Pancreatic Tissue Engineering Using Agarose Based Nano-Composites and Human Umbilical Cord Derived Mesenchymal Stem Cells

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By

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Under the Supervision of:

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CERTIFICATE

This thesis is submitted under Regulation 8.20 (a) of the Academic Regulations for Doctoral Programmes which allows a faculty member of the Institute/Professional to do Ph.D. research without the benefit of a supervisor.

This is to certify that the thesis entitled **"Pancreatic Tissue Engineering Using Agarose Based Nano-Composites and Human Umbilical Cord Derived Mesenchymal Stem Cells"** submitted by **NUPUR RAJEEV KUMAR** ID No **2012PHXF0010G** for award of Ph.D. Degree of the Institute, embodies my original work.

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Abstract

Diabetes Mellitus (DM) is a chronic disease affecting millions of people worldwide. It is characterized by reduced insulin secretion (type 1) or insulin sensitivity (type 2) or both. Type 1 diabetes mellitus is an auto immune condition resulting from destruction of pancreatic β cells causing decreased insulin secretion. Current treatment includes lifelong insulin therapy which has serious disadvantages and complications. An alternative therapeutic treatment is transplantation of intact human pancreas. However, this approach is limited owing to shortage of donors and the need for long-term immunosuppressive therapy.

Various studies have highlighted the transplantation of islet of Langerhans as a potential treatment of type 1 diabetes. The major limitations associated with this approach include immune rejection and scarcity of donor islets. To alleviate these limitations, research has been focusing on use of tissue engineering for creating functional pancreatic constructs. Tissue engineering is an amalgamation of various fields such as engineering, life science and material science. This subject intends to create biological substitutes for replacing damaged tissue and regeneration. Major components of tissue engineering or tissue engineering triad comprises of scaffolds, cells and bioactive molecules.

Scaffold is an essential element as they act as an extracellular matrix for cell attachment and proliferation. Various polymeric scaffolds have shown promising results for their application in pancreatic tissue engineering. But neither natural nor synthetic polymers have been extensively studied in this respect. As a result of which, we are still waiting for the creation of an appropriate scaffold that can act as a perfect environment for the growth of pancreatic cells. In recent years, research has shifted towards understanding the role of nanostructures present in the extracellular matrix. The nanostructures have shown to provide structural stability and as they provide increased surface area, aids in cellular attachment and proliferation. Therefore, embedding nanoparticles within the scaffold can improve their overall function.

We have formulated a blend of agarose and chitosan coated silver nanoparticles (AG-CHNp scaffolds) crosslinked by glutaraldehyde using freeze drying technique to produce a nanocomposite with spongy gel-like properties ideal for tissue engineering of pancreas. The scaffold was tested for physical, chemical, mechanical and biological parameters and was found to be highly biocompatible with good swelling ratio and appreciable degradation capability. It also showed additional anti-bacterial activity against both Gram-positive and Gram-negative bacteria. Further, AG-CHNp scaffolds demonstrated excellent biocompatibility

with HeLa (human cervical carcinoma cell line), MiaPaCa2 (human pancreatic carcinoma cell line) and HEK (human embryonic kidney cell line) cells and have showed their sustained growth. With the aim of studying the application of AG-CHNp scaffolds for pancreatic tissue engineering, the scaffolds were tested against mice primary pancreatic cells. The scaffold showed long term compatibility with the pancreatic cells. It showed successful attachment and proliferation of the cells for a period of 40 days which was well characterized at transcriptional and translational levels.

Finally, the scaffold was used for differentiation of mesenchymal stem cells isolated from human umbilical cord into insulin producing cells. Various cell types have been tested for pancreatic tissue engineering out of which allogenic and xenogeneic islets have shown great promise. But sacristy of donors, limits their role in this field. Hence, differentiation potential of mesenchymal stem cells was studied. Our results show successful differentiation of cells into pancreatic lineage which was characterized by reverse transcriptase PCR, western blotting and immunofluorescence.

We believe that the scaffolds formulated, shows exceptional qualities like biocompatibility, biodegradability and hemocompatibility. It also helps in long term survival and proliferation of mice pancreatic cells. Therefore, with these current results, the novel AG-CHNp scaffold looks very promising in the field of pancreatic tissue engineering.

Contents

Abbreviations

NCDs	Non-communicable diseases
WHO	World Health Organization
IDF	International Diabetes Federation
DM	Diabetes Mellitus
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
IDDM	Insulin Dependent Diabetes Mellitus
ECM	Extracellular matrix
PEG	Polyethylene glycol
PLA	Poly lactic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
NHS	N-hydroxysuccinimide
PLG	Poly (lactide-co-glycolide)
PVP	Poly (vinylpyrrolidone);
PU	Polyurethane
EGF	Fibroblast growth factor
FGF	Fibroblast growth factor
INS-1	Insulinoma cell line
HEK	Human Embryonic kidney
MiaPaCa2	Human Pancreatic carcinoma
PDMS	Polydimethylsiloxane
BM-MSCs	Bone marrow derived mesenchymal stem cells
RIN-5	Rat insulinoma cells
ECs	Endothelial cells
VEGF	Vascular endothelial growth factor
IDE1	Inducer of definitive endoderm 1
iPSCs	Induced Pluripotent stem cells
GLP-1	Glucagon like factor-1
SOD	Superoxide dismutase

CAT	Catalase
ROS	Reactive oxygen species
XRD	X-ray diffraction
FTIR	Fourier transformation Infrared spectroscopy
TGA	Thermogravimetric analysis
DMA	Dynamic mechanical analysis
SEM	Scanning electron microscopy
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NADP	Nicotinamide adenine dinucleotide phosphate
FCC	Face centered cubic
AG-CHNp scaffolds	Agarose-chitosan coated silver nanocomposite scaffold
HBSS	Hank’s balanced salt solution
DTZ	Dithizone
PBS	Phosphate buffer saline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
PBST	Phosphate buffer saline with 1% Tween 20
HRP	Horseradish peroxidase
BSA	Bovine serum albumin
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
SSC	Side scatter
2D	Two dimensional
DMSO	Dimethyl sulfoxide
3D	Three Dimensional
RT-PCR	Reverse transcriptase PCR
KRB Buffer	Krebs-Ringer bicarbonate buffer

ESCs	Embryonic stem cells
MSCs	Mesenchymal stem cells (MSCs
UC	Umbilical cord
MEM	Minimal essential media
ITS solution	Insulin-transferrin-serine solution
q-PCR	Real time PCR
PDX-1	Pancreatic and duodenal homeobox 1
ISL-1	Insulin gene enhancer protein ISL-1

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Chapter 1
Review of literature

1.1 Introduction

Non-communicable diseases (NCDs) or chronic disease are medical conditions which are not caused by infectious agents. These diseases stay for longer durations and are of slower progression. Four major types of NCDs are: cardiovascular diseases, chronic respiratory diseases, cancer and diabetes (WHO, 2013). NCDs account for 70% of deaths worldwide ("Non-Communicable Diseases", 2018). Diet and lifestyle play an important role in maintaining proper physical and mental health (WHO, 2003). For centuries, infectious diseases have been considered as the main killer around the world. But with non-communicable diseases (NCDs) taking the front seat, it is estimated that by year 2020 NCDs will cause seven out of ten deaths in developing nations

Diabetes Mellitus (DM) is a metabolic disorder which is characterized by chronic hyperglycemia. This condition primarily affects the islets of Langerhans situated in the pancreas and is mainly attributed to the defects in insulin secretion or action of insulin on cells or both. Initially labeled as a disease of rich countries, diabetes has shown a tremendous increase in the past few years, even in middle income nations. According to WHO's global diabetes report 2016, a total of 422 million people across the world are currently suffering from diabetes (WHO, 2016). International diabetes federation report (2015) states that one in every eleven individuals worldwide is suffering from this debilitating disease and the report also suggests that one in every ten individuals will suffer from diabetes by the year 2040 (IDF, 2015).

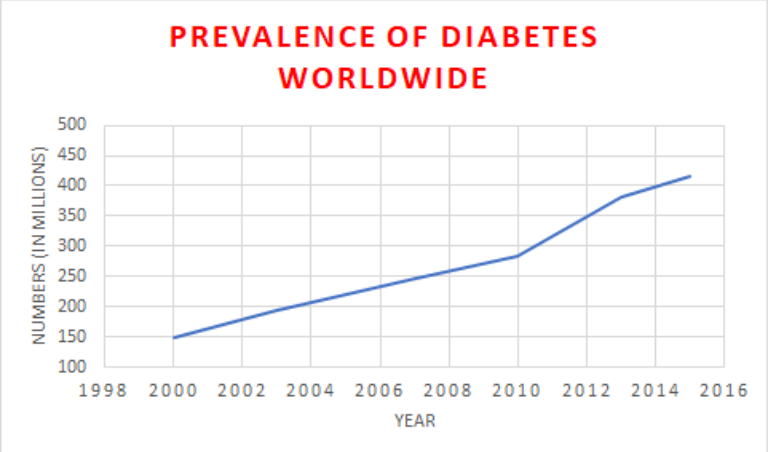


Figure 1.1 Worldwide prevalence of diabetes in the past two decades (Source for data: IDF Diabetes Atlas; http://www.diabetesatlas.org).

The report also ranks china first in relation to the number of diabetic patients (between the age of 20 and 79 years) followed by India and the USA (IDF, 2015).

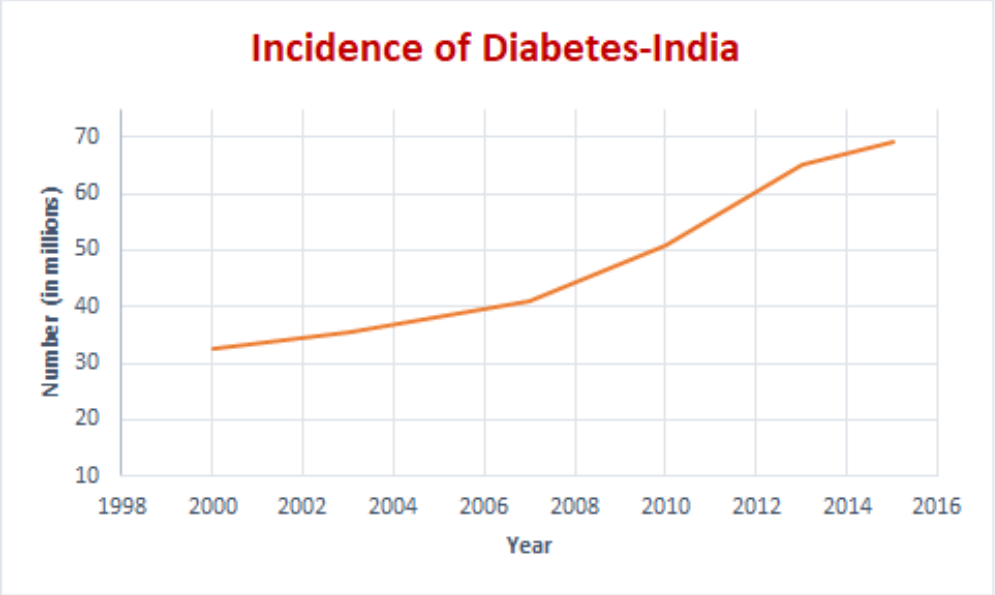


Figure 1.2 Prevalence of diabetes in India (Source: IDF Diabetes Atlas, http://www.diabetesatlas.org)

Diabetes mellitus is classified as one of the metabolic disorders characterized by a chronic hyperglycemic condition. This state is mainly attributed to defects in insulin secretion or to the action of insulin in cells or both. Most cases of diabetes are one of two types: type 1 diabetes (T1D) and type 2 diabetes (T2D). There are additional types such as gestational diabetes (GD) and maturity onset diabetes of the young (MODY).

1.2 Pancreas: Anatomy

The pancreas is a soft lobulated "J"- shaped exo-endocrine gland deeply located in the upper part of the abdomen. Pancreas is 12-15 cms in length and weighs around 80-90gms. It is divided into four main parts starting from the head which lies in the curve of the duodenum followed by neck, body and ending near the hilium of spleen with the tail. The exocrine portion of the pancreas synthesizes and secretes digestive enzymes and bicarbonate. This portion mainly comprise of acinar and ductal cells associated with connective tissue, vessels and nerves. Acinar cells secrete the enzymes whereas bicarbonate is secreted by the epithelial cells lining small pancreatic ducts. The endocrine part of the pancreas is packed together into islets of Langerhans cluster. These clusters consist of alpha cells, beta cells, PP cells, epsilon cells and delta cells. The alpha cells comprise of 20% of the total islets and they secrete glucagon. Glucagon is a catabolic hormone responsible for mobilization of glucose, fatty acids, and amino

acids from storage to blood. The beta cells cover up the major mass of islets approximately 75%. They are responsible for secretion of Insulin. Insulin is an anabolic hormone which accumulates glucose, fatty acids and amino acids in cells and tissues. Elevated blood sugar levels stimulate the secretion of insulin. Both insulin and glucagon play a significant role in regulation of carbohydrate, protein and lipid metabolism. Accounting for a total of 4% of islets, delta cells secretes peptide the hormone somatostatin. Pancreatic somatostatin inhibits the release of both glucagon and insulin. Epsilon cells secrete ghrelin, which inhibits insulin release and regulates other endocrine secretions. Lastly, PP cells accounts for a total of 1% of islets and secretes pancreatic polypeptides which inhibits pancreatic enzyme secretion.

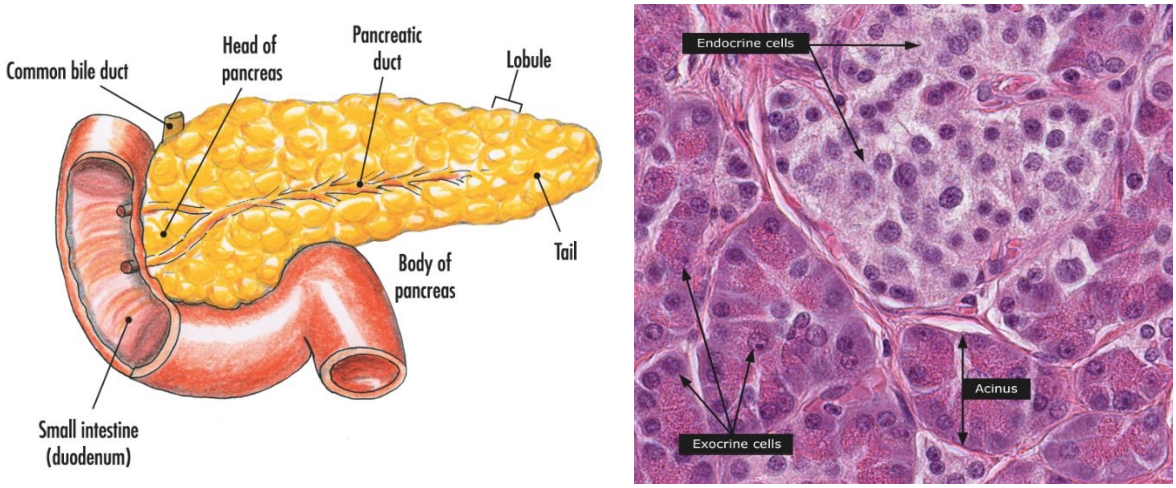


Figure 1.3 Anatomy and Histology of the Human pancreas

(a) Gross Anatomy of Pancreas (Courtesy: anatomy-medicine.com). (b) Histology of pancreas (Courtesy: https://www.proteinatlas.org/learn/dictionary/normal/pancreas/detail+1/magnification+1)

1.3 Types of Diabetes

1.3.1 Type 1 Diabetes (T1D): Also known as Insulin Dependent Diabetes Mellitus (IDDM). T1D is mainly caused by loss of ability of beta cells to secrete insulin, which in turn leads to the increase in blood glucose level. It is an autoimmune disorder in which antibodies are generated against pancreatic proteins (Van Belle et al, 2011). The auto antibodies such as Islet cell cytoplasmic antibodies (ICCA), Islet cell surface antibodies (ICSA) or Specific antigenic targets of islets selectively damage beta cells leading to the inability of pancreas to secrete insulin. Different factors, including genetics and some viruses contribute to type 1 diabetes (Butalia et al, 2016). Although type 1 diabetes usually appears during childhood or

adolescence, it also can develop in adults. More than 85% of the people who do develop type 1 diabetes do not have a parent or sibling with the disease. Type 1 Diabetes can be sub categorized into Type 1A and Type 1B depending on the cause of the disease. Type 1A has an autoimmune cause whereas Type 1B has an idiopathic cause (American Diabetes Assocation, 2011). 70-90% of population suffering from type 1 diabetes have immunological self-reactive autoantibodies (Type1A) and rest are with ambiguous pathogenesis (Type1B).

Several genes have been identified which are associated with the development of diabetes. There are at least 40 diabetic susceptibility loci known till date (Noble et al, 2012). IDDM 1, the major histocompatibility complex on chromosome 6 has been associated with around 40-50% of Type 1 Diabetes patients. Other loci include IDDM 2 (the insulin gene locus on chromosome 11), PTPN 22 (the protein tyrosine phosphatase gene) with a mutation at LYP (the lymphocyte-specific phosphatase gene) on chromosome 1 associated with susceptibility to multiple autoimmune disorders etc. Type 1 diabetes is associated with several other autoimmune conditions such as Celiac disease, Hashimoto's disease, Graves' disease, Addison's disease and Pernicious anaemia (Krzewska, 2016).

1.3.2 Type 2 Diabetes (T2D): Also known as Non-Insulin Dependent Diabetes Mellitus (NIIDM). It is the polygenic disorder where target tissue become insensitive to insulin. Type 2 Diabetes is generally associated with increase in insulin resistance. Type 2 D is also dependent on life style, overweight and obesity are high risk factors for T2DM. Normally onset of T2D is usually after 40 years and hence know as disease of middle aged, and elderly (Mann et al, 2017; Raj et al, 2009). But these days T2D is also seen in children as young as 10 years old (Pettitt et al 2009). The onset of T2D is due to a complex interaction between environmental factors and genetic component. The hereditary component of T2D are dependent on multiple genes dispersed all along the genome unlike T1D where the genetic component was concentrated in the HLA region (Ali, 2013). There are more than 150 DNA variations associated with the development of T2D.

1.3.3 Monogenic diabetes

Both T1D and T2D are polygenic diabetes as their occurrence is related to multiple genes. Normally, polygenic diabetes runs within the family and can be diagnosed by various biochemical tests. Polygenic diabetes sums up to almost 98% of the total number of diabetics around the world. Monogenic form of diabetes on the other hand, is caused by single gene

abnormality. Monogenic diabetes comprises of Maturity-onset diabetes of the young (MODY), Neonatal diabetes and syndromic forms of diabetes.

1.3.3.1 Maturity-onset diabetes of the young (MODY)

MODY is a monogenic form of diabetes characterized by early onset of the disease and autosomal inheritance. This can be caused by mutations in gene encoding transcription factors associated with beta-cell functioning (Anık et al, 2015). Presently, there are around 13 different genes which have shown their association with MODY. Out of these, six gene mutations are most commonly seen. These includes Hepatocyte nuclear factor-1-alpha (HNF1A), Hepatocyte nuclear factor-4-alpha (HNF4A), Pancreas/duodenum homeobox protein-1 (PDX1), Hepatocyte nuclear factor-1-beta (HNF1B), BETA2/Neurod1 and glucokinase (GCK) (Antosik et al, 2016). All the mutation limits the production of insulin by beta cells. Differential diagnosis between MODY and T1D or T2D is necessary as the disease surfaces like their polygenic counterparts. Direct sequencing is usually performed for diagnosing MODY with maximum sensitivity. The treatment of MODY depends upon the severity of the hyperglycaemia which is also related to the gene mutation. For example, GCK mutation can cause mild hyperglycaemia which may not require treatment whereas HNF4A and HNF1A cause gradual beta-cell dysfunction which requires Sulfonylureas for treatment (Anık et al, 2015).

