation, provides protection from autoimmunity in diabetic I patients and avoid the use of toxic effects of Immunosuppressants (Nair and Aloysious, 2011). Studies with microencapsulation and macro encapsulation strategies have shown positive outcomes; however encapsulation results in central necrosis of the islets in the long term(Chandra et al., 2011)(Vos et al., 1999). Muthyala et al have shown that the combined use of imunoisolation membranes and scaffold had a positive hypoglycaemic effect for 90 days in vivo and demonstrated viable cells upon retrieval of the implant(Muthyala et al., 2011). In our preliminary studies, the immune reaction and poor hypoglycaemic effect were observed. Hence, we adopted a proven immunoisolation strategy of encapsulation of tissue-engineered construct in alginate gels considering the space limitation in the omental pouch of the diabetic rat model, for our *in vivo* studies(Schneider et al., 2005). Alginate gel encapsulation has the advantage of large surface area to volume ratio enables nutrient and oxygen exchange

The site of implantation is considered favourable if it provides insulin drainage to the portal vein, allows rapid revascularization of transplanted islets and prevents islet loss(Berman et al., 2009a). The omentum is rich with arteries and lymph vessels and allows vascularisation of the implant(Berman et al., 2009b), in

addition, the transplanted islets are protected from high blood pressure which is a limitation in portal vein transplantation model(Berman et al., 2009b)(Uzunalli et al., 2015).

The large lattice scaffold was used for *in vivo* analysis as it could retain the mature islets and formed the appropriate size of ILCs from mesenchymal stem cells that showed better insulin secretion. The ability of differentiated ILCs on large lattice scaffolds encapsulated in alginate to reverse hyperglycemia in diabetic rats was compared to the rat mature islets seeded in large lattice scaffolds and alginate encapsulated. The mature rat islets in the scaffold showed a greater decrease in blood glucose levels in comparison to the effects of using stem cell differentiated ILCs in the scaffold. The intraperitoneal blood glucose tolerance tests conducted, at 30 days post-transplantation, as shown in figure 4.42 of chapter 4, shows that mature islet in scaffold and ILCs in scaffold showed glucose clearance and reached a plateau level in 2 hours compared to diabetic rats, however, mature islet group had better clearance of glucose compared to the ILC group. The islets preserved their morphology and were viable cells after retrieval. The differentiated ILC groups showed islet-like clusters with their spherical morphology along with elongated cells with fibroblast shape. Insulin positive cells were identified in both groups. However, the insulin detected in the serum of animals implanted with ILC in scaffolds was lower than the animals implanted with mature islets in the scaffold. Animals implanted with the tissue-engineered constructs survived until the endpoint of the study i.e 60 days post-transplantation compared to the diabetic controls which survived for 15 to 30 days after induction of diabetes. The scaffold could retain the

morphological integrity of the mature rat islets which might have contributed to the better function of this group. The lower insulin production and the resultant decrease in blood glucose control can be circumvented by increasing the number of scaffolds with ILCs to be implanted in the animal models. Reports have showed that the production of insulin by stem cell differentiated ILCs are lower compared to mature pancreatic islets albeit they help in survival of the animal longer and normal glucose control achieved by optimising the number of these constructs to be implanted(Lumelsky et al., 2001)(Kroon et al., 2008b) (Karaoz et al., 2013). External blood vessels were observed to be formed to the surface of the tissue-engineered construct during the period *in vivo,* however, small vessels growing towards the core of islet clusters in the construct was not observed. The islets are surrounded by larger blood vessels with capillaries reaching the inner core of the islets, this high vasculature of islets is important for oxygen, nutrient supply to islet cells and may support islet neogenesis(Nyman et al., 2008). The vasculature is crucial for islet survival *in vivo* and the loss of vasculature during isolation is one of the main causes in loss of graft function in islet transplantation(Speier et al., 2008). The large lattice scaffold with stem cell differentiated ILCs have shown a reduction in hyperglycemia, prolonged survival of implanted rats and engraftment with growing blood vessels provides promising results for the future generation of transplantable tissue-engineered islets.