1.3.3.2 Neonatal Diabetes Mellitus

Neonatal diabetes mellitus (NDM) is a rare disorder seen in infants before six months of age. It is associated with mutations in genes responsible for beta cell development such as glucose kinase or K_{ATP} channel etc (Ashcroft et al, 2012). Currently 20 genes associated with NDM have been identified (Suzuki et al, 2014). Symptoms of NDM includes thirst, dehydration and frequent urination. Many new-borns with NDM are smaller than normal new-borns, a condition known as intrauterine growth restriction (American diabetes association, 2018). Also, some infants also show shunted growth and may remain underweight as compared to other infants of same age and sex. NDM can be further classified into transient neonatal diabetes mellitus (TNDM) and permanent neonatal diabetes mellitus (PNDM) (Hattersley et al, 2017). In about 50% of the NDM cases, the condition remains lifelong, this is known as PNDM. In the rest of the cases, diabetic condition is resolved after few months, state known as TNDM. TNDM patients do have a higher chance of relapse in their adolescence or adulthood (Thomas et al, 2015).

1.4 Treatment options for diabetes

Currently available treatment options include external insulin administration for T1D patients and use of drugs to combat insulin resistance in T2D patients. Certain T2D patients also require insulin administration. Apart from these alternative treatment methods, whole organ pancreas transplantation and islets transplantation has been attempted in several patients.

1.4.1 External Insulin administration/Insulin therapy

The discovery of insulin dates back to 19th century. Before insulin therapy, diabetes was a dreaded disease owing to its high mortality rate. In 1923, Two Canadian scientists Dr Frederick Banting and Professor John Maceod received Nobel prize for the discovery of insulin (Alpert, 2016). After this discovery, there has been multiple modifications in the source and structure of insulin. From adding protamine to insulin structure (to alter absorption and prolong its action) to using insulin from pork and beef origin which was later substituted with humanised insulin, have improved the outcome for diabetics around the world (Greene et al, 2015). Though there has been improvement in insulin therapy, still millions of people inject them with insulin multiple times a day, which not only is inconvenient but also has side effects and is not cost effective.

1.4.2 Use of Pharmaceutical agents

Various classes of drugs have been used for management of diabetes either individually or in combination with each other. These includes Biguanides, Sulfonylureas, Megalitinides, Thiazolidinediones, Alfa gluocosidase inhibitors, Incretin Based Therapies, Dipeptidyl - Peptidase lV inhibitor

1. **Biguanides**: These class of drugs includes betformin, phenformin, metformin, etc. Among which metformin is the most common drug used to manage diabetes. Metformin works on inhibiting a mitochondrin specific isoform of glycerophosphate dehydrogenase which further reduces glycerophosphate- Dihydroxyacetone phosphate (DHAP) shuttle. These events lead to increased ratios of glycerophosphate to DHAP, NADH to NAD, lactate to pyruvate and inhibition of hepatic gluconeogenesis thereby managing blood glucose (Hundal et al, 2000; Galligan et al, 2016). It also helps in peripheral glucose clearance.

2. **Sulfonylureas (SUs)**: Glybenide, glybenclemide, glipecide are the common sulfonylurea drugs used for the treatment for T2D. They stimulate insulin secretion from pancreatic B cells by targeting the potassium (K_{ATP}) channel which plays a major role in maintaining the B cell

membrane potential. SUs or glucose inhibit the K_{ATP} channel and depolarizes the B cell membrane. This triggers the opening of Ca^{2+} channels which leads to the high influx of Ca^{2+} inside the cell thereby causing release of the insulin containing secretary granules (Proks et al, 2002). Hypoglycemia is the most common and serious issue which limits the use of SUs as a therapeutic drug. Prolonged use of SUs may also leads to stroke, myocardial infarction, injury and death (Shorr et al, 1996).

3. **Megalitinides**: Repaglinide and nateglinide acts on same potassium (K_{ATP}) channel in the B cells of pancreas which leads to the increased stimulation for insulin secretion but has a different binding site. It is usually given before meals and is metabolized in liver (Olokoba et al, 2012). Repaglinide is five times more potent than glibenclamide(SUs) in stimulating insulin secretion (Fuhlendorff et al, 1998) but this drug is most effective during early stage of diabetes and also needs the complementation of metformin (Blicklé , 2006).

4. **Thiazolidinediones**: They are the first drug molecules which are known to increase the sensitization of insulin to the cells. Troglitazones, rosilitazones pioglitazones and others are known to activate the nuclear transcription factor, Peroxizome -Proliferator activated receptor ϒ (PPARϒ). It is essential for normal adipocyte differentiation and proliferation as well as fatty acid uptake and storage (Yki-Järvinen, 2004). PPARϒ regulate transcription of varitey of genes encoding proteins involved in glucose homeostasis and lipid metabolism. There are also some questionable effects of thiazolidinediones which restricts its use. Rosiglitazone is known to increase the risk of myocardial infarction and death from cardiovascular diseases (Nissen et al, 2005; Nissen et al, 2007). But these findings are yet to be proven.

5. **α glucosidase inhibitors**: Acarbose, Voglibose, and Miglitol are known to tackle with hyperglycemic condition by increasing the glucose tolerance as they prevent immediate breakdown of carbohydrates (Kawamori et al, 2009). These are generally avoided for patients with renal impairment. It has also shows some side effects such as diarrhoea (Olokoba et al, 2012).

6. **Incretin based therapies**: Incretin hormones (glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)) stimulates insulin release from the pancreatic beta cells in response to enteric glucose load. GLP-1 binds to Glp-1 receptor(GLP-1R) thereby increasing the glucose sensitivity of β cells, recusing them from apoptosis and triggering their proliferative pathways, which leads to the expansion of β cell population which in in turn secretes more insulin. GLP-1 also has role in suppression of α cells of pancreas which

lowers the hepatic glucose production (Kim et al, 2008; Lovshin et al, 2009). GLP-1 has some adverse effects so GLP-1 mimetics/ GLP-1R agonists are introduced. Some of them are Exenatide, Liraglutide, Albiglutide, Taspoglutide,etc. But these therapeutics also have severe shown side effects like nausea, vomitting, immunogenic response, pancreatitis which may lead to morbidity and death (Lovshin et al, 2009).

7. **Dipeptidyl - Peptidase lV inhibitor**: Dipeptyl peptidase is a ubiquitous enzyme, that rapidly inactivates GLP-1 and GIP. Drugs that inhibit Dipeptyl peptidase have shown improve β cell function and glycemic control. Some of the examples include, Vildagliptin as a monotherapy or in combination with metformin and Sitagliptin as a monontherapy or in combination with metformin (Pratley et al, 2007). Potential effects on glycemic control and β cell functions are still in speculations.

1.4.3 Alternative treatment options

With the disadvantages involved with use of insulin, its analogues and other pharmaceutical agents, attempts have been made to search for other more suitable options. These include whole pancreas transplantation, islet transplantation, tissue engineering of pancreas etc.

1.4.3.1 Whole pancreas transplantation and Islet transplantation

One of the long-term therapy includes pancreatic and islet transplantation. Simultaneous pancreas-kidney transplantation is performed in patients with severe complications due to diabetes such as end stage nephropathy (Niclauss et al, 2014; Niclauss et al, 2016). First pancreatic transplantation was performed in 1966. Though initially pancreatic transplantation was not very successful, but with advances in surgical techniques and immunosuppression, there has been significant improvement in its efficiency (Niclauss et al, 2016). In the year 2000, The “Edmonton protocol” for islet transplantation was a breakthrough in terms of providing long-term relief to diabetics. Edmonton protocol for transplantation requires, as many as 2 to 3 donor pancreases to procure enough islets for a single T1DM patient becoming insulin dependent (Shapiro et al, 2000). However, there are complications associated with immune rejection and/or with chronic immunosuppressive treatment. Ten years hence, follow-up studies have also proven that multiple infusions of the cells are required per patient due to a loss of function of the cells over time (Ryan et al, 2005; Shapiro et al, 2006). The shortage of suitable donors has also been a big obstacle thereby reducing its wide application.

1.4.3.2 Tissue engineering

Tissue engineering is an amalgamation of pluridisciplinary fields encompassing engineering, material science and life sciences that aims to produce biologically viable substitutes for tissue and organ regeneration (Sengupta et al, 2014). It aims to create biological substitutes that can replace defective or damaged tissues which will help in restoring or maintaining tissue function. It provides an alternative to bridge the ever-growing gap between demand and supply of organs for transplantation (Kumar et al, 2018). Major research efforts have been focused on *in situ* tissue engineering approach which aims to leverage the innate regenerative potential of human body in order to enable tissue regeneration at the site of injury using bioactive molecules-based cues (Li et al, 2014). Scaffolds along with cells and bioactive molecules form the tissue engineering triad (Asghari et al, 2017). Correct combination of these three components aid in development of a substitute for growing damaged tissues.

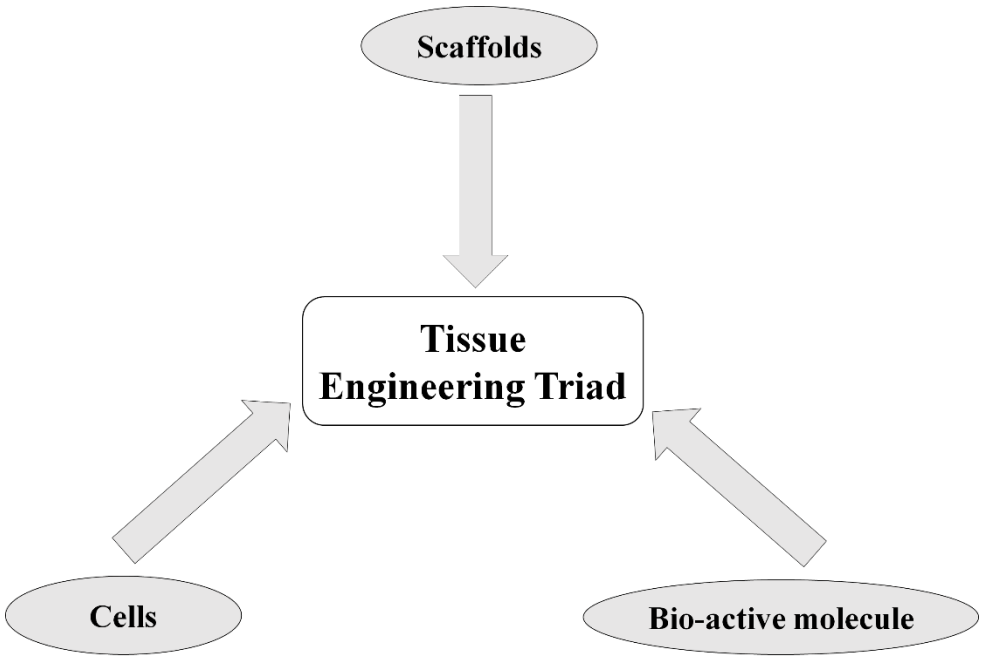


Figure 1.4: Tissue engineering triad showing components for engineering a functional tissue construct.

1.4.3.2.1 Scaffolds

Tissue engineering relies heavily on synthesizing a 3D scaffold which acts as a template for regeneration of tissues. Scaffolds are three-dimensional constructs with the prime function of being able to mimic the physico-chemical properties of natural extracellular matrix (ECM) (Asghari et al, 2014). For applications in the field of tissue engineering, scaffolds need to be

able to provide structural and mechanical support to the cells as well as elevate regeneration by effectual delivery of therapeutic molecules (Lee et al, 2014). Extracellular matrix plays a very important role structurally as well as functionally. It provides shape to the entire organ by providing meshwork on which cells can grow. ECM also secretes various growth factors, provides mechanical strength and enable cellular communication (Diekjürgen et al, 2017). Moreover, ECM helps in gases and nutrient distribution and aids in cellular migration, tissue organization etc (Baker et al, 2012). Scaffolds can be both artificial (formed by various types of polymers and scaffolding techniques) or decellularized. Decellularization of organs does have advantages over the scaffold fabrication as the decellularized scaffolds will posses structure closer to the natural ECM. But non-availability of organs limits their application.

1.4.3.2.2 Cells

Another component of the triad- cells play a major role in designing strategy for tissue engineering of organs. The cells used should be able to integrate in the tissue and secrete various growth factors and cytokines which in turn will help in tissue regeneration (Castells-Sala et al, 2013). Major cell types used for tissue engineering includes: Autologous, Allogenic, Xenogeneic, embryonic stem cells and adult stem cells. Allogenic and xenogeneic cells can be good choice but with difficulty in cell availability their usage became limited. Stem cells are undifferentiated mass of cells having capability of self- renewal and differentiation into multiple lineages (Tesche et al, 2010). There are two broad categories of stem cells: Embryonic stem cells (ESC) and adult stem cells (ASC). Embryonic stem cells are isolated from the inner cell mass of the blastocyst stage of embryos. At this stage, the cell fates have not yet been fully determined and hence these cells offer a wide variety of differentiation possibilities. Though great work has been done to identify factors responsible for differentiation of ESCs into pancreatic cells, the ethical issues surrounding the usage of ESCs have made scientists search for more viable options. The differentiation capability of mesenchymal stem cells derived from human placenta and umbilical cord has been studied extensively. Another alternative considered is induced pluripotent cells first produced by Yamanaka *et al* (Takahashi et al, 2006; Yu et al, 2007). Their autologous character and differentiation potential give iPSCs advantages over other cell types, but the exact molecular mechanism associated with the reprogramming is yet to be clearly understood.

1.4.3.2.3 Bio-active molecules

Bio-active molecules play a crucial role in maintaining various aspects of tissue engineering such as proliferative capacity of the cells, chemotaxis, wound healing, differentiation potential etc (Castells-Sala et al, 2013). Previously, molecules such as growth factors, cytokines have been used in *in-vitro* assay. But their direct application to cells can cause degradation due to cell secreted enzymes. Therefore, matrix bound molecules can show better effect due to their constant renewal and slower release (Tayalia et al, 2009). Also, the type of bio-active molecule used depends upon the organ concerned.

1.5 Scaffolding Approaches

Various scaffolding approaches have been used for manufacturing scaffolds. Technique for scaffold construction is based on various factors, that includes organ of concern, choice of polymers, pore size required etc (Dhandayuthapani et al, 2011). Scaffolding techniques include freeze drying, solvent casting, gas foaming, electrospining, micromolding, etc. Freeze drying or lyophilization is one of the most commonly used fabrication technique. This procedure works on the principle of sublimation where the polymer is mixed with a solvent and frozen. Later this solvent is removed by the process of lyophilization. Lyophilization reduces the pressure of the system which will allow the water in the frozen polymer solution to sublimate directly from solid to gas phase thereby giving porous and interconnected structures (Subia et al, 2010; Asghari et al, 2014).

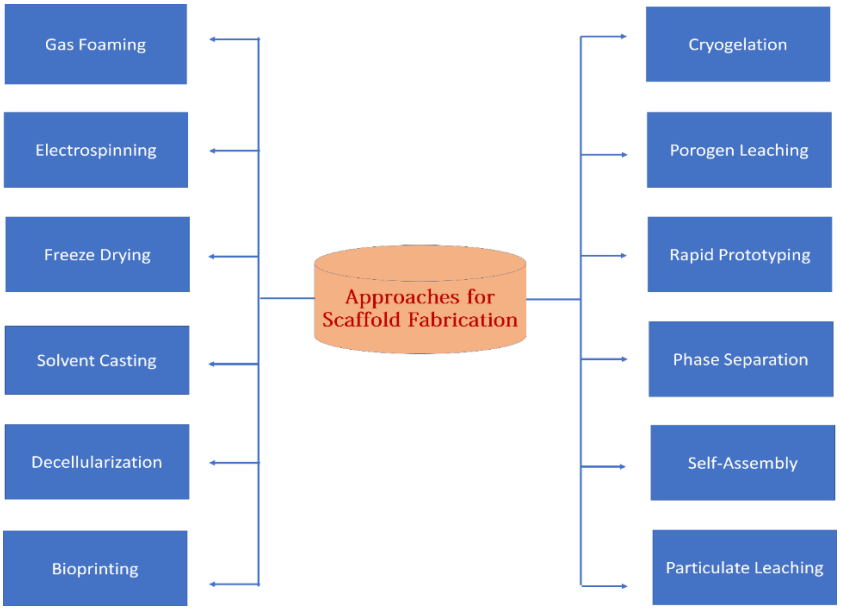


Figure 1.5: Fabrication techniques for scaffold preparation. The selection of the appropriate scaffolding approach depends on the scaffold requirements and tissue-specific considerations.

1.6 Polymers

Polymers can be categorized as natural or synthetic polymers depending on their origin. Naturally occurring polymers like polysaccharides (chitosan, alginate, hyaluronic acid), inorganic polymers (hydroxyapatite) and natural proteins (collagen, fibrin, silk) exhibit several benefits like low toxicity, biocompatibility and enzymatic degradation (Asghari et al, 2014; Asti et al, 2014). Natural polymers also contain bioactive motifs, which help establish cell-scaffold interactions, thus, enhancing tissue functionality (Lin et al, 2015). The downsides associated with natural polymers include temperature sensitivity, immunogenicity and source-dependent heterogeneity (Lee et al, 2014).

The second family of polymers, the synthetic polymers, includes alpha-hydroxy acids such as poly lactic acid (PLA), poly-glycolic acid (PGA), poly lactic-co-glycolic acid (PLGA) copolymers, and polycaprolactone (PCL) (Asti et al, 2014; Makadia et al, 2011). Synthetic polymers have found wide applications in the field of tissue engineering owing to their tuneable physico-chemical properties. The polyester family of synthetic polymers provide controllable and reproducible material properties like elasticity and degradability, which are very useful in tailoring matrices with desired functions (Lin et al, 2015). Lower possibility of infections and immunogenicity risks give synthetic polymers an edge over the natural polymers (Gentile et al, 2014). Taking advantages from both classes of materials, recent work has focused on synthesizing hybrid scaffolds with both natural and synthetic components.

Polymer	Merits	Demerits
Collagen	Biocompatible and Biodegradable	Low mechanical strength
Gelatin	Biocompatible Biodegradable Non-antigenic and Non-immunogenic	Low mechanical strength
Fibrin	Self-assembly Soft elasticity Low toxicity to cells Good attachment, proliferation and migration properties	Immunogenicity
Agarose	Biodegradability Soft tissue like mechanical properties Rapid gelling capacity	Low cell attachment and proliferation
Alginate	Non-toxic approach to encapsulate cells Excellent gelling properties	Limited cell adhesion
Silk	Excellent mechanical strength Good Biocompatibility Water based processing Ease of chemical modification Biodegradability	Stimulates host immune response

Table 1.1: Advantages and Disadvantages of natural polymers for tissue engineering applications.

Polymer	Merits	Demerits
PGA	Biocompatible Tuneable degradation rate Stable three-dimensional structures	Increased release of acidic degradation products Inflammatory response Rapid *in vivo* absorption
PLA	Biocompatible and biodegradable Long half-life Tailorable physico-chemical properties	Hydrophobic nature with low biomimetic and cell adhesion properties
PLGA	High-biocompatibility Non-toxic biodegradation Tuneable mechanical strength Biodegradation rate	Poor protein absorbance, cell affinity and surface characters like hydrophilicity
PCL	Biodegradable Low melting point Remarkable blend-compatibility, versatile mechanical properties and viscoelastic properties	Hydrophobic nature, limited bio-regulatory activity and susceptible to bacteria-mediated degradation
PDMS	High biocompatibility Excellent oxygen solubility Ideal for slow release of compounds	Hydrophobic surface, low cell adhesion
PEG	Low immunogenicity, tissue-like elasticity, well-defined chemistry	Biologically inert, does not support cell growth

Table 1.2: Advantages and Disadvantages of synthetic polymers for tissue engineering applications.