5. 6 Cell-ECM interactions

Islets are highly regulated by cell-ECM interactions. It is proved beyond doubt that such interactions are essential for maintaining architecture, survival,

higher insulin secretion, migration, differentiation and maturation from foetal stage. However, most of the research in islet tissue engineering has focussed on the growth factor cocktail that influences the differentiation of stem cells to ILC. The evidence of the importance of cell-ECM interactions has surged interest in improving the scaffold to obtain functional tissue constructs. *Invitro* cell-ECM interaction studies have been complicated by difficulties in obtaining pure ECM preparations, attributing outcomes to specific matrix components. Islets have multiple types of cells with the unique arrangements in tissue with various receptors that can bind to multiple binding sites present on a matrix protein. A combinational effect of each receptor-ligand interaction produces the final cellular outcome. The ECM of the islet tissue varies between species and foetal to mature tissue. `At foetal stage, vitronectin, fibronectin and collagen iv are the major components of ECM, however, very little fibronectin and vitronectin are present in mature islet ECM(Cirulli et al., 2000). Foetal beta cells express αvβ1, αvβ5 and α1β1 integrins through the development stage to maturation, however, α1β1 are downregulated after maturation, which suggests that foetal cells interact with collagen iv via α1β1 for migration to form the correct architecture of the islets. Other integrins αvβ3 and αvβ5 have an important role in the maturation of progenitor cells from ductal epithelium are expressed at the early development stage and downregulated thereafter. Islets express different types of integrins whose expression and interaction with matrix proteins are heavily regulated by the developmental stage. Hence we hypothesised that matrix components vitronectin, fibronectin and collagen IV expressed at an early developmental stage when coated on large lattice scaffold surface may enhance the differentiation and insulin secretion of Islet like clusters.

Fibronectin, a high molecular weight glycoprotein, contains multiple adhesion sequences mainly RGD and PHSRN that binds with integrins such as α5β1, α3β1 expressed in islets(Kreis and Vale, 1999). Vitronectin is a small molecular weight glycoprotein that interacts with collagen iv in the matrix and expresses RGD sequences that enable islet binding(Koivunen et al., 1994). It is reported that α3 and α5 integrins in developing pancreatic tissue colocalise with adhesion sequences on fibronectin which in turn inhibits apoptosis in islets. RGD sequences on fibronectin and vitronectin are important in enabling cell migration during early islet differentiation. Collagen IV forms planar hexagonal networks in the basement membrane of islets that can interact with islet cells via integrins α3β1 or indirectly by binding to matrix glycoproteins.

Covalent modification of the scaffold surface with ECM molecules is an effective method to incorporate biomimetic property without denaturation of the bioactive molecule as experienced with methods such as co-electrospinning(Hartman et al., 2010). Sodium hydroxide treatment is a simple method to increase the carboxyl and hydroxyl groups on the surface of the scaffold and the alkali treatment does not result in cytotoxic products(Yeo et al., 2010). The matrix of developing pancreas changes during the maturation process and it may not be appropriate to use a single ECM molecule to promote differentiation as each molecule in ECM has a combinatorial effect on each other. Hence, we compared the effect of each ECM molecules (collagen iv, fibronectin, vitronectin) to the combination of all three ECM molecules immobilised on the surface of the large lattice scaffold on the insulin secretion of the differentiated ILC. The matrix components were immobilised by

crosslinking using EDC and NHS chemistry. NHS is a heterobifunctional linker in the crosslinking reaction and it provides steric freedom thus helps to retain specific activity of the immobilised molecules(Ratner et al., 2004). The high surface area and interconnected pores of the electrospun large lattice scaffold enable higher peptide binding on the surface all through the depth of the scaffold as observed in eosin and picosirius red staining(Zhang et al., 2007). XPS analysis confirms the crosslinking with the presence of N1 peaks on the surface of the scaffolds. The mesenchymal stem cells were viable and maintained their spindle morphology in culture indicating the process of immobilisation of ECM molecules has not compromised the biocompatible nature of the scaffold.