1.7 Pancreatic tissue engineering

Though multiple attempts have been employed for tissue engineering of pancreas, there are various problems associated with engineering the tissue construct. First and foremost being the organ itself. Pancreas comprises of both an exocrine (ductal and acinar cells) and endocrine portion (islets of Langerhans). Though β cells are the major players in the glucose metabolism, other small members of the islets family also play a crucial part in its overall functioning (O'sullivan et al, 2010). Therefore, working with multiple cells types together for proper working of islets makes the process of islets engineering even more convoluted. Another

important factor is angiogenesis. Although islets comprise of only 1-2% of the total pancreatic mass, it takes up as much as 15-20% of the absolute pancreatic blood supply (Phelps et al, 2015). With this huge requirement of blood supply, choice of transplantation site becomes very important so that the transplanted construct receives sufficient blood supply for survival. With this huge requirement of blood supply, choice of transplantation site becomes very important so that the transplanted construct receives sufficient blood supply for survival. Various sites for transplantation have been examined. These includes peritoneal cavity (Brady et al, 2013), hepatic portal vein (Marchioli et al, 2016), subcutaneous space (Luan et al, 2014), subcapsular space of kidney (Jalili et al, 2011; Kodama et al, 2011) etc. Some sites like omental pouch (Pareta et al, 2014) and mesentery (Phelps et al, 2015) have shown promising results but the problem of hypoxia and shortage of blood supply is yet to be resolved. Lastly, the islets itself are a cause of concern for pancreatic engineering. Islets have a very poor viability and stability in-vitro (O'sullivan et al, 2010). Limited supply of islets makes it even harder to work with them.

To reduce the above-mentioned risk involved in pancreatic transplantation, new strategies have been developed for islet transplantation. These includes

1. Immunoisolation of islets to minimize the need for long term immunosuppression.
2. Transplantation of β cells derived from *in vitro* differentiation of stem cells to improve donor tissue source.
3. Mimicking the islet niche and native interactions in the capsules to improve efficacy of islet transplantation.
4. Natural and synthetic polymers as means of transplantation to enhance the efficacy of islet survival

Various categories of cell type have been used for pancreatic tissue engineering. It ranges from using allogenic and xenogeneic source of islets to alternative sources such as embryonic stem cells, and mesenchymal stem cells (Luan et al, 2014; Gazda et al, 2014, Vegas et al, 2016; Khorsandi et al, 2015). Though allogenic and xenogeneic islets have shown promising results, issues like fewer availability and poor stability post isolation have limited their role in the field. Stem cells on the other hand is taking the centre stage in current research scenario as it has shown promising differentiation potential (Montanya, 2004). While embryonic stem cells are banned in various countries due to the ethical issues surrounding it, mesenchymal stem cells

isolated from different sources has been widely studied (Aloysious et al, 2014; Khorsandi et al, 2015).

After identifying the appropriate cell type to be used, it is also important to decide the culture environment i.e. whether to culture pancreatic cells individually or co-culture them with other cell types. Transplantation of only β cells has displayed limited success. On the other hand, few groups have shown that β cells co-cultured with other cell types such as fibroblasts, mesenchymal stem cells have improved viability, functionality and insulin secretion ((Jalili et al, 2011; Kim et al, 2017; Hamilton et al, 2017). However, the major problem associated with islet transplantation is substantial cell loss post isolation and transplantation caused due to hypoxia induced apoptosis, loss of suitable microenvironment and immune rejection (Luo et al, 2013; Zheng e al, 2012; Wang et al, 2013). Therefore, current research has also been actively focusing on the use of encapsulation which will prevent the transplanted cells to come in direct cross fire of the host's immune system. Moreover, growth factors and angiogenic factors have also been encapsulated within the construct which can aid in maintaining the viability of transplanted construct (Opara et al, 2010).

Finally, creation of appropriate scaffold that can act as a natural environment for the cells is of utmost priority. Various polymers have been tried and tested for their application in scaffold design. Both natural and synthetic polymers have been tested for tissue engineering of pancreatic construct. These polymers include Natural polymers: Agarose, collagen, gelatin, Fibrin, alginate, silk. Synthetic Polymers: Polyglycolic acid, Polylactic acid, PEG, PLGA, PCL and PDMS

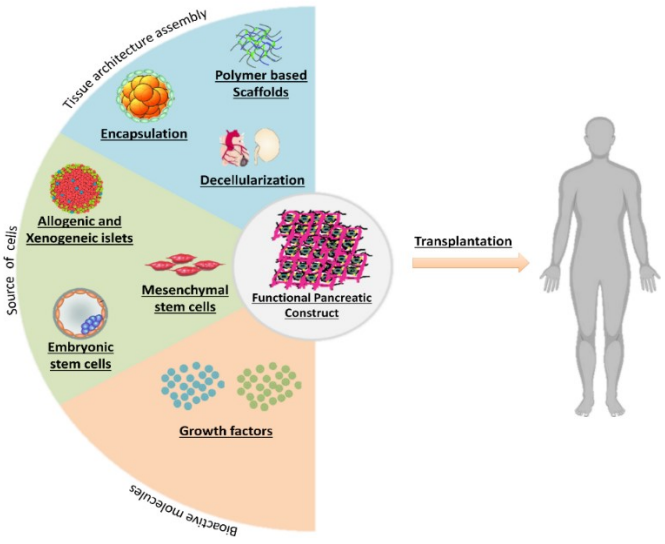


Figure 1.6: Outline of advances in pancreatic tissue engineering**.**

1.8 Natural polymers

1.8.1 **Collagen**

Collagen is a structural basement membrane protein and a widely used biomaterial for cell attachment and proliferation (Ramshaw et al, 2009). Being a part of the extracellular matrix, collagen has found wide usage in tissue engineering. It has been used for engineering heart valve (Tedder et al, 2011), lungs (Dunphy et al, 2014), bone (Zhou et al, 2015) etc. Several reports across the world have indicated the use of collagen for pancreatic tissue engineering. One report showed incorporation of fibroblast in Type-1 collagen gels. Before solidification, islets were also embedded in the collagen gel. Collagen provides the ECM for islet's growth and fibroblast maintains the matrix integrity. This scaffold showed improved cell survival and insulin secretion. Importantly, incorporation of fibroblast reduced the number of islets required to reverse diabetes in transplantation (Jalili et al, 2011). Another study combined other basement membrane protein (laminin and heparin sulphate proteoglycan) along with collagen to form gels to which islets were embedded. These cells not only showed better proliferation, attributed to the reduced caspase-3 expression but also improved cell survival (Xu et al, 2011).

Another report used scaffolds containing rat tail collagen cross linked with EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) -NHS (N-hydroxysuccinimide) and containing combination of chondroitin-6-sulfate, chitosan and mouse laminin to incorporate neonatal porcine islets. The islets survived up to 28 days indicated by positive insulin and glucagon staining. Also, matrix didn't show any signs of inflammation and scaffold could maintain its shape and size for over 28 days (Ellis et al, 2011). Another study illustrated early restoration of euglycemia post transplantation (from 17 to 3 days) relative to controls using PLG scaffolds coated with collagen IV, laminin and fibronectin (Yap et al, 2013). The collagen-IV modified scaffold showed improved islet survival, enhanced islet metabolism and better glucose induced insulin secretion.

Collagen alone does not provide the necessary mechanical strength required for pancreatic tissue. Hence, a combination of other polymers such as chitosan, chondroitin-6-sulphate and laminin or crosslinking has been used to improve the scaffold (Ellis et al, 2011). Almost all the studies mentioned above show that incorporating collagen with other basement membrane proteins tends to improve islet survival and function.

1.8.2 **Gelatin**

Gelatin, a natural product generated from hydrolysis of collagen has offered great potential as a scaffolding material (Hoque et al, 2015). Being a natural polymer and having beneficial properties of biocompatibility, biodegradability, lack of antigenicity and immunogenicity, gelatin based scaffolds have shown promising results for tissue engineering of cartilage (Chen et al, 2016), bone (Maji et al, 2016), skin (Han et al, 2014) etc. Various groups across the globe have shown effective use of gelatin and its blends for engineering islets. Collagen being a component of basement membrane of ECM in adult human pancreas gives gelatin an advantage over other polymers. One of the major properties required for the pancreatic tissue engineering is good mechanical strength. Gelatin alone does not fulfil this criterion hence various blends of gelatin with other polymers have been used for the same.

Gelatin has been used for encapsulating rat pancreatic islets grown on poly glycolic acid scaffold. These engineered islets were transplanted to Streptozotocin (STZ) induced diabetic nude mice. Diabetic mice maintained normal glycemia till 120 days after transplantation with the islets showing potential to secrete exogenous insulin (Kodama et al, 2009). Muthyala *et al* used gelatin to synthesize 3D porous interpenetrating polymer network (IPN) scaffolds along with poly (vinylpyrrolidone) (PVP) using the cross linkers glutaraldehyde and 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) by freeze drying method (Muthyala et al, 2010). IPN scaffold displayed ideal properties for tissue engineering with good mechanical strength. Out of the many scaffolds synthesized, one of the scaffolds (gelatin-PVP-semi IPN) showed good growth of viable β cells even up to 30 days (Muthyala et al, 2010). Further the authors presented that a combination approach consisting of mouse islets grown on the gelatin-PVP-semi IPN scaffold encapsulated in a PU–PVP–semi-IPN microcapsule (capsule made up of polyurethane–extrusion grade Tecoflex 60D (TFPU) and PVP coated with semi IPN solution) showed diabetes reversal and maintenance of euglycemia in rat models for up to 90 days (Muthyala et al, 2011). Previous studies have used gelatin in combination with dextran to produce three scaffolds (DEXGEL). Sodium meta periodate was used to incorporate aldehyde group in dextran which could link with amine group of gelatin thereby negating the use of additional cross linker. DEXGEL served as a platform for differentiation of adipose stem cells into islet like clusters. These islets exhibited higher level of insulin secretion as compared to 2D culture systems (Aloysious et al, 2014).

1.8.3 **Fibrin**

Fibrin, a protein involved in blood clotting has been used widely for tissue engineering applications owing to the properties such as self-assembly and soft elasticity (Janmey et al, 2009). Fibrin hydrogels have shown various impressive properties such as low toxicity to cells, cell attachment, proliferation and migration (Yasuda et al, 2010; Riopel et al, 2015). Fibrin gels have been used to chemically differentiate human endometrial stem cells into pancreatic β cells using activin A, nicotinamide, FGF (Fibroblast growth factor) and EGF (Epidermal growth factor) (Niknamasl et al, 2014). Insulin secretion was found to be higher in 3D fibrin gel enclosed with differentiated cells as compared to their 2D counterparts. Khorsandi *et al* have shown differentiation of bone marrow derived mesenchymal stem cells into insulin producing cells using 3D culture by fibrin glue (Khorsandi et al, 2014). Previously, long term proliferation of rat insulinoma cells line (INS-1) on fibrin gels had showed increased insulin secretion in response to glucose stimulation (Riopel et al, 2013). Even with these advantages of using fibrin gels for pancreatic tissue engineering, there are some unaddressed issues associated with it.

Fibrin has also shown to significantly improve insulin secretion in diabetic mice which were transplanted with fibrin cultured islets. These mice had shown highly vascularized islets along with improved viability (Kim et al, 2012). This shows the importance of fibrin not only in maintaining cell viability of islets, but also their angiogenesis. Though there has been lot of improvement using fibrin for islets proliferation, one of the major drawbacks of using fibrin is the possibility of immune response *in vivo* (Janmey et al, 2009). Furthermore, the potential application and risks associated with fibrin for islets proliferation and transplantation is yet to be established.

1.8.4 **Agarose**

Agarose, a naturally occurring polysaccharide, is one of the most used polymers in the field of tissue engineering. Its favourable properties such as biodegradability, soft tissue like mechanical properties, strong and rapid gelling capacity make it an ideal candidate for soft tissue engineering (Kohane et al, 2008). Agarose gels have been used as a gene delivery vehicle (Kohane et al, 2008), scaffold for implantation surgery (Varoni et al, 2012), cartilage tissue engineering (Bhat et al, 2010), liver tissue engineering (Tripathi et al, 2015) etc.

For islet engineering, agarose-agarose islet macrobead was used to encapsulate porcine islets. This macrobead was xenotransplanted in pancreatectomized dogs. This along with Pravastatin (mild anti-inflammatory agent) therapy showed prolonged functionality and biocompatibility

of the islets (Gazda et al, 2014). Luan *et al* transplanted islets in a prevascularized subcutaneous space. Induction of blood vessels was performed using freeze dried agarose rods comprising of bFGF and heparin. 1500 islets were transplanted in the prevascularized subcutaneous tissue without using any immunosuppressing agents. This transplantation reverted hyperglycaemia and showed long term allogenic islet graft survival (Luan et al, 2014). One report used agarose microwells made up of polydimethylsiloxane (PDMS) moulds for formation of primary islet aggregates, pseudo islets with pre-defined proportions. It was found that dissociated islets when aggregate in a controlled environment can lead to change in the core mantle arrangement of α and β cells which modifies itself after implantation under the kidney capsule. After transplantation, these islets behaved almost like the native islets thereby emphasising the importance of cell to matrix interaction, appropriate size and shape of islets for maintaining their structure and function *in vivo* (Hilderink et al, 2015). Another study used size-controlled pseudo islets from rat pancreas on agarose gel based microwells. The micromolds were synthesized using soft lithography with different diameters (100, 300, 500 μm). These small islet aggregates showed better insulin secretion and cell survival as compared to medium and large aggregates. Also, native tissue-like cellular organization was seen in both small and medium islets (Ichihara et al, 2016). This study highlighted the role of using size dependent islets for transplantation. Recently, a novel approach of combining agarose gel scaffolding with bone marrow derived mesenchymal stem cells (BM-MSCs) showed improved insulin secretion as compared to controls where islets were grown on only agarose gels. This study highlighted the role of BM-MSCs and agarose gels in improving the overall functionality of islets. It is suggested that BM-MSCs provide growth factors and paracrine signalling and agarose gels allows cells to absorb nutrients in all directions which helps in better islet function (Kim et al, 2017).

1.8.5 **Alginate**

Microencapsulation, as mentioned above is the process of entrapping cells or tissues within polymeric membrane which will act as immunosuppressive barrier (Calafiore et al, 2014). Currently, a lot of research has been done in microencapsulating β cell grafts, which will allow easy transplantation, immunoprotection and use of non-human islets (Zhu et al, 2015). One of the most commonly used polymer for microencapsulation is alginate. Alginate is a polysaccharide isolated from brown sea weed (Steele et al, 2014). It has gained tremendous popularity after Lim *et al* used alginate beads encapsulated with islets as artificial pancreas (Lim et al, 1980). From then, usage of alginate has come a long way. Alginate hydrogels have

found application as beads, delayed gelation systems, macroporous scaffolds, 3D printed scaffolds etc (Andersen et al, 2015). Though alginate beads allow rapid and non-toxic approach to encapsulate cells, property of limited cell adhesion possess a huge disadvantage for its application in tissue engineering. Therefore, for islets tissue engineering, alginate has been mainly used for microencapsulation. A breakthrough in encapsulation research came in 2010 when Opara *et al* suggested a multi-layer model of bioartificial pancreas containing two alginate layers separated by a semi-permeable membrane made up of poly-l ornithine. The inner layer was used to encapsulate islets and the outer layer was for angiogenic proteins. These microcapsules were implanted in the omental pouch of the rat. They observed that use of alginate beads for controlled delivery of growth factors can initiate blood vessel formation thereby improving the graft viability and function (Opara et al, 2010). Gelation of alginate takes place by presence of ions (Ca^{2+} or Ba^{2+}). But these ionically bound alginate hydrogel may not be able to withstand the mechanical stress associated with implantation. Hence, alginate was modified by incorporating a carboxylic group on alginate backbone and then covalently linked to modified PEG (Phosphine group at the end) by using Staudinger ligation. This hydrogel showed better stability and cell attachment than the alginate controls (Hall et al, 2011). The microencapsulation system proposed by Opara *et al* was improved with thick and crosslinked outer alginate layer. This helped in maintaining the stability of system for a longer period and this microcapsule remained intact even after 90 days of transplantation. This work also further highlighted omental pouch as a potential implantation site for islet transplantation (Pareta et al, 2014). Furthermore, Richardson *et al* showed stage wise directed differentiation of alginate encapsulated human embryonic stem cells into islet like cells. Clear viable colonies were evident after differentiation and maturation. Encapsulated cell differentiation resulted in strong maturation marker expression and improved hormone secretion as compared to their 2D counterparts (Richardson et al, 2014). Additionally, 3D bio plotting has been used to formulate alginate-gelatin porous scaffold which can be used as extrahepatic islets delivery system. Bioplotting is a technique which causes extrusion polymers to create custom engineered scaffolds. When islets were removed from the hydrogel, they showed full functionality (Marchioli et al, 2015). This study is one of the most recent reports on use of 3D bio plotting for islets engineering. Another recent study displayed the use of a modified form of alginate i.e (triazole-thiomorpholine dioxide (TMTD) alginate for islets implantation. This group also highlighted the fact that size of the microspheres also affects the immunological response to the implants. Human embryonic stem cells derived β cells (SC-β cells) encapsulated in 1.5 mm TMTD alginate spheres showed better glycaemic control than the convetionally used 500 μm

alginate spheres. This is the first study reporting long term glycaemic control in immune competent mice containing SC-β cells (Vegas et al, 2016). This report highlights the role of alginate and its derivatives as an immunoisolatory device in a xenotransplantation setting. Alginate encapsulated islets have also been used for clinical applications for patients with type 1 diabetes by various groups. They have shown long term stability of capsule in the body with continuous reduced dependence on exogenous insulin (Haitao et al, 2015). But a perfect site of implantation which overcomes all the disadvantages is yet to be determined (Calafiore et al, 2014).

1.8.6 **Silk**

Silk protein which is commonly used in textile industry is produced by silk worms and spiders. The fibrous protein in it native form consists of a component sericin which can elicit an inflammatory response (Edgar et al, 2016). This can be removed by the process of alkali or enzyme based 'degumming'. Apart from textiles silk has also found application in tissue engineering and drug delivery. Silk offers various outstanding properties which amplifies its role as a biomaterial. One of them being excellent mechanical strength. Silk's mechanical strength is higher than the Kevlar, which is used as a reference point in high performance fibre technology (Kundu et al, 2013). Apart from this it has good biocompatibility, water based processing, ease of chemical modifications, biodegradability etc (Kearns et al, 2008; Kundu et al, 2013). Silk can be moulded in any form like films, electro-spun fibres, hydrogels, scaffolds and particles.

In the field of tissue engineering, silk and its combination with other polymers has been used for wound healing (Kanitkar et al, 2014), bone (Shao et al, 2016), tendons and ligaments (Naghashzargar et al, 2015), urethra (Wei et al, 2015), cartilage (Kundu et al, 2013; Singh et al, 2016) etc. Silk has also been widely used for pancreatic tissue engineering.

Silk hydrogels have been used to encapsulate mice islets. These hydrogels provided a 3D environment in which the islets could maintain their viability and functionality. In the normal pancreas, islets are surrounded by ECM containing collagen, laminin and fibronectin which helps in cell adhesion and proliferation. To mimic similar situation, extracellular proteins and secondary stromal cells were incorporated in silk hydrogel which showed enhanced islet function (Davis et al, 2012). Another study showed that oral ingestion of silk fibroin hydrolysates helps in maintaining pancreatic β cell integrity and improves insulin secretion by increasing the β cell mass in hyperglycemic mice (Do et al, 2012). Co-encapsulation of β cells

and mesenchymal stem cells (MSCs) using silk hydrogels has also been explored. Though silk is a magnificent biomaterial, it still might stimulate host inflammatory response which is deleterious for islets growth. But presence of MSCs helps in reducing this effect because of its immunomodulatory and angiogenic properties. This multi-dimensional approach has shown good results in terms of graft's functionality and revascularization with an undesirable drawback of bone differentiation (Hamilton et al, 2017). Recently, Kumar *et al* microencapsulated silk scaffold with alginate and agarose. This scaffold showed sustained growth for rat insulinoma cells (RIN-5). Rat β cells also showed better growth on the 3D scaffold as compared to its 2D counterpart which was confirmed by expression of primary pancreatic genes (Kumar et al, 2017).

1.9 Synthetic Polymers

1.9.1 **Polyglycolic acid**

Polyglycolic acid (PGA) is a US Food and Drug Administration approved biocompatible polymer obtained by ring cleavage polymerization of glycolide. PGA hydrolyses *in vivo* to give glycolic acid, which is a metabolite in the citric acid cycle, thus, resulting in low toxicity (Athanasiou et al, 1996); Chun et al, 2008). PGA has wide applications in the field of tissue engineering due to its tuneable degradation rate and intrinsic nature to form stable three-dimensional structures (Asti et al, 2014). However, PGA undergoes rapid absorption *in vivo*, causing failure of the scaffold. Also, inflammatory responses are provoked owing to increased release of acidic degradation products. Combination of PGA with several copolymers like PLGA or PEG has been shown to enhance its physical and mechanical properties (Asti et al, 2014). PGA has been widely used to make bioresorbable sutures and cartilage regeneration (Asghari et al, 2016).

A hybrid scaffold of collagen and PGA with basic fibroblast growth factor has been developed to promote wound healing in type 2 diabetic mice. This hybrid matrix had enhanced compression strength, thus suppressing wound contraction, while also inducing angiogenesis and granulation tissue formation (Nagato et al, 2006). Another study by Chun *et al* showed the growth of islet cells on PGA scaffold functionalized with a layer of poly-l-lysine, which enhanced the surface activity and adhesion capacity of PGA scaffold, promoting cell proliferation. The PGA scaffold was also shown to provide superior nutrient absorption and metabolite excretion to the cultured islets, providing apt microenvironment for their growth and survival. The cultured islets exhibited enhanced viability, better morphology and improved

glucose-stimulated insulin secretion (Chun et al, 2008). The viability of PGA-islet grafts transplanted into the leg muscles of rats with streptozotocin-induced diabetes has also been investigated. This scaffold provided a compatible 3D microenvironment with visible adhesive growth of islets on the scaffold, adequate blood supply and nutrients. The results showed higher insulin secretion and significant decrease in blood glucose concentration in rats transplanted with PGA-islet grafts as compared to control (Song et al, 2009). Recently Li *et al* used PGA scaffolds for increasing the efficacy of islet coating by endothelial cells (ECs). Coating of ECs on the islets has been shown to improve revascularization and reduce initial inflammatory response. Due to the presence of PGA scaffolds, enhanced coating efficiency of ECs on the islets was observed. The islet functionality was also improved with enhanced high glucose-stimulated insulin release. The authors thus, suggested the use of PGA scaffold in pre-transplant culturing of islet cells and ECs (Li et al, 2017).

1.9.2 **Polylactic acid**

Polylactic acid (PLA) is a US Food and Drug administration approved aliphatic polymer with wide applications in the field of biomedical devices and tissue engineering (Fonte et al, 2015). PLA hydrolyses *in vivo* to give lactic acid, which gets incorporated into the citric acid cycle and is naturally excreted, thus making PLA biocompatible, biodegradable in nature (Sabek et al, 2016). However, numerous surface treatments need to be implemented to hydrophobic PLA to impart enhanced biomimetic and cell adhesion properties (Farina et al, 2017). PLA has tuneable and versatile physical and chemical properties and can be moulded to take on a myriad of shapes, including microspheres, scaffolds, sutures, and nanoparticles (Tyler et al, 2016). Taking advantage of its long half-life, PLA has been extensively used in fabrication of long-term implantable devices for therapeutic applications (Sabek et al, 2016). PLA and its copolymers have vast applications in skin grafting and bone, spinal cord and nerve regeneration (Tyler et al, 2016).

In the field of pancreatic tissue engineering, the potential therapeutic application of polylactic acid microspheres has been studied in the treatment of diabetic periodontitis. The microspheres, loaded with 25-hydroxyvitamin D3 were shown to inhibit inflammatory response and bone loss in rats with diabetic periodontitis (Fonte et al, 2015). PLA-PEG based nanoparticles have also been used as a means for subcutaneous delivery of insulin. Nanoparticles loaded with 50 IU of insulin load per kg were shown to control the blood glucose level, thus, reviving normoglycemia in diabetic rats. These biodegradable nanoparticles were proved to be non-

toxic in nature and thus qualifying as potential candidates for parenteral insulin therapy (Sabek et al, 2016). Kasujo *et al* described the application of PLA based porous capsules to obtain a vascularized microenvironment for extra-hepatic islet transplantation. The bioartificial cavity showed numerous vessels, guided infiltration of host's connective tissue cells and vascular endothelial cells with no significant infiltration by inflammatory cells, hence serving as a favourable microenvironment for islet transplantation (Kasujo et al, 2015). A three-dimensional delivery system has been developed which can be used for encapsulation and implantation of pancreatic cells. The PLA based nanogland provided support to islet-like aggregates, derived from differentiation of human mesenchymal stem cell, enhancing their viability and maintaining their function *in vitro*. The nanogland was shown to provide steady secretion of insulin, thus, having potential implication for diabetic cell therapy (Sabek et al, 2016). Recently, a 3D printed encapsulation system has been formulated using polylactic acid for subcutaneous implantation of pancreatic islets. Surface treatment was employed to functionalize the system and it was implanted with VEGF enriched platelet gel, to help enhance vascularization. This study highlighted the transcutaneous refillability and potential retrievability of the graft, emphasizing on its application in the field of diabetic cell therapy (Farina et al, 2017).

1.9.3 **Polylactic-co-glycolic acid**

Polylactic-co-glycolic acid (PLGA) is a US Food and Drug Administration approved co-polymer obtained by ring-opening co-polymerization of lactide and glycolide (Pan et al, 2012). PLGA has been widely used in varied forms like films, porous scaffolds, hydrogels, or microspheres for biomedical, tissue engineering and drug delivery purposes owing to its high biocompatibility and non-toxic biodegradation (Gentile et al, 2014). An added advantage to the physico-chemical properties of PLGA is the tuneable mechanical strength and biodegradation rate achievable by altering the PLA: PGA ratio (Makadia et al, 2011). Nonetheless, PLGA has poor surface characters like hydrophilicity, protein absorbance and poor cell affinity (Zhao et al, 2016). Numerous surface modulation strategies like surface immobilization, physical adsorption of bioactive molecules, plasma treatment and incorporation of other biocompatible materials into the PLGA matrix have been applied to make the interface between PLGA and its environment more biomimetic and to enhance the cell affinity (Gentile et al, 2014).

Recently, biocompatible PLGA scaffolds have been fabricated using 3D printing to be used for tissue engineering (Mironov et al, 2017). Electrospun PLGA-based hybrid nano fibrous

membranes and scaffolds have been widely used for skin, bone, nerve and soft tissue engineering applications (Zhao et al, 2016).

In the field of pancreatic tissue engineering, micro porous, biodegradable PLGA has been successfully utilized as a platform for islet transplantation in mouse models (Blomeier et al, 2006). Salvay *et al* explored the effects of PLGA scaffolds with adsorbed ECM components on the survival of transplanted islets. It was noted that adsorption of these proteins on the scaffold enhanced the efficacy of islet grafts and significantly decreased the time taken for the reversal of diabetes in type 1 diabetes mice (Salvay et al, 2008). The effect of integration of ECM components on long-term *in vitro* maintenance of human pancreatic islets, cultured in a micro fabricated PLGA scaffold has also been investigated. The PLGA scaffold provided for a viable niche, with the *in vitro* cultured islets displaying insulin release profiles characteristic of native islets (Daoud et al, 2011). Kheradamand *et al* demonstrated the use of PLGA scaffolds as an extra-hepatic site for islet transplantation. The addition of ethylcarbodiiminde(ECDI)-fixed donor splenocyte infusions to the PLGA scaffolds enhanced the efficacy of tolerance induction *in vivo*, and indefinite normoglycemia was maintained in diabetic mice models (Kheradamand *et a*l, 2011). Bioresorbable PLGA microspheres have been designed for encapsulation and sustained administration of β-cell proliferative compounds to intact mouse islets in culture. The improved bioavailability of the mitogen to the β-cells *in vivo* can lead to increased β-cell proliferation, which has been proposed to have therapeutic applications in restoration of blood glucose levels in diabetic patients (Pasek et al, 2016). Recently, Liu *et al* highlighted fabrication of artificial islet tissues using fibroblast-modified PLGA membrane for differentiating pancreatic stem cells into insulin producing cells. This construct secreted insulin and was shown to reduce blood glucose levels in diabetic nude mice. The modified PLGA membrane showed higher compatibility, better proliferation and increased viability of pancreatic stem cells, and enhanced histocompatibility with nude mice (Liu et al, 2017).

1.9.4 **Polycaprolactone**

Polycaprolactone (PCL) is a hydrophobic, biodegradable and a US Food and Drug Administration approved polymer prepared by ring-opening polymerization of ε-caprolactone in the presence of SnO_2 and heat (Asghari et al, 2016). PCL has gained an edge in the field of biomedical research owing to its low-melting point, remarkable blend-compatibility and viscoelastic properties. PCL has wide applications in the drug-delivery systems, as surgical sutures as well as scaffolding material for tissue engineering due to its properties like tuneable

degradation rates and mechanical properties (Mkhabela et al, 2014). Drawbacks associated with PCL include hydrophobicity, limited bio regulatory activity and susceptibility to bacteria-mediated degradation (Asti et al, 2014). To enhance favourable cellular responses, various functional groups have been incorporated onto the polymer, making it more hydrophilic and biocompatible (Mkhabela et al, 2014). PCL and its copolymers like PCL-PEG and PCL-PLA copolymers have been shown to have varied applications for cartilage, bone and peripheral nerve regeneration (Asghari et al, 2016).

Nanofibrous PCL scaffolds have been used for differentiation of human iPSCs (Induced Pluripotent stem cells) into definitive endoderm cells using IDE1 (inducer for definitive endoderm 1). Electrospun PCL scaffold exhibited more pores, decreased toxicity and reduced thickness of the nano fibres, providing more surface for cellular proliferation and attachment (Hoveizi et al, 2014). A composite using polycaprolactone (PCL)/polyacrylic acid (PAAc) hydrogel have found application in oral delivery of the drug, Gliclazide which is used in the treatment of type 2 diabetes. The balance of hydrophobic PCL with hydrophilic PAAc provided the control of swelling property of the hydrogel. The PCL/PAAc hydrogel offered a controlled release of the drug and was shown to enhance its bioavailability, resulting in reduced glucose levels (Bajpai et al, 2015). PCL also found application in diabetic wound healing. Gholipour-Kanani *et al* blended PCL with chitosan to avoid the use of chemical cross-linkers and achieve a nanofibrous scaffold with sustainable integrity in aqueous media. This polycaprolactone-chitosan-poly (vinyl alcohol) (PCL:Cs:PVA) scaffold was further proved to promote diabetic wound healing owing to their biocompatibility and structural similarities to the native ECM (Gholipour-Kanani et al, 2016). Another report showed the application of curcumin-loaded poly(ε-caprolactone) (PCL)/gum tragacanth (GT) (PCL/GT/Cur) nanofibers in the field of wound healing. The antibacterial nanofibrous membranes enhanced the healing process owing to simulation of native ECM, presence of curcumin and GT, and enhanced mechanical stability of scaffold due to the presence of PCL. Tissue engineered scaffolds were also shown to decrease blood glucose levels in the rat models (Ranjbar-Mohammadi et al, 2016). A current finding highlighted the use of heparanized ring shaped PCL scaffold functionalized with VEGF for containing islets in an alginate core. Vascularization was successfully induced throughout the scaffold due to the presence of immobilized VEGF. The embedded islets were shown to maintain their viability and functionality; responding normally to glucose stimulations, at the same time possessing plausible immune protection properties. The scaffold demonstrated improved revascularization and can be proposed as potential approach for subcutaneous islet

transplantation (Marchioli et al, 2016). Recently, Smink *et al* demonstrated the use of a modified PCL, poly (D, L-lactide-co- ε-caprolactone) (PDLLCL) to create a scaffold which acted as an artificial and retrievable subcutaneous transplantation site for pancreatic islets. PDLLCL was shown to not interfere with islet viability and functionality. Also, islets cultured on PDLLCL exhibited comparatively more insulin granules and lower release of immune system provoking double-stranded DNA, thus, suggesting PDLLCL as suitable scaffold having potential application for treatment of type 1 diabetes (Smink et al, 2017).

1.9.5 **Polydimethylsiloxane**

Polydimethylsiloxane (PDMS), a silicon based organic polymeric compound has been commonly used as a surfactant, stamp resin for soft photolithography etc. Its superior properties make it a better choice for tissue engineering than the other synthetic equivalents. PDMS has high biocompatibility, biostability, excellent oxygen solubility which makes it a perfect candidate for implantation (Pedraza et al, 2013). PDMS was used to construct a macroporous scaffold via solvent casting and particulate leaching method. PDMS has a hydrophobic surface which is ideal for slow release of compound but does not support cell adhesion. Therefore, fibronectin was added onto the surface of PDMS scaffold to make it hydrophilic. Islets loaded onto PDMS scaffolds and implanted in the omental pouch showed good islet retention and long term normoglycemia. Interestingly, islets on the scaffold showed enhanced viability and function under low oxygen tension when compared to 2D controls (Brady et al, 2013). Another study seeded a fibrin-platelet derived growth factor hydrogel loaded with islets onto the PDMS scaffold and transplanted it into the mice. This system helped to reduce the time required to attain normoglycemia and displayed increased vessel branching (Jiang et al, 2017). Recently PDMS scaffold has also been used for incorporation of anti- inflammatory agents such as dexamethasone and Fingolimod (Jiang et al, 2017; Zawalich et al, 2013). Dexamethasone was added in various quantities in PDMS scaffolds. Low concentration of dexamethasone showed improved islets engraftment, but higher concentrations were found to be detrimental as it alters glucose induced insulin secretion by supressed activation of the PLC/protein kinase C signalling system (Jiang et al, 2017; Zawalich et al, 2013). Fingolimod exhibited persistent release but at a very low concentration (0.1% w/w) which do not have any significant effect on the islets (Frei et al, 2018).

1.9.6 **Polyethylene glycol**

Polyethylene glycol (PEG) is one of the most popular synthetic polymer used for tissue engineering applications. PEG is a less immunogenic material, has tissue-like elasticity and well-defined chemistry which gives it an edge over other polymers for islets engineering (Kozlovskaya et al, 2012). PEG has been used in the form of scaffold and encapsulating agent for islets transplantation. As PEG is biologically inert, it doesn't not support any form of cell growth. Hence to use PEG as a scaffold, it must be augmented with another co-polymer. Mason *et al* used collagen fibrils in PEG hydrogels and studied it effect on encapsulated embryonic pancreatic precursor cells. Cells on these scaffolds showed high glucose responsiveness and had improved level of insulin gene expression (Mason et al, 2009). Furthermore, PEG scaffold was also supplemented with fibrin ribbons which was used for co-culturing endothelial cells and islets together (Mason et al, 2010). Endothelial cells were encapsulated within the fibrin ribbons and islets in the PEG hydrogel. Their results suggested optimum growth for both cell types, penetration of endothelial cells into the hydrogel and improved vascularization. One of the major problems associated with islet transplantation is the requirement of large number of islets. To overcome this issue, surface modifications of islets are currently being utilized. The main objective of this technique is to reduce the number of islets required for transplantation (Kozlovskaya et al, 2012). GLP-1 or glucagon like factor-1 is produced by the L cells of distal ileum and is a insulinotropic ligand. Kizilel *et al* directly immobilized GLP-1 on the surface of islets by layer-by layer assembly of biotin-PEG-NHS, Streptavidin, and biotin PEG-GLP-1. Coated islets showed better insulin secretion than the control islets in response to high glucose which proves the efficiency of this technique. This study also addressed the issue of donor shortage as it required lower number of transplanted islets to achieve normoglycemia (Kizilel et al, 2010). Another study using PEG as an encapsulating agent developed a device which had rat islets growing on an acellular scaffold and encapsulated in a PEG/VA semi permeable membrane. This device was incorporated in diabetic rats and showed reduction in insulin requirement which lasted for 2 weeks. This device restored partial insulin secretion. For achieving complete euglycemia optimal islet number to be transplanted needs further investigation (De Carlo et al, 2010). PEG based hydrogel microwells have also been developed using photolithography. MIN6 β cells were seeded on the microwells and were maintained for 5 days after which they were retrieved and encapsulated. This PEG based microwell consistently demonstrated successful formation of MIN6 aggregates and the encapsulated MIN6 aggregates showed better insulin secretion and positive expression of intracellular

binding protein E-cadherin as compared to single cell encapsulations (Bernard et al, 2012). One of the major causes of islet loss after transplantation is hypoxia which affects the longevity of the implant. Therefore, to facilitate short oxygen supply to the islets, PEG stabilized haemoglobin had been used as an artificial oxygen carrier (Chae et al, 2002). But this system does not have a long-term use as continuous conversion of haemoglobin to methaemoglobin by autoxidation and free radical damage is deleterious to the cells. Therefore, PEG based haemoglobin conjugates cross linked with antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) has been used (Nadithe et al, 2012) and demonstrated excellent protection against free radicals and oxygen induced stress in RINm5F cell line. The viability of RINm5F was higher and reactive oxygen species (ROS) generation had reduced for cells treated with conjugates. Their results also showed sustained or increased insulin release from treated islets under partial oxygen pressure situations. This study has given wonderful insight in using PEG based conjugates for preventing hypoxia induced graft failure. Another attempt to prevent post transplantation islets loss was made by Golab *et al* where they immunoprotected the islets by coating them with Treg cells conjugated with Biotin-PEG-SVA (succinimidyl valeric acid ester). This was found to be better than previously used Biotin-PEG-NHS for coating pancreatic cells with Treg cells and showed only slightly better insulin secretion (Golab et al, 2014). PEG hydrogels have also been used for encapsulation of islets with rate bone marrow derived mesenchymal stem cells, GLP-1 and ECM based cell adhesion ligands. It has shown to improve insulin secretion and mesenchymal stem cells have also shown immunomodulatory effects. The overall functionality of islets had increased seven-fold compared to islets alone (Bal et al, 2017). As mentioned before, angiogenesis plays a major part in maintaining islets functionality. Pancreatic islets only comprise of 1-2% of the total pancreatic population but receive as much as 15-20% of the total pancreatic blood supply (Ballian et al, 2007). Therefore, maintaining similar angiogenic effect post transplantation is absolutely required. Phelps *et al* created PEG hydrogels with mild maleimide-thiol cross-linking. These scaffolds were further modified by addition of RGD motif for cell adhesion and VEGF for vascularization (Phelps et al, 2015). This study highlighted the use of mesentery as a transplantation site which is far less invasive than hepatic portal transplantation. This scaffold and the delivery strategy indicated beneficial outcomes in terms of vascular invasion and insulin secretion. This research also negated injection of islets into the blood stream which can cause immediate inflammatory reaction. Recently, a blend of synthetic PDMS and natural PEG polymer was used for transplantation of islets in epididymal fat pad (Rios et al, 2016). Islets were mixed with PEG and then casted in photolinked PDMS moulds. As the islets were encapsulated in PEG and

PDMS, they were protected from the immediate inflammatory attack from the immune system. One interesting finding in this study was the glucose tolerance test which showed normoglycemia within 90 minutes like mice with normal pancreata.