The differentiated ILCs showed a higher expression of insulin to glucose stimulation in large lattice scaffolds coated with the combination of ECM proteins compared to two-dimensional cultures. Narayanan et. al. showed similar results with RIN5F cell lines when cultured on a matrix of fibronectin, collagen iv and laminin(Narayanan et al., 2013). However, in our study, insulin secretion was not significantly higher compared to the scaffold coated with collagen IV. A higher positive expression of insulin when mature islets are cultured in collagen iv matrix have been reported in the literature(Daoud et al., 2010)(Salvay et al., 2008). Our results are comparable to the literature that vitronectin enables attachment and motility during early development via integrins $\alpha_V\beta_v$ and $\alpha_V\beta_1$ has been shown that this may result in reduced insulin content in adult cells(Kaido et al., 2006).

The soluble factors in media trigger the initial differentiation process while the three-dimensional scaffold and ECM matrix is important in directing the

differentiation to spherical functional islets. The use of a defined matrix compared to an undefined ECM matrix such as matrigel eliminates the effect of unknown factors that may lead to the formation of a mixed population of cells. The defined matrix provides control of the differentiation strategy to be developed as a viable clinical option. The combinatorial effect of growth factors, scaffold architecture and ECM molecules direct the differentiation to pancreatic islet phenotype. In our future study, the combinatorial effect of ECM molecules in different ratios and its mechanism in directing the differentiation to ILC would be focussed. The results of this study shape our future research direction in developing a defined system of differentiation to form a tissue-engineered islet construct physiologically similar to mature islets.

CHAPTER 6

SUMMARY AND CONCLUSION

Diabetes mellitus is a group of metabolic disorders classified into two groups, type 1 and type 2, based on the specific absence of secreted insulin and insulin resistance respectively. It is characterised by hyperglycaemia, leading to long term damage to organs mainly heart, eyes, kidneys, nerves and blood vessels. The disease imposes a huge economic burden on patients and countries worldwide

Currently, diabetes is treated with oral hypoglycemic medications and insulin injections. These treatment modes are associated with serious side effects such as hypoglycemia. The drugs and insulin injections fail to mimic the physiologic pattern of glucose sensing and rhythmic release of insulin into the bloodstream to control and maintain the blood glucose level within a narrow range. They fail to prevent the progression of secondary complications in patients. Transplantation of the whole pancreas or Islet of Langerhans aims to achieve the physiological pattern of glucose sensing and insulin release to keep the glucose concentration within normal range and prevent secondary complications. However, this method of treatment has been limited to patients with chronic conditions due to the shortage of donor pancreas and the requirement of two or more cadaveric donor organs to obtain the required number of islets to achieve euglycaemia. The requirement for lifelong immunosuppression and the toxic effect of the immunosuppressant reduce the viability of the transplanted islets and hence limit the success of the treatment. The absence of extracellular

matrix in the isolated islets is another factor that negatively impacts the survival of transplanted islets. The extracellular matrix is highly important for spatiotemporal support and signaling to maintain cytoarchitecture, viability and function of the islets. Hepatic portal vein used as the site for transplantation impacts the transplanted islets by blood mediated immune response and delay in vascularisation of the implant.

The current study on "In Vitro And In Vivo Assessment Of Adipose Stem Cell-Derived Islet Like Cells On A Novel 3D Scaffold" was based on the hypothesis that three-dimensional biomimetic nanofibrous scaffolds could enhance stem cell differentiation to islet-like clusters and maintain its survival and functionality when transplanted *in vivo.*

Three-dimensional nanofibrous polymer scaffolds of different porosity were developed that support the growth and differentiation of stem cells to islet-like clusters. Stem cell differentiated islets form the preferred alternative cells to overcome the shortage of mature islets for transplantation. The scaffolds provide mechanical support and help maintain the spherical cluster morphology of the islets and the pore size of the scaffolds controls the cluster size of the differentiated islets formed. Hence, islet clusters of the desired size could be developed for transplantation. The sizes of the clusters impact the outcome of the transplantation as bigger islets undergo apoptosis from the center due to hypoxia. The Large lattice scaffold could also support the *in vitro culture* of mature islets and help maintain their viability. Thus the present work emphasise the importance of cell-matrix and cell-cell interaction for viability and function of the islets.