1.10 Gaps in existing research

1. Non-availability of pancreatic cells has led to search of newer alternatives. Embryonic stem cells and adult stem cells have been studied. As major ethical issues surround the usage of embryonic stem cells, mesenchymal stem cells are a better option. Differentiation potential of mesenchymal stem cells has not yet been explored to its complete potential. MSCs have been used for differentiation into various cell types but their role in pancreatic differentiation still needs to be studied.
2. As the scaffold acts as a natural environment for the growth of cells, characteristics like biocompatibility, biodegradability, vascularization, low toxicity and non-immunogenicity becomes indispensable. Various polymers have been tried and tested for their application in scaffold design. However, both natural and synthetic polymers fail to address all the major requirements for an ideal scaffold for pancreatic tissue engineering.
3. Nanocomposites have shown great promise in tissue engineering of bones, skin regeneration etc. but their ability in pancreatic tissue engineering has been sparsely investigated.

Therefore, taking all this lacuna into consideration, following objectives were formulated

- Isolation and characterization of mesenchymal stem cells from human placenta and designing three dimensional (3D) scaffolds for tissue engineering.
- Differentiation of mesenchymal stem cells into pancreatic cells in 2D as well as in 3D (scaffolds).
- To study the interaction of different pancreatic cells on scaffolds and characterization of different tissue constructs.

Chapter 2

Synthesis and characterization of agarose-chitosan coated silver nanoparticle composite scaffold

2.1 Introduction

Tissue engineering relies heavily on synthesizing a 3D scaffold which could act as a template for regeneration of tissues. In order for the cells to assemble and function properly it is important that the 3D scaffold acts as a natural microenvironment. Numerous scaffolds have been synthesized using different biomaterials, but regardless of the tissue to be grown on it number of key factors determines the suitability of the scaffolds for application (O' Brien, 2011). The most important of them being biocompatibility of the scaffold, allowing the cells to adhere, grow and migrate. Secondly the scaffold should possess time dependent degradation thereby replacing the artificial tissue over time (Fleischer et al, 2013). Furthermore, the scaffolds should possess the architecture with interconnected pores and with high porosity allowing diffusion of nutrients and wastes (Phelps et al, 2009). As scaffolds are vital elements of tissue engineering, a variety of fabrication techniques have been employed for making them. These techniques include freeze drying, solvent casting, gas foaming, electrospining, micromolding, etc. (Dhandayuthapani et al, 2011). Freeze drying or lyophilization is one of the most commonly used fabrication technique. This procedure works on the principle of sublimation where the polymer is mixed with a solvent and frozen. Later this solvent is removed by the process of lyophilization. Lyophilization reduces the pressure of the system which will allow the water in the frozen polymer solution to sublimate directly from solid to gas phase thereby giving porous and interconnected structures (Subia et al, 2010; Asghari et al, 2017).

For our research, agarose and chitosan coated silver nanocomposite was synthesized using freeze drying technique. Agarose is a naturally occurring polysaccharide isolated from red purple sea weed. Agarose form strong gels even at low concentrations due to its specific chemical structure. Its applications in pancreatic tissue engineering has been discussed in detail in Chapter 1. One of the major disadvantages associated with agarose is the fact that it lacks the ability of cell attachment, which limits its role in this field (Varoni et al, 2012). Therefore, a blend of agarose with other polymers have been tried for tissue engineering applications. Chitosan is a positively charged linear polysaccharide composed of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine. It is the most commonly used polymer in the field of tissue engineering because of its remarkable properties which includes biodegradability, biocompatibility, non-immunogenic, anti-bacterial properties, etc (Cao et al, 2009; Li et al, 2013; Hajiabbas et al, 2015; Cao et al, 2005). Chitosan forms secondary interactions (via hydrogen bond) with other polymers as it has polar groups present in its

chemical structures. Therefore, chitosan has found application in drug delivery systems, nutrition supplement and in wound healing. Chitosan blended with other polymers have been used in regeneration of almost all the organs including liver, kidney, pancreatic islets, heart, bone, etc (Trivedi et al, 2014; Patel et al, 2011; Yalçın et al, 2008; Bacakova et al, 2014; Mao et al, 2003; Liu et al, 2011; Aziz et al, 2012).

2.2 Materials

2.2.1 **Scaffold preparation**

- Chitosan (Sigma Aldrich chemical Co. (St. Louis, MO))
- Agarose (UltrapureTM) (Invitrogen, USA)
- Silver nitrate (Sigma Aldrich chemical Co. (St. Louis, MO))
- Glutaraldehyde solution (25%, for synthesis) (MERCK, USA)
- Sodium hydroxide, Acetic acid and DMSO (Fischer Scientific, USA)

2.2.2 **Cell culture reagents**

- Dulbecco's modified eagle's media (DMEM) (Hi-media, India)
- Foetal bovine serum (FBS) (Hi-media, India)
- Trypsin solution (Hi-media, India)
- Phosphate buffer saline (PBS) (Hi-media, India)
- 3-(4,5, di methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT (Hi-media, India)

2.2.3 **Cells**

- HeLa (Human cervical carcinoma)
- MiaPaCa-2 (Human pancreatic carcinoma)
- HEK (Human embryonic kidney)

2.2.4 **Bacterial Cultures**

- Staphylococcus aureus
- Bacillus subtilis
- Escherichia coli
- Klebsiella pneumonia

2.3 **Methods**

2.3.1 **Synthesis of chitosan coated silver nanoparticles**

Chitosan-coated silver nanoparticles were synthesized using a pre-optimised protocol (Jena et al, 2012). Chitosan was prepared at a concentration of 1 mg/ml at 45 °C with constant stirring. 0.1 N sodium hydroxide followed by 100mM silver nitrate was added to the mixture on a continuous basis. Immediate colour change to dark yellow suggested the formation of chitosan coated silver nanoparticles. The suspension was then allowed to settle and later centrifuged at 2000 rpm. The pellet was washed with distilled water twice, dried and dissolved in 0.1% acetic acid.

2.3.2 **Nanoparticle characterization**

In conjunction with the colour change, various other parameters were used to characterize chitosan-coated silver nanoparticles. Synthesized nanoparticles were characterized by UV spectroscopy using Shimadzu UV-2450 spectrophotometer. For transmission electron microscopy (TEM), a drop of aqueous solution of chitosan coated silver nanoparticles was placed on the carbon-coated copper grids. The samples were dried and kept overnight under a desiccator before loading them onto a specimen holder. TEM measurements were performed on JEM-2100, HRTEM, JEOL, JAPAN operating at 200 kV. The size distribution and zeta potential of nanoparticles were determined by Dynamic Light Scattering (DLS) using Zeta sizer nano-ZS Malvern Instruments, UK at Room Temperature. 1ml of sample was mixed well by vortexing, poured into disposable sizing cuvette for size distribution analysis. Three different peak readings, size versus percentage intensity were plotted. Three such readings were taken, and average size distribution was determined. Similarly, zeta potential was analyzed with help of clear disposable zeta cell. Average of three readings was taken to determine zeta potential distribution.

2.3.3 **Preparation of agarose-chitosan coated silver nanocomposite scaffold (AG-CHNp)**

Agarose was dissolved in water to prepare a 3wt% polymeric solution. The chitosan coated silver nanoparticles were synthesized with the Agarose solution. Different amounts of chitosan dissolved in acetic-acid aqueous solution was added to Agarose and allowed to mix for 15 min under constant stirring. The nanoparticles were synthesized as described above, leading to an immediate colour change into brownish yellow. Finally, the scaffold was prepared by using a cross linker, i.e. glutaraldehyde (1%) for one hour. The synthesized scaffolds would be denoted

as AG-CHNp1, AG-CHNp2, AGCHNp3 and AG-CHNp4 according to the increasing concentration of chitosan used. The synthesized scaffolds were frozen at -80°C for 24 h, followed by lyophilization for 16–18 h. Freeze-drying will cause sublimation of ice crystals directly into vapour phase which will in turn cause formation of porous structures **(Figure 2.1)**.

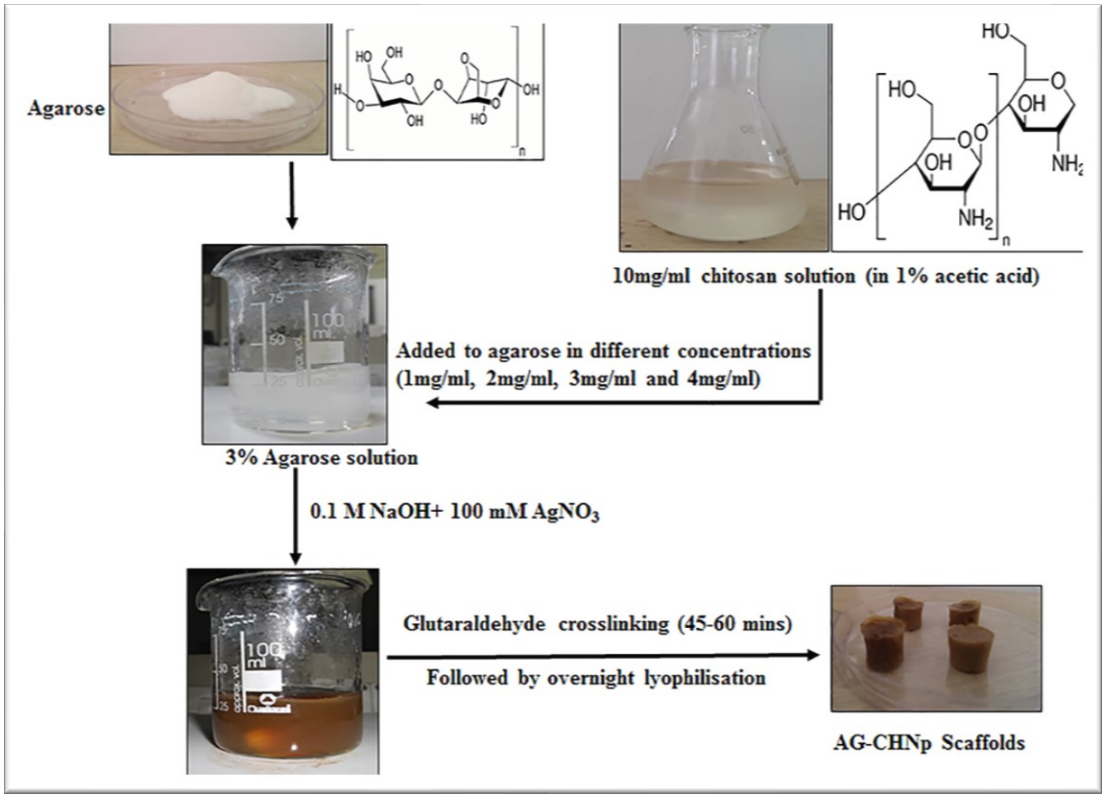


Figure 2.1: Schematic representation showing synthesis of Agarose-chitosan coated silver nanoparticle composite scaffolds.

2.3.4 **Characterization of AG-CHNp scaffolds**

AG-CHNp scaffolds were characterized at physical, mechanical and biological level for compatibility and usage. Following are the techniques used for characterization of scaffolds:

Physical characterization	**Biological Characterization**	**Mechanical Characterization**	**Other characterization**
1. X-ray diffraction 2. FTIR 3. UV-Visible spectroscopy	1. MTT Assay 2. Hemocompatibility assay 3. Anti-bacterial activity 4. DAPI staining	1. Dynamic Mechanical analysis 2. Thermogravimetric analysis	1. Swelling profile 2. In-vitro degradation assay

Table 2.1 Characterization of AG-CHNp scaffolds

2.3.5 **Physical Characterization**

The synthesized scaffold was characterized by several physio-chemical methods. UV-visible spectroscopic analysis of the scaffold just before gelation was performed to confirm the synthesis of embedded nanoparticles. The crystalline structure of the scaffolds was studied using X-ray diffraction. The XRD patterns were generated using Rigaku MiniFlex II at room temperature operating at a voltage of 30 kV. The readings were taken at 2h angle range from 5 to 50˚. For analyzing the chemical bonding and functional groups present within the scaffold, FTIR analysis was performed using Shimadzu IR Affinity-I with the help of an attenuated total reflectance (ATR) accessory. The FTIR spectrum was analyzed from 400-4000cm^{-}1.

2.3.6 **Biological Characterization**

2.3.6.1 **MTT Assay**

To assess the biocompatibility of the scaffolds, MTT assay was performed using HeLa cell line. Scaffolds were placed in an uncoated 24 well plate and sterilized using increasing concentration of ethanol followed by UV radiation for 20 minutes. The scaffolds were then equilibrated with complete media to facilitate gaseous exchange for 2-4hours. Following this, HeLa cells were seeded at a concentration of 1X10^5 cells per well and incubated for a period of 16 days at 37˚C with 5% CO_2. For control, same cell density was seeded in poly-lysine

coated 24 well plates. Media was changed every alternate day. Media was removed from the well and the scaffolds were given a gentle PBS wash before addition of MTT at 0.5mg/ml for 3-4 hours. After the incubation, media was aspirated out and the MTT crystals were dissolved with DMSO and incubated for 15-20 mins for color development. Absorbance was measured at 570nm to calculate number of viable cells.

2.3.6.2 **Hemocompatibility assay**

Fresh human blood was collected in 15ml centrifuge tube containing sodium citrate (10:1). The blood was then diluted with normal saline (8ml blood + 10ml saline). To study hemolysis, scaffolds were cut into 5mmX5mm size and placed in a tube containing normal saline solution and incubated for 30 minutes at 37˚C. Diluted blood was added to the tube and incubated for 60 minutes at 37˚C. The positive control had diluted blood added to sodium carbonate solution which caused hemolysis and the negative solution had blood in normal saline solution. Following the incubation, all the tubes were centrifuged for 5 minutes at 3000 rpm. The supernatant was transferred to the cuvette and readings were taken at 545nm.

Percentage hemolysis was calculated with the following formula

$$\frac{\text{OD (test)- OD (Negative Control)}}{\text{OD (Positive Control)- OD (Negative control)}} \text{X100}$$

2.3.6.3 **Anti-bacterial activity**

Scaffolds were also analyzed for their anti-bacterial activity against both gram positive and gram-negative bacteria by resazurin assay. The scaffolds were casted in a 96 well plate (approximately 150µl) and different concentration of bacterial cells (10^4, 10^5 and 10^6 cfu of bacteria) were added. The plate was incubated at 37˚C overnight. Following day, resazurin dye was added to all the wells and incubated for 3-4 hours.

2.3.6.4 **DAPI staining**

To visualize the attachment and proliferation of cells on the scaffolds, two cell lines (MiaPaCa2 and HEK) were seeded on the scaffolds and incubated at 37˚C with 5% CO_2 humidity for a period of five days. On the day of experiment, the media was aspirated out and the scaffolds were washed with PBS. Later the cells were fixed with 2.5% glutaraldehyde and dehydrated using ethanol gradient. Various sections of the fixed scaffold were cut, and the best obtained

sections were stained with DAPI containing mounting media for 15-20mins. Following incubation, sections were observed under fluorescence microscope using excitation (405nm) and emission wavelength (450nm) of DAPI.

2.3.7 **Mechanical Characterization**

2.3.7.1 **Dynamic mechanical analysis**

The storage and loss modulus of the scaffolds was examined by dynamic mechanical analyser using Universal VA.5A TA instruments.

2.3.7.2 **Thermal gravimetric analysis (TGA)**

TGA was performed using Shimadzu DTG-60. The samples were heated from 30 to 600°C with a heating rate of 10°C/minute.

2.3.8 **Other characterization**

2.3.8.1 **Swelling Profile:** The degree of swelling for a scaffold was studied using phosphate buffer saline (pH=7.4). The dry weight of scaffolds was measured before immersing in PBS solution for various time intervals (days 1, 3, and 7). On the respective day, the scaffolds were removed and excess solution was drained out by blotting onto a filter paper. Following which, the wet weight of the scaffolds was measured and swelling ratio was calculated as follows

$$\text{Swelling Ratio} = \frac{\text{Wet weight - Dry weight}}{\text{Dry Weight}}$$

2.3.8.2 ***In-vitro*** **degradation profile**

Initial weight of the scaffolds was noted down (Wr_i) and then were incubated in phosphate buffer saline containing lysozyme (10,000 units/ml) at 37°C for different time periods (7,14,21,28 days). Scaffolds were respectively removed, washed with deionized water and lyophilized. The final weight of the scaffolds was noted (W_f).

Percentage degradation was calculated using the following formula

$$\text{Rate of degradation} = \frac{W_i - W_f}{W_i} * 100$$

2.3.8.3 **Scanning electron microscopy**

The microstructures of the prepared scaffolds were examined by SEM (FEI Nova Nano FESEM 450; FEI, Hillsboro, OR). Sample preparation is same as used for DAPI staining. All the samples were gold coated and scanning was carried at voltage of 10 kV (for visualizing the surface of the scaffolds) and 5 kV (for visualizing MiaPaCa and HEK cells on the scaffolds).

2.4 **Results**

2.4.1 **Synthesis and characterization of chitosan coated silver nanoparticles**

Results for characterization of nanoparticles are shown in **Figure 2.2**. Ultraviolet visible spectroscopy showed a prominent and characteristic peak at 420nm, suggesting the formation of chitosan coated silver nanoparticles. Shape, size and crystalline nature of the synthesized nanoparticles were determined by TEM studies. TEM micrographs showed monodispersed and spherical nanoparticles. Size distribution profile of the synthesize nanoparticle were determined by dynamic light scattering. Three peaks corresponding to 23.12, 353.1 and 4841 nm were obtained suggesting that most of the particles synthesized were below 500nm. But a fine peak at 4800nm suggested agglomeration of a small proportion of nanoparticles. The degree of stability of the colloidal dispersion and degree of surface potential as measured by zeta potential was found be positive 46.

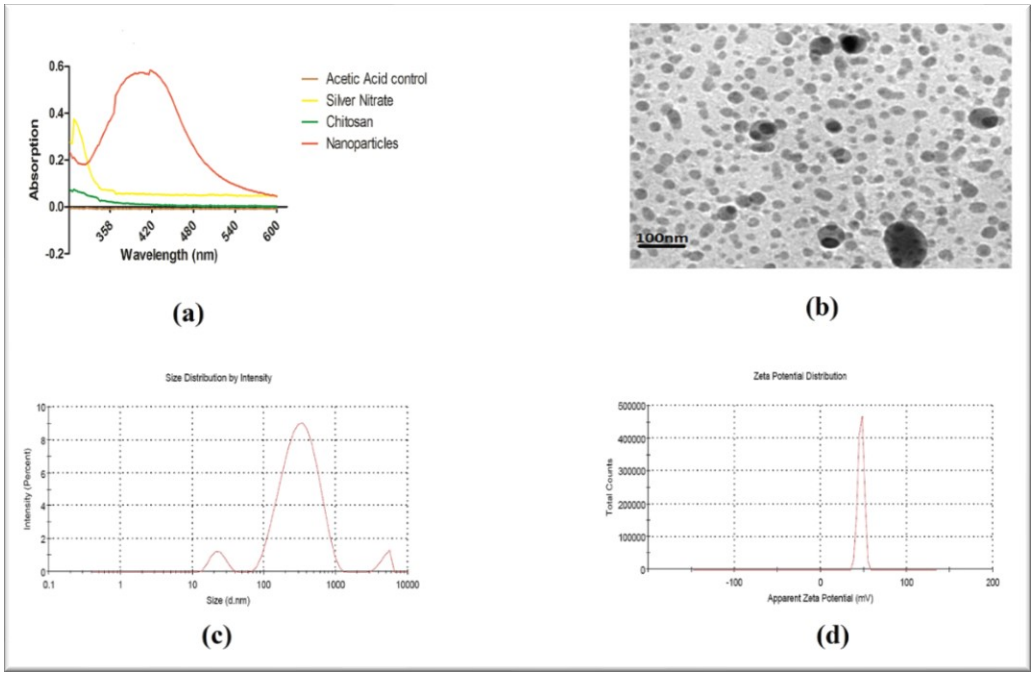


Figure 2.2: Characterization of chitosan coated silver nanoparticles. (a) UV visible spectroscopy analysis of the nanoparticles. (b) TEM micrographs showing spherical nature of the nanoparticles. (c) Dynamic light scattering to show size distribution profile of the nanoparticles. (d) Zeta potential showing degree of surface potential.

2.4.2 **Preparation and physical characterization of AG-CHNp scaffolds**

Using a 3% polymeric solution of Agarose and varying concentration of chitosan coated silver nanoparticles as the base four scaffolds were synthesized. The chitosan nanoparticles were synthesized inside the scaffolds to obtain a uniform and porous nanoparticle base. The synthesis was confirmed by UV spectroscopy and visible colour change **(Figure 2.3(a))**. Glutaraldehyde, shown to be an effective crosslinking agent for stabilization of several kinds of biomaterials was used to obtain a cross-linked Agarose scaffold. To identify the components of the synthesized scaffold and its crystallinity, powder XRD was performed. Obtained peaks were analyzed to identify individual components of the synthesized scaffold. A peak at 2θ=20° was identified to be of chitosan, which corresponds to previous literature and the single peak indicates its semi-crystalline nature **(Figure 2.3(b))** (Nazemi et al, 2012). Pure agarose also exhibits a single peak at 2θ=20°. This can be due to the amorphous nature of agarose. XRD pattern for silver nitrate shows distinct diffraction peaks at around 25°,37°,45°,70° and 76° which are characteristics of (110), (111), (200), (220) and (311) planes of Face centred cubic (FCC) silver, respectively (Trivedi et al, 2014; Govindan et al, 2012). Presence of peak at 2 θ= 20˚ and silver nitrate peak in the AG-CHNp scaffolds confirms the presence of chitosan coated silver nanoparticles.

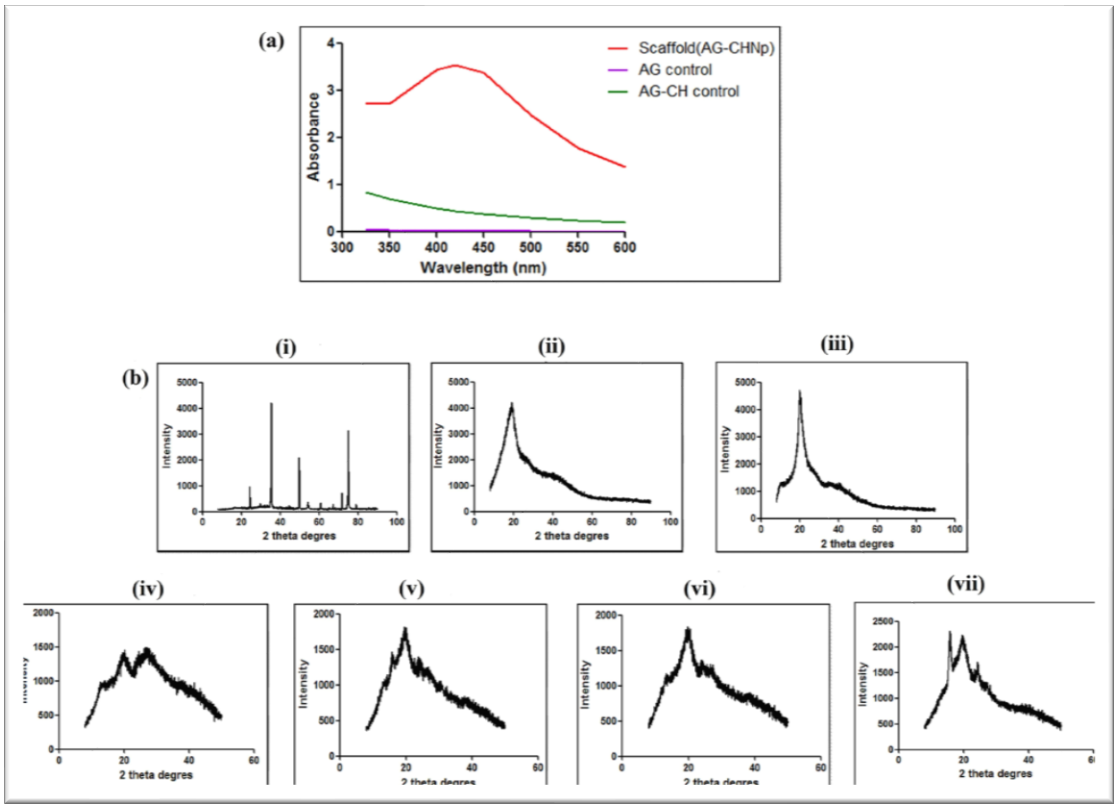


Figure 2.3: Physical characterization of AG-CHNp scaffolds. **(a)** UV-visible absorption spectrum of AG-CHNp scaffolds. **(b)** X-ray diffraction analysis of the AG-CHNp scaffolds and controls.

In the FTIR spectrum, Agarose showed characteristic peaks at around 3300 cm^{-1} attributed to –OH stretching, 2913 cm^{-1} attributed to –CH axial deformation, 1083 cm^{-1} for –C-O axial deformation and 946 cm^{-1} for 3,6 anhydrogalactose **(Figure 2.4)** (Varoni et al, 2012; Tripathi et al, 2015). Chitosan showed their characteristic peaks at around 3400 cm^{-1} (-OH and $–NH_2$ groups), 1553 cm^{-1} and 1370 cm^{-1} (amino groups) and 1080 cm^{-1} (O-C-O group) (Diab et al, 2012). The presence of combination of functional groups from both the polymers is clearly visible in spectrum of all the four AG-CHNp scaffolds. The presence of peak at around 1645 cm^{-1} (C=N) can be attributed to glutaraldehyde crosslinking within the composite (Trivedi et al, 2014; Tripathi et al, 2015).

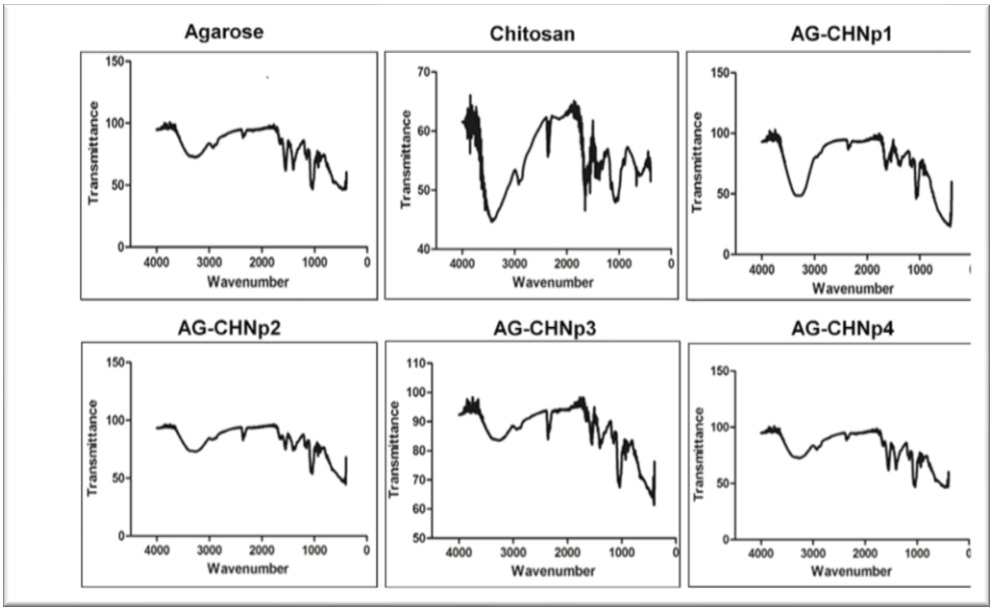


Figure 2.4: FTIR spectrum of all the four AG-CHNp scaffolds and controls.

2.4.3 **Biological Characterization**

2.4.3.1 **MTT Assay**

Metabolically active cells contain dehydrogenase enzymes which reduces yellow coloured MTT salt (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to NADP and NADPH. The resulting purple formazan crystals are solubilized in DMSO and quantified spectrophotometrically at 570nm.Thus, an increase in optical density helps to assess cell's proliferative capacity within the scaffold. The viability of HeLa cells was studied for a period of 16 days **(figure 2.5)**. As compared to the 2D control, all the four scaffolds showed increased and sustained growth of cells for a longer period. In the 2D controls, HeLa cells grew and reached confluence by day 6. With no more surface area available, there is immediate reduction in the cell number owing to cell death. On the other hand, as the scaffolds have 3D morphology and provide better surface area for growth, there is a visible sustained growth during all the time points. Furthermore, AG-CHNp4 showed better growth of cells as compared to all the other scaffolds. This could be attributed to higher concentration of chitosan in AG-CHNp4.

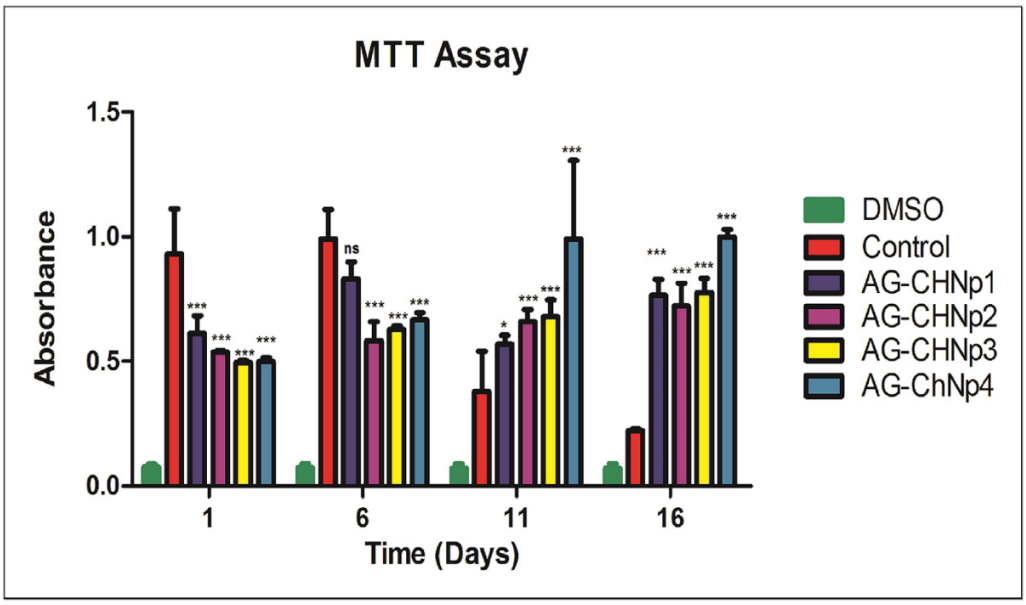


Figure 2.5: MTT assay showing cell viability and proliferation of HeLa cells on AG-CHNp scaffolds.

2.4.3.2 **Hemocompatibility Assay**

The degree of hemocompatibility of the biomaterials refers to the degree of mutual interaction between the components of the scaffolds and blood. Though the scaffolds were found to be porous, it is important that the scaffolds should not alter the integrity of blood when they come in contact with it. To check the degree of hemolysis of the synthesized scaffolds, the scaffolds along with appropriate controls were incubated with equal amount of diluted blood. Hence, hemocompatiblity of AG-CHNp scaffolds was analyzed and is shown in **Table 2.2**. Hemolysis is calculated in terms of percentage and can be placed in one of the following categories (Pal et la, 2006):

1. Highly hemocompatible (<5% hemolysis)
2. Hemocompatible (5-10% hemolysis)
3. Non- hemocompatible (>20%)

Sample	O.D at 545nm	Percentage Hemolysis	Remarks
Positive control	0.785	100	Complete hemolysis
Negative control	0.0125	0	No hemolysis
AG-CHNp1	0.0255	1.68	Highly Hemocompatible
AG-CHNp2	0.031	2.39	Highly Hemocompatible
AG-CHNp3	0.020	0.97	Highly Hemocompatible
AG-CHNp4	0.020	0.97	Highly Hemocompatible

Table 2.2: Hemocompatibility Assay of AG-CHNp scaffolds.

2.4.3.3 **Anti-bacterial Activity**

Different concentrations of bacteria were treated with scaffolds along with necessary controls and resazurin was added to observe color change. Live bacterial cells convert resazurin dye into resorufin, which is pink in color. If bacterial growth is not there, resazurin does not get converted and hence will remain blue in color. All the four scaffolds showed strong anti-bacterial activity against both gram positive and gram-negative bacteria **(Figure 2.6)**.

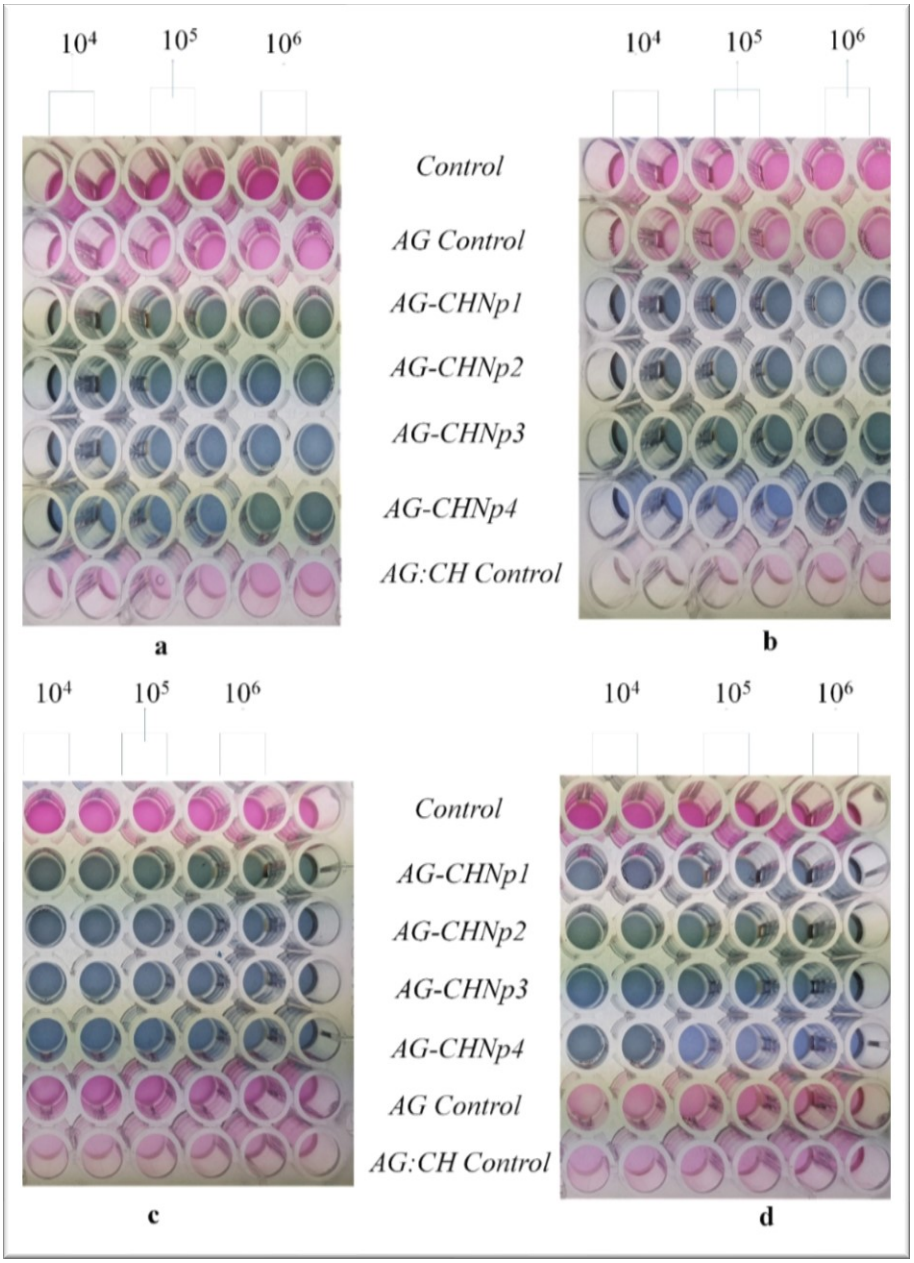


Figure 2.6: Estimation of anti-bacterial activity of AG-CHNp scaffolds using resazurin assay. a) *Escherichia coli2345*, b) *Bacillus subtilis*, c) *Klebsiella pneumoniae* & d) *Staphylococcus aureus737*.

2.4.3.4 **DAPI staining**

The cell adhesion and attachment property were analyzed using DAPI staining of MiaPaCa2 and HEK cell lines **(Figure 2.7)**. As DAPI is a nuclear stain, rounded morphology of cell's nucleus was visible. Furthermore, post attachment cells were found to growth in an optimal and sustained manner.

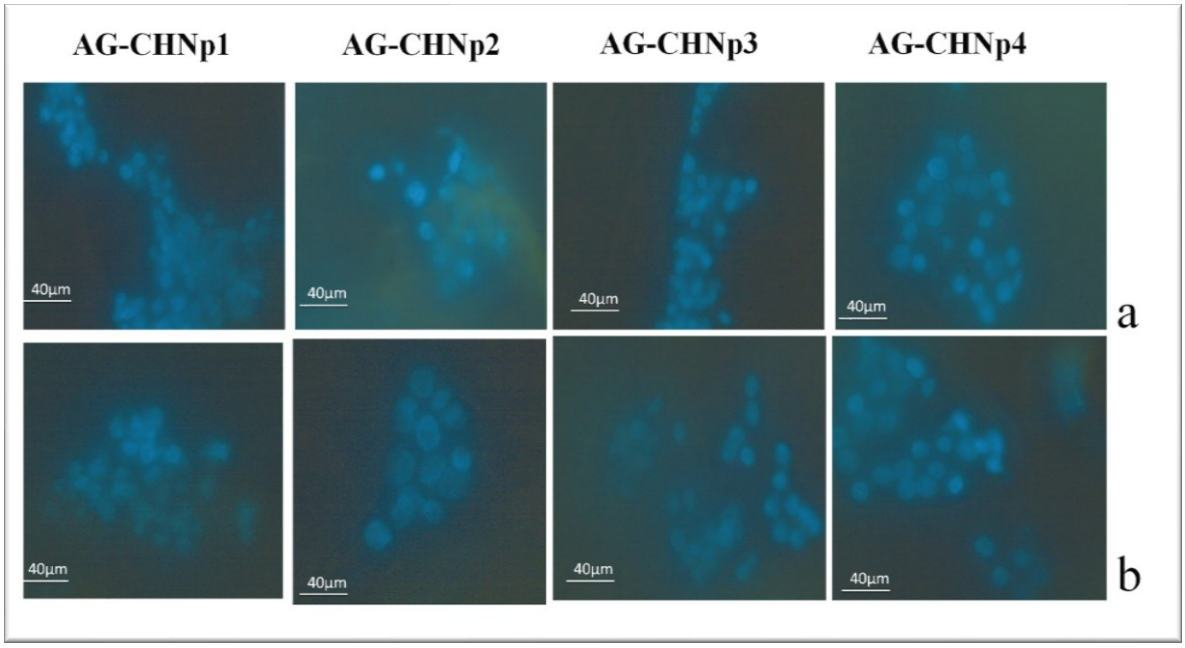


Figure 2.7 DAPI stained sections with MiaPaCa2(a) and HEK(b) cells.