The tissue-engineered construct of the study comprised of adipose-derived mesenchymal stem cells differentiated to ILC and seeded on -large lattice 3D

scaffold. The construct was further coated with alginate to avoid immune rejection. *In vivo* analysis of the tissue-engineered construct demonstrated that they could reduce the blood glucose concentration of the diabetic rat models and prolong the life of the recipient animals. The tissue-engineered construct was accepted without immune rejection. Histology of the retrieved implants showed that the islets were viable and exhibited spherical morphology. They were positive for insulin expression which could be analysed in the blood samples as well. The omental pouch has helped to revascularise the implant as blood vessels could be identified to grow in the implants. Omentum is a potential site for future clinical transplantation as it helps vascularisation of the implant.

The three-dimensional nanofibrous porous scaffolds were immobilised with extracellular matrix components specifically seen during the development stage in foetal pancreas for further maturation of stem cell differentiated islet clusters. Collagen IV, vitronectin, fibronectin and combination of all molecules were immobilised on three-dimensional scaffolds. AMSC were seeded on the modified scaffolds and differentiated to islet-like clusters (ILC) Positive increment in insulin release was observed in the scaffolds with all the three components as the effect of cell-ECM interact is combinatorial. The results of the current study show potential for future development as tissue-engineered islet constructs for therapy.

Future Directions

- In the current study, the number of tissue-engineered constructs that could be implanted with respect to the size of the omentum of diabetic rats was limited. An increase in the number of stem cells differentiated islet construct could further improve the glycemic control of the diabetic animal model.
- The *in vivo* effectiveness of ECM immobilised tissue construct for glycemic control in larger animal models have to be analysed for future development as a therapeutic option.

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LIST OF PUBLICATIONS

- D. Vaikkath, **R. Anitha**, B. Sumathy, P. D. Nair, A simple and effective method for making multipotent/multilineage scaffolds with hydrophilic nature without any postmodification/treatment, Colloids Surf. B Biointerfaces 2016, *141*, 112. doi:10.1016/j.colsurfb.2015.12.041 ISSN 0927-7765

- **Rakhi Anitha**, Dhanesh Vaikkath, Sachin J Shenoy, Prabha D Nair, Tissue Engineered Islet-Like Cell clusters Generated from Adipose Tissue-Derived Stem Cells on 3D electrospun scaffolds can reverse diabetes in an experimental rat model and the role of porosity of scaffolds on cluster differentiation. **,** Journal of Biomedical Materials Research, 2019, doi: 10.1002/jbm.a.36854. ISSN:1552-4965

- Rakhi Anitha, Prabha D Nair, Conjugation of ECM proteins on 3D nanofiber scaffold and its effect on the differentiation of mesenchymal stem cells to islet-like clusters. **Manuscript under review**

PAPERS PRESENTED AT CONFERENCES

- Rakhi A.,Dhanesh Vaikkath, Prabha D. Nair (2013).A novel three dimensional nanofibrous scaffold for differentiation of stem cells into Islet like clusters. Tissue Engineering And Regenerative Medicine International Society Annual Conference, Atlanta, USA. (Poster presented)

- Rakhi A., Dhanesh Vaikkath, Prabha D. Nair (2014).Porous nanofibrous scaffold with a potential for Islet tissue engineering. International conference on polymeric biomaterials, bioengineering and biodiagnostics, New Delhi, India (Poster presented)

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Achievements

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- **DBT-JRF 2010-** Rank 50 on all India basis
- **GATE** 2010
- **Junior Research Fellowship** (2011-2013) by Council of Scientific and Industrial Research (CSIR), New Delhi, India
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- DBT travel Grant to attend TERMIS-AM 2013 conference at Atlanta, Georgia, USA
- Teacher Investigator, SPYTiS-II, student's project scheme, KSCSTE, Kerala

APPENDIX

A-1

PREPARATION OF KREB'S RINGER BICARBONATE HEPES (KRBH) BUFFER

Sodium chloride - 114mM

Sodium bicarbonate - 29.5mM

Potassium chloride - 4.4mM

Magnesium sulphate - 1mM

Calcium chloride - 1.28mM

HEPES - 10mM

Bovine serum albumin- 0.1%

Adjust the pH to 7.4