2.4.4 **Mechanical Characterization**

2.4.4.1 **Dynamic mechanical analysis**

When a specific amount of force is applied to a scaffold, it tends to deform which can cause changes in its storage modulus and loss modulus. A scaffold should be able to provide adequate mechanical strength so that it can sustain the wear and tear in the body. For soft tissues, mechanical strength in the range of 0.4-350MPa has been found to be suitable (Hollister, 2005). The results were shown in the form of graph plotted between storage modulus, loss modulus and percentage strain against applied force. The value of all the four AG-CHNp scaffolds was found be in the range of 5-8MPa **(Figure 2.8)**. A decrease in elastic deformation (indicated by storage modulus) and viscous response of the scaffold (as indicated by loss modulus) is evident. The strength of the scaffold was found to increase with strain, thereby showing the strain hardening effect.

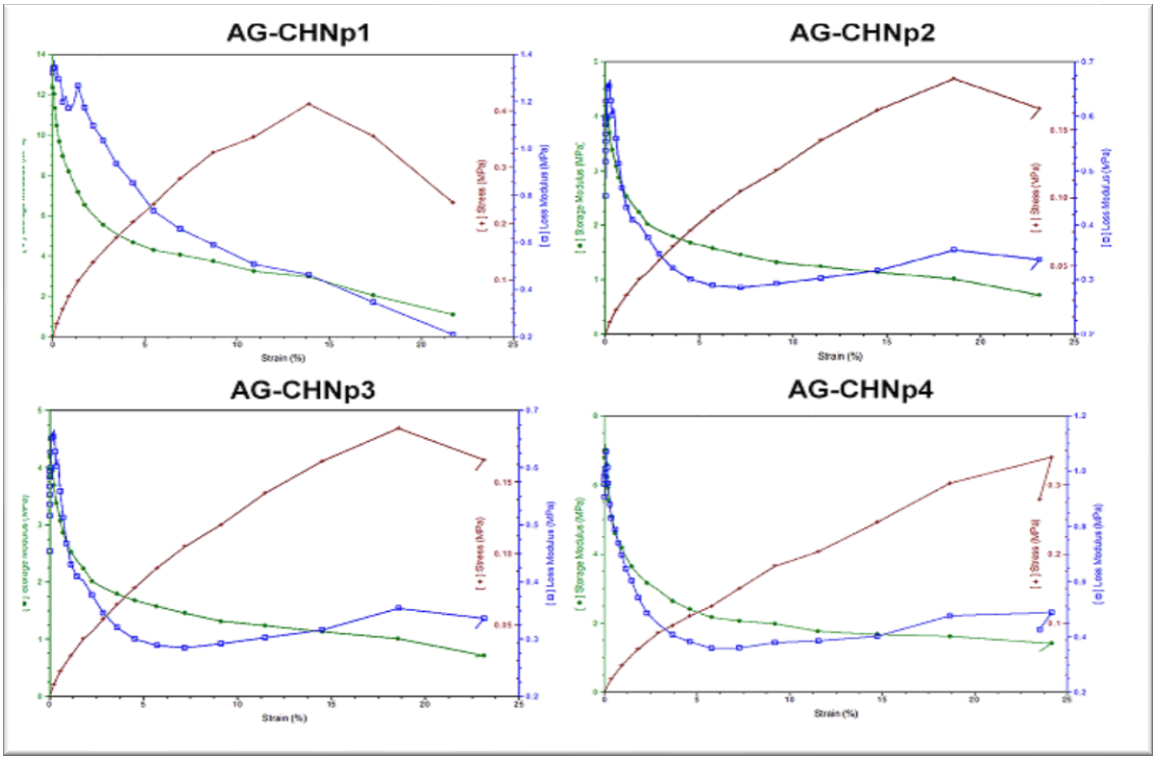


Figure 2.8: Dynamic mechanical analysis of the AG-CHNp scaffolds.

2.4.4.2 **Thermogravimetric Analysis**

Thermal stability of the compounds was assessed by Thermogravimetric analysis, with the profile showing two prominent stages **(Figure 2.9).** The profile shows two prominent stages. First transition occurs between 75-100˚C, which can be attributed to moisture vaporization causing the weight loss. The second transition occurs between 200-250˚C. This can be due to decomposition of both the polysaccharide polymers i.e. agarose and chitosan. The profile also suggests improved thermal stability of the scaffolds as compared to the controls as there is a significant difference in the complete degradation rates which can be attributed to the presence of chitosan coated silver nanoparticles.

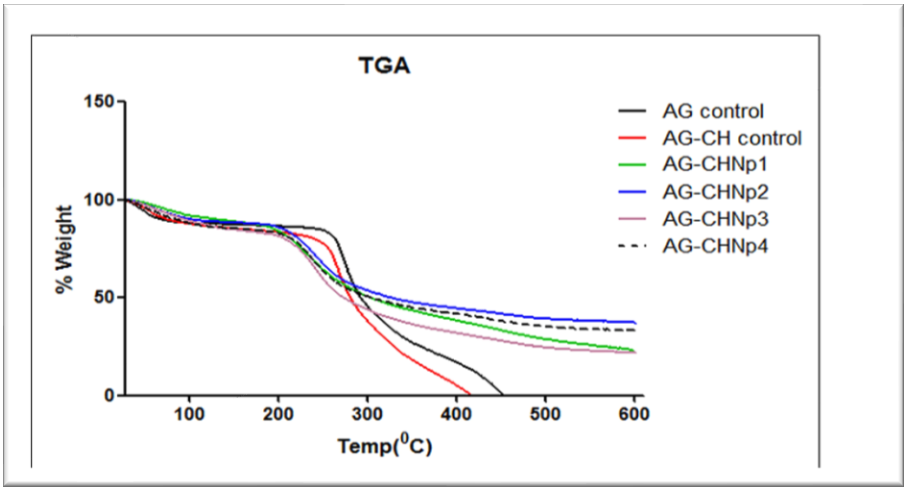


Figure 2.9: TGA profile of AG-CHNp scaffolds and agarose control.

2.4.5 **Other Characterizations**

2.4.5.1 **Swelling Profile**

Dry weight of the scaffolds was noted and then soaked in PBS for a period of 7 days **(Figure 2.10)**. Readings were taken at three different time intervals and swelling ratio was calculated. All the samples showed increase in swelling till the final time point. The scaffolds showed lesser swelling ratio as compared to the control. This can be due to strong interaction between agarose and chitosan coated silver nanoparticles. This strong interaction can result in the formation of additional cross links which can restrict the entry of water (Tripathi et al, 2015; Vimala et al, 2011). The strong water retaining capacity of the synthesized scaffolds could be attributed to their hydrophilicity and a 3-D architecture.

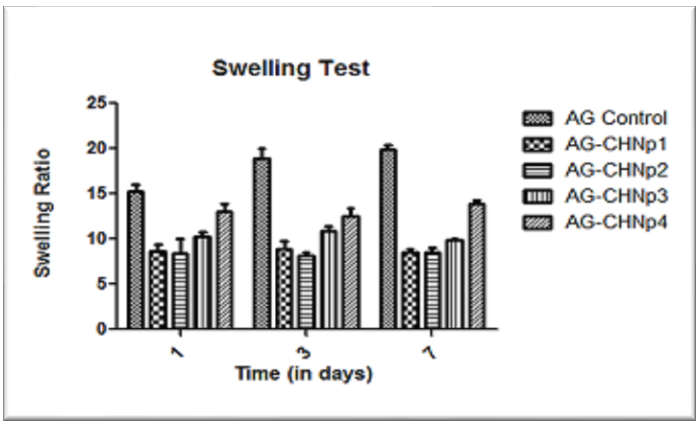


Figure 2.10: Swelling profile of AG-CHNp scaffolds and agarose control.

2.4.5.2 ***In-vitro*** **degradation profile**

Rate of degradation was measured using phosphate buffer saline (pH 7.4) containing lysozyme for a period of 28 days **(Figure 2.11)**. The degradation rate was measured in terms of change in dry weight of the scaffolds at a given time interval. All the scaffolds showed a gradual rate of degradation with time and were about 35-39% after 4 weeks of incubation. The degradation can be primarily because of lysozyme action on the macromolecules and degrading it to smaller chains. When the scaffold is implanted in the body, cells grow on its surface and simultaneously secretes its own extra cellular matrix i.e. rate of degradation should match the rate of tissue formation. Therefore, it is important that the scaffolds degrade with time and degradation should be optimum. The above result suggests that these formulated scaffolds are biodegradable and have an optimum rate of degradation which can allow cells to secrete their own extracellular matrix.

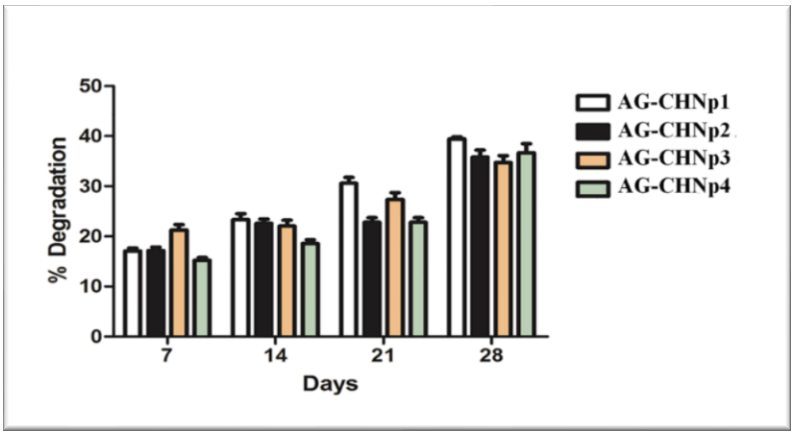


Figure 2.11: In-vitro degradation profile of AG-CHNp scaffolds.

2.4.5.3 Scanning electron microscopy

The scaffolds were designed in such a manner to accommodate cell attachment, growth and migration. The porous structure allows for diffusion of nutrients and removal of toxic compounds. To ascertain the gross morphology of the 3D structure and to determine the pore

size SEM analysis was performed **(Figure 2.12)**. The scaffolds were found to have an average pore size of 175–300 µm. Pore size between 100 and 500 µm is important for cell attachment, nutrient and oxygen diffusion (Kock et al, 2012). Scaffolds with similar pore size have been used before for tissue engineering of pancreas, liver, etc (Borg et al, 2011).

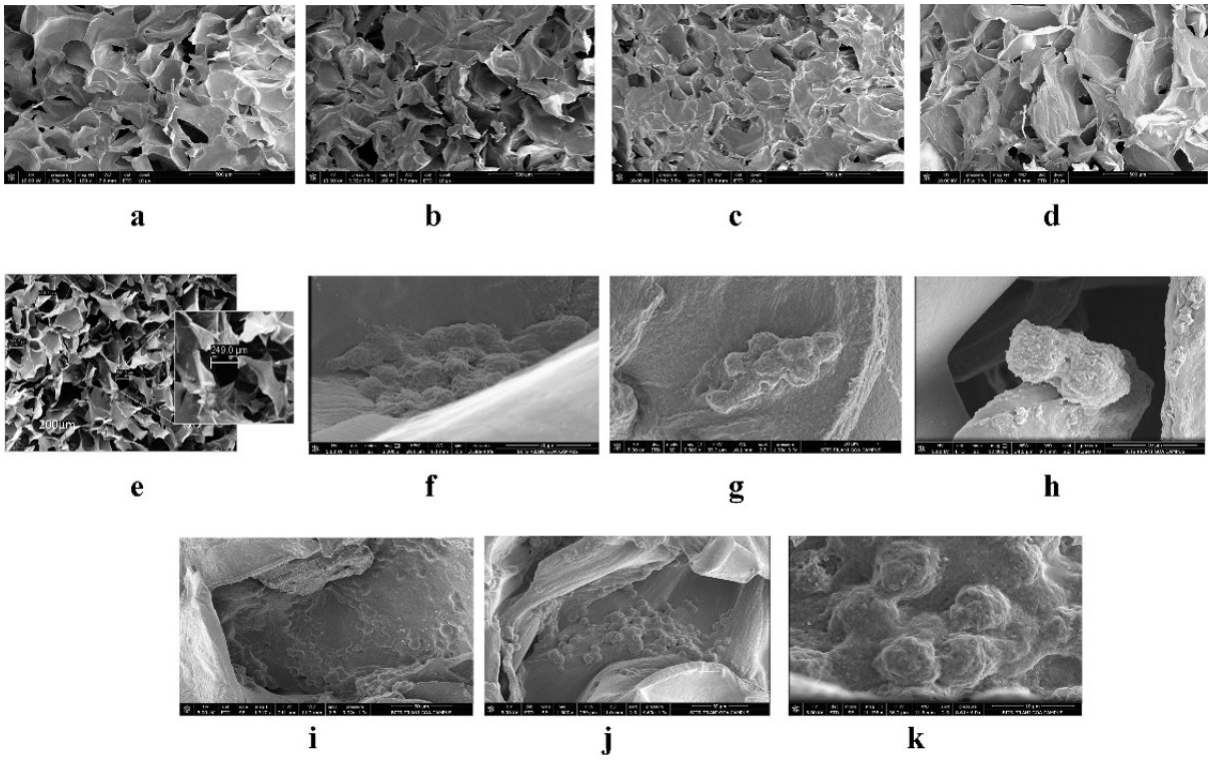


Figure 2.12: Scanning electron micrographs of AG-CHNp scaffolds. SEM micrographs of all the four scaffolds showed interconnected pores and an average pore size of 175-300 µM (a) AG-CHNp1, (b) AG-CHNp2, (c) AG-CHNp3, (d) AG-CHNp4, (e) SEM image of AG-CHNp scaffold showing the pore, (f),(g),(h) Image showing HEK cells growing the on the scaffold, (i),(j),(k) Image showing MiaPaCa2 cells growing on the scaffold.

2.5 **Discussion**

Most of the tissue engineering applications demand the need of biodegradable materials with potential to serve multiple purposes. The most common approach for tissue engineering is seeding cells onto a biomaterial matrix. The design of the scaffold prior to cell application is of prime importance. As mentioned before, various polymers have been tester for the same. Hydrogel-based nanocomposite scaffolds have found application in multiple tissue engineering work along with drug delivery as they allow controlled release of drugs, and other growth factors required for cell growth (Dvir et al, 2011; Albani et al, 2013). In the current study, a blend of agarose and chitosan coated silver nanoparticles was optimized using freeze drying technique to produce a nanocomposite with spongy gel-like properties ideal for tissue engineering of soft tissues. Though nanostructures are an important component of the

extracellular matrix of any organ, use of nanoparticles for preparation of composites has been sparsely studied. A simple and efficient preparation protocol was optimized which had shown to give soft, elastic nanocomposite using freeze drying. The scaffold was characterized for various physical, chemical, mechanical and biological parameters **(Figure 2.13)**.

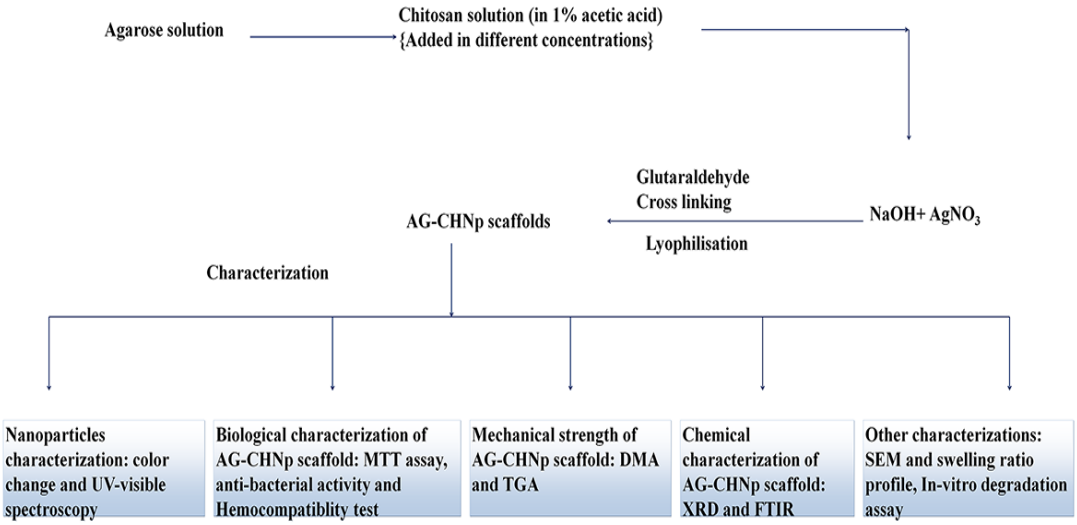


Figure 2.13: Schematic representation of fabrication and characterization of AG-CHNp scaffolds.

The scaffolds showed good swelling ratio, excellent hemocompatibility, appreciable anti-bacterial activity against both Gram positive and Gram-negative bacteria. The scaffolds also showed good biocompatibility with HeLa, MiaPaCa2 and HEK cells and have showed their sustained growth. With suitable mechanical strength, these scaffolds can be used for tissue engineering of soft tissues such as pancreas, kidney heart, liver, etc.

Chapter 3

Pancreatic tissue engineering using agarose-chitosan coated silver nanocomposite scaffold and mice pancreatic cells

3.1 **Introduction**

Tissue engineering is an amalgamation of pluridisciplinary fields encompassing engineering, material science and life sciences that aims to produce biologically viable substitutes for tissue and organ regeneration (Sengupta et al, 2014). Scaffold requirement differs for the type of tissue being engineered. Various types of polymeric scaffolds have been tested for pancreatic tissue engineering but not many have shown long term viability of cells.

Islets are small clusters of endocrine cells with a diameter of 50–200 μm. Various mammalian islets that are used for research comprise of a similar set of cells which differ in composition and cytoarchitecture. Various evidences corroborate that functionality of the islets profoundly relies on their interaction with the extracellular matrix. Insulin secretion, islets survival and proliferation have shown to be regulated by the interactions with the ECM (Pinkse et al, 2006; Beattie et al, 2002). Matrix interactions have also shown influence on islets development and β-cell differentiation (Beattie e t al, 1997; Kaido et al, 2004). Therefore, it is important to construct a scaffold which can provide favourable environment for the islets to adhere, sustain and survive for longer period of time.

AG-CHNp scaffolds synthesized using freeze drying technique has been already tested against various cell lines (HEK, MiaPaCa2 and Hela) for their attachment and proliferation. But to use the scaffold for pancreatic tissue engineering, AG-CHNp scaffolds should also have good compatibility with normal pancreatic cells. To achieve this, the pre-constructed AG-CHNp scaffolds were tested against mice primary pancreatic cells. Out of the four scaffolds (AG-CHNp1, AG-CHNp2, AG-CHNp3 and AG-CHNp4) AG-CHNp4 gave most promising results, hence for present study, only AG-CHNp4 scaffold were used.

3.2 **Materials**

3.2.1 **Pancreatic primary cell isolation**

- Diethyl ether (MERCK, USA)
- Collagenase XI (Sigma Aldrich chemical Co., St. Louis, MO)
- 70-micron filter (Hi-media, India)
- Calcium chloride (Hi-media, India)

3.2.2 **1X Hank's balanced salt solution**

- Potassium chloride (Hi-media, India)
- Potassium dihydrogen phosphate (MERCK, USA)
- Sodium chloride (Sigma Aldrich chemical Co., St. Louis, MO)
- Sodium bicarbonate (Hi-media, India)
- Disodium hydrogen phosphate (MERCK, USA)
- D-Glucose (Hi-media, India)
- Ultrapure water

3.2.3 **Pancreatic cell growth media**

- RPMI 1640-GlutaMAX (Gibco^{TM})
- Fetal bovine serum (Brazilian origin, Gibco^{TM})
- 100X Penicillin-Streptomycin (GibcoTM)

3.2.4 **Dithizone staining**

- Dithizone (Sigma Aldrich chemical Co. (St. Louis, MO))
- Dimethyl sulfoxide (DMSO) (Thomas Baker Pvt. Ltd)
- Phosphate buffer saline (PBS) (Hi-media, India)

3.2.5 **RNA isolation and Real time PCR(q-PCR)**

- Trizol (Takara, Japan)
- Chloroform (Fischer Scientific)
- Isopropyl alcohol (Fischer Scientific)
- Ethanol (Hi-media, India)
- Reverse aid cDNA synthesis kit (Thermo Fisher Scientific, USA)
- SYBR® Green PCR master mix (Applied Biosystems, USA)
- Nuclease free water (Takara, Japan)

3.2.6 **Western Blotting**

3.2.6.1 **Lysis buffer for protein isolation**

- 1M Tris (Sigma Aldrich chemical Co. (St. Louis, MO))
- 2M Sodium chloride (Hi-media, India)
- 0.1M Ethylenediaminetetraacetic acid (EDTA) (Fischer Scientific, USA)
- 100 mM Dithiothreitol (DTT) (Sigma Aldrich chemical Co. (St. Louis, MO))
- 1% Triton X100 (Hi-media, India)
- Sodium orthovanadate (Sigma Aldrich chemical Co. (St. Louis, MO))
- 10% glycerol (Hi-media, India)
- Phenylmethane sulfonyl fluoride (PMSF) (Sigma Aldrich chemical Co. (St. Louis, MO))

3.2.6.2 **Protein estimation and SDS-Polyacrylamide gel electrophoresis (PAGE)**

- Bradford's reagent (Sigma Aldrich chemical Co. (St. Louis, MO))
- 30% acrylamide and bis-acrylamide solution (Sigma Aldrich chemical Co, USA)
- 10% Sodium Dodecyl Sulfate (SDS) (Sigma Aldrich chemical Co, USA)
- 10% Ammonium persulfate (APS) (Sigma Aldrich chemical Co, USA)
- TEMED (Bio-Rad)
- Stacking gel buffer: 0.5 mol Tris-HCl, pH 6.8 (Sigma Aldrich chemical Co, USA)
- Resolving gel buffer: 1.5 mol Tris-HCl, pH 8.8 (Sigma Aldrich chemical Co, USA)
- Ultra-pure water
- 1X SDS-PAGE Running buffer (Sigma Aldrich chemical Co, USA)
- SDS-PAGE sample loading buffer

3.2.6.3 **Protein transfer and detection**

- 1X western blot transfer buffer (Sigma Aldrich chemical Co, USA)
- Methanol (Thomas Baker)
- Immun-blot PVDF (Bio-rad, USA)
- Membrane blocking buffer: 5% Non-fat milk in 1X PBS
- Membrane washing buffer: 1% Tween20 in 1X PBS

- **Primary antibody**: PDX-1 (Santacruz biotechnology sc-390792; 1:2500)
  Pax-4 (Santacruz biotechnology sc-98941; 1:2500)
  Glucagon ((Santacruz biotechnology sc-514592; 1:2500)
  Insulin (Santacruz biotechnology sc-98941; 1:2500)
  β-actin (Cell signalling 4970; 1:5000)
- **Secondary Antibody**: Goat anti rabbit IgG HRP conjugated (Merck 6.2114E14)
  Anti-mouse IgG HRP linked antibody (Cell signalling 7076)
  Alexa Fluor -633 Goat anti- mouse secondary antibody (Invitrogen)
- Immobilon Western Chemiluminescent HRP Substrate (MERCK, Millipore)

3.2.7 **Cell culture Reagents**

- MTT (Hi-media, India)
- DAPI mounting media (Sigma Aldrich chemical Co.)
- Glutaraldehyde solution (25%, for synthesis) (MERCK)
- Phosphate buffer saline (PBS) (Hi-media, India)

3.2.8 **Immunofluorescence**

- Paraffin
- Xylene (Fischer Scientific, USA)
- Bovine serum albumin (BSA)
- PDX-1 Primary antibody (Santacruz biotechnology)
- Alexa Fluor -633 Goat anti- mouse secondary antibody (Invitrogen, USA)

3.3 **Methods**

3.3.1 **Isolation of pancreatic cells from mice**

Primary pancreatic cells were isolated using reported protocols (Li et al, 2009; Kumar et al, 2017). Female Balb/c mice (age 6-8 weeks) were used for pancreas harvesting. Isolation was carried out under the approval of Institutional animal ethics committee (registration number: 1577/PO/Re/S/2011/CPCSEA), Kalinga Institute of Industrial Research (KIIT), Bhubaneshwar, India. Briefly, mice were euthanized by diethyl ether. An incision was made in the abdominal area to expose the liver and intestine. The pancreas was then dissected and placed in a 50 ml centrifuge tube containing collagenase XI prepared in Hank's balanced salt solution (HBSS). The centrifuge tube was then placed in 37°C for 15 minutes with occasional

tapping to get cells in suspension. After the incubation, the tube was shaken vigorously to disrupt the pancreas completely. As the solution turned homogenous, the digestion was ceased using 1mM calcium chloride. Further, the solution was filtered through a 70-micron filter to remove any tissue debris. The solution was centrifuged at 1000 rpm for 60 seconds at 4°C. The pellet was washed with 1X HBSS and resuspended in media containing complete media.

3.3.2 **Dithizone staining**

Dithizone (DTZ) staining was performed for mice pancreatic primary cells for characterization of beta cells (Shiroi et al, 2002). 50mg dithizone was dissolved in 5ml dimethyl sulfoxide (Thomas Baker Pvt. Ltd) and filter sterilized to prepare the stock solution. Working solution was prepared by adding 10ul DTZ stock solution to 1ml media. The cells were washed 1X phosphate buffer saline (PBS) and working solution was added to the cells. The cells were incubated with the working solution for 15 minutes. Post incubation, media was removed, and cells were washed with 1X HBSS. The stained clusters were examined under the microscope.

3.3.3 **Real time PCR**

Total RNA was isolated from mice pancreatic cells using Trizol reagent as per manufacturer's instructions. Following extraction, 1µg of RNA was reverse transcribed to cDNA using Reverse aid cDNA synthesis kit. The synthesized cDNA was used as template to perform Real time PCR (q-PCR) to check the expression of pancreatic markers (Glucagon, pancreatic amylase, insulin and PDX-1) using gene specific primers. 1ul of cDNA was applied in a total reaction mixture of 10 µl containing 5ul of SYBR® Green PCR master mix, 1ul each of forward and reverse primers and 2ul of nuclease free water. This reaction was carried out for 40 cycles in Insta Q96 (Hi-media, India).

3.3.4 **Western blotting**

Western blot analysis was performed to study the expression of various pancreatic cell markers. Cells were harvested by adding 100 µl lysis buffer and stored in -80 ^{0}C overnight. The lysates were thawed on ice, briefly vortexed for 30 secs thrice and centrifuged at 13,000 g. for 30 mins. After estimation with Bradford assay, 30ug of protein was run on 12% SDS-PAGE and transferred onto PVDF membrane for 70 mins at 80 V. The membranes were blocked with 5% skimmed milk for 2 hours at room temperature. After blocking, the membranes were incubated overnight with primary antibody at 4 ^{0}C. Post incubation, the membranes were washed thrice with 1X PBS containing 1% Tween 20 (PBST). The washed membrane was probed with HRP

tagged secondary antibody for 2 hours at room temperature. Finally, the membranes, after washing with 1X PBST, were developed on X-ray film using chemiluminescent solvents.

3.3.5 **Flow cytometry analysis**

Cells were washed with incubation buffer after harvesting from 60mm dishes (0.5% BSA in 1X PBS) and fixed using 2% formaldehyde for 15 minutes. This was followed by permeabilization with 0.1% Triton X in 1X PBS for 30 minutes. Cells were then incubated with primary antibody for PDX-1 and Insulin for 1 hour at room temperature. Cells were further incubated with FITC tagged secondary antibody for 30 minutes at room temperature. Stained cells were subjected to flow cytometric analysis using BD FACS Canto II cytometer and analysed using FACS Diva software. Multiple gating strategies were used to gate the correct events. Forward scatter (FSC) area vs height measurement was done to remove clumps for single cell analysis, single cells falling along a diagonal were chosen for further analysis. Forward scatter (FSC) and Side scatter (SSC) were used to gate viable and single cells events. Gating was done to exclude debris and dead cells from analysis by categorizing low-FSC events as debris, and events with low FSC and high SSC as dead cells. A compact cell population was thus gated based on size and granularity of the registered events on the scatter plot. Gated PDX-1 and insulin positive cells were determined as proportionate shift in population.

3.3.6 **MTT Assay**

AG-CHNp scaffolds have been previously studied for their biocompatibility against various cell lines. To check for biocompatibility of mice pancreatic primary cells, AG-CHNp4 scaffolds were placed in 24 well uncoated plates. The scaffolds were sterilized using ethanol gradient. This was followed by exposure to UV radiation for 20 minutes. Complete media was added to the sterilized scaffolds and incubated for 4 hours to promote equilibration of scaffolds for adequate gaseous exchange. Furthermore, the mice pancreatic primary cells were seeded onto the pre-sterilized scaffolds and on coated 24 well plate (which acts as 2D control) and incubated at 37°C with 5% CO_2 humidified chamber for a period of 40 days. Half media was changed every 5 days. On the day of the experiment, media was removed from the well and washed with PBS. MTT solution (0.5 mg/ml) was added for 3–4 hours. Following the incubation, DMSO was added and incubated for 20 minutes. The purple color developed was measured at 570 nm to calculate cell viability.

3.3.7 **DAPI Staining**

For microscopic analysis of the attachment and growth of mice pancreatic primary cells on AG-CHNp scaffolds, DAPI staining was performed. The cells were seeded on the AG-CHNp4 scaffolds and incubated at 37°C with 5% CO_2. One the day of the experiment, the scaffolds were washed with PBS and incubated overnight with 2.5% glutaraldehyde for fixation. Following day, scaffolds were rinsed with PBS and dried using ethanol gradient. DAPI mounting media was added to section of fixed scaffolds and incubated for 25 minutes in dark. The slides were observed using inverted fluorescence microscope (Excitation: 405 nm and emission: 450 nm).

3.3.8 **Immunofluorescence**

Immunofluorescence was performed to confirm the presence of PDX-1, Insulin and glucagon positive cells. The pancreatic primary cells were seeded onto the scaffolds for a period of 40 days. On the day of experiment, the media was removed, and the cells were fixed with 2.5% glutaraldehyde overnight. Post fixation, sample dehydration was performed using ethanol gradient, cleared using xylene and were paraffinized. For immunofluorescence, the embedded scaffolds were sectioned, deparaffinized and rehydrated. Furthermore, the sections were permeabilized using 0.1% triton X-100 and blocked using 1% BSA for 1 hour at room temperature. The sections were incubated with primary antibody (PDX-1, Insulin and glucagon) at 4°C overnight. Post incubation, sections were washed with 1X PBS and probed with fluorophore tagged secondary antibody for 2 hours at room temperature. After incubation, the sections were washed with 1X PBS, counterstained with DAPI mounting media for 15 minutes and observed using inverted fluorescence microscope (Olympus, BX61).

3.3.9 **Qualitative estimation of Insulin secretion**

For qualitative estimation of basal level of insulin secretion, western blotting was performed. Mice pancreatic primary cells were seeded on AG-CHNp4 scaffolds. The media which was added for the growth of cells (Spent media) were collected on various time points. The spent media were then centrifuged to remove any cellular debris. Freshly prepared complete media was used as control. The media (60µg) was run on 12% SDS PAGE gel and was transferred to a PVDF membrane. The membranes were blocked at room temperature using 5% skimmed milk, followed by overnight incubation with the primary monoclonal anti-Insulin antibody. Post incubation, the membrane was washed with PBST and incubated with HRP tagged

secondary antibody at room temperature. After the incubation, the membrane was washed with 1X PBST and chemiluminescent detection was carried out.

3.3.10 **Glucose challenge test**

In order to further evaluate the functionality of pancreatic cells growing on the scaffolds, glucose stimulated insulin release was recorded. Mice pancreatic primary cells were seeded on AG-CHNp4 scaffolds and grown for a period of 45 days. On the day of experiment, media was removed, and cells were washed with 1X PBS. Furthermore, the cells were incubated with Krebs-Ringer Bicarbonate (KRB) buffer containing 3.3mM glucose for 1 hour. Post incubation, the buffer was removed, and cells were washed with 1X PBS. Likewise, cells were then incubated with KRB buffer containing 17mM glucose for one hour and the buffer was collected After glucose challenge, insulin secretion was qualitatively analysed by chemiluminescent detection using western blotting.

3.4 Results

3.4.1 **Characterization of cells isolated from mice**

3.4.1.1 **Dithizone staining**

The isolated primary cells from mice were characterized using Dithizone (DTZ). Dithizone is a sulphur containing compound which has specific affinity for zinc ions. Therefore, DTZ is used to stain zinc containing beta cells (Bai et al, 2015). **Figure 3.1(a)** shows DTZ stained primary cells. The cells were stained crimson red suggesting the presence of beta cells.

3.4.1.2 **Gene expression profile**

To characterize the isolated cell preparation, a PCR based technique was employed to quantify the fold change in gene expression levels of signature pancreatic tissue markers. Isolated cells were grown on tissue culture plates for 4 days, harvested, total RNA isolated and c-DNA was synthesized. Expression levels of insulin, PDX-1, Glucagon and Amylase transcripts were determined using gene specific primers **(Table 3.1)**. We found approximately 200-fold change in mRNA transcript levels of Insulin, one of the most important hormones secreted by pancreas participating in carbohydrate metabolism **(Figure 3.1(b))**. Similarly, higher levels of Pancreatic and duodenal homeobox 1 (*PDX-1*), a transcription factor necessary for β cell maturation was observed. However, we observed extremely low transcript levels of Amylase relative to the reference gene. The transcript levels were normalized with housekeeping gene

GAPDH and the fold changes were calculated. For primer specificity confirmation melting curve analysis was done for each set of primers.

Gene	Forward Primer	Reverse Primer
GAPDH	GTGAAGGTCGGTGTGAACGG	GATGCAGGGATGATGTTCTG
Glucagon	CACTACCAGGGCACATTCACC	ACCAGCCACGCAATGAATTCCTT
PDX-1	GCGGTGGGGGCGAAGAGCCGGA	GACGCCTGGGGGCACGGCACCT
Insulin	TTCTTCTACACACCCAAGAC	CTAGTTGCAGTAGTTCTCCA
Pancreatic amylase 2	TGGCGTCAAATCAGGAACATGG	GGCTGACAAAGCCCAGTCATCA

Table 3.1: List of primer sequences for mice pancreatic markers

(a) **(b)**

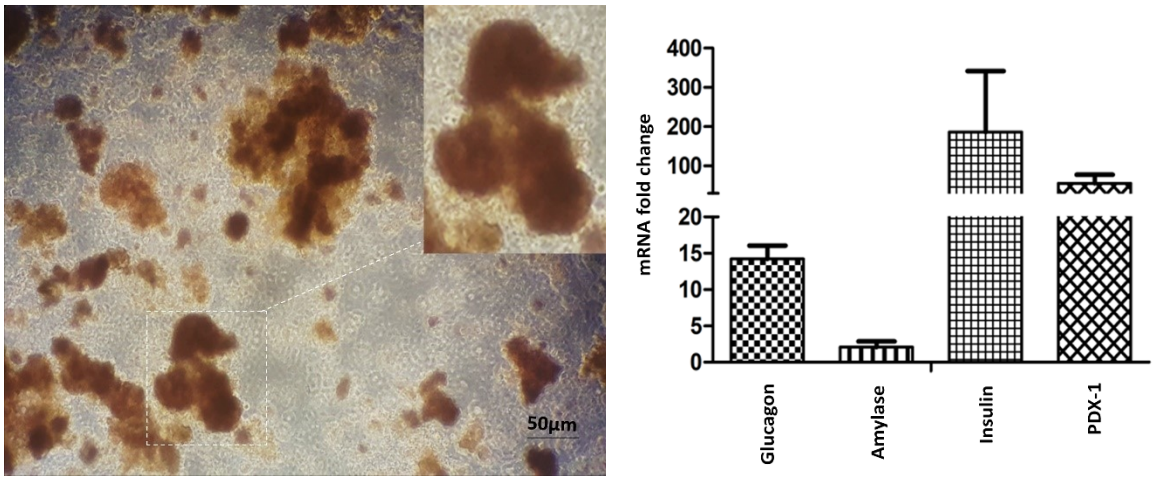


Figure 3.1: Characterization of mice primary pancreatic cells. (a) Dithizone staining showing crimson red stained beta cells. (b) Relative expression of various pancreatic genes after four days of primary culture. Gene expression was relative to GAPDH.

3.4.1.3 **Western Blotting**

To confirm the mRNA level expression, western blotting was performed. The isolated proteins were transferred onto a PVDF membrane and expression levels for PDX-1, PAX-4, Glucagon and Insulin were studied using specific monoclonal antibodies. Western blotting analysis showed positive expression of PDX-1, PAX-4, Insulin and Glucagon **(Figure 3.2(a))**. Although the expression of glucagon and insulin was considerably higher than transcription factors PDX-1 and PAX-4. Furthermore, bands were also quantified using densitometric program in ImageJ image processing software and then was normalized with β-actin.

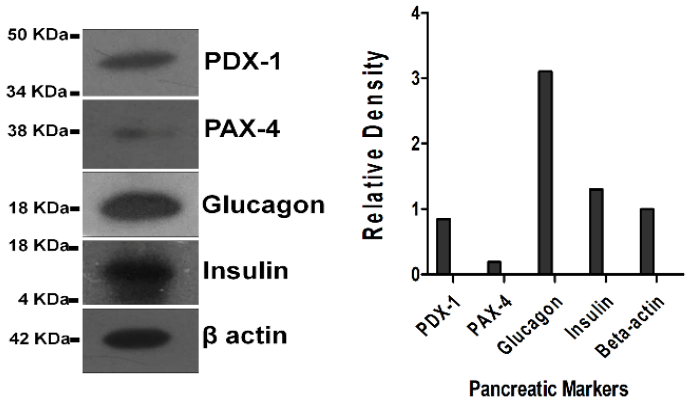


Figure 3.2 (a). Characterization of mice primary pancreatic cells. Western blot analysis detected the expression of Glucagon (18KDa), PDX-1 (34-50KDa), PAX-4 (38KDa) and Insulin (4-18KDa) in primary mice pancreatic cells.

3.4.1.4 **Flow cytometry**

The isolated mice pancreatic primary cell population was also characterized using flow cytometry to find out the percentage of PDX-1 and insulin positive cells. The cells were processed on the 4th day of culture. They were fixed and probed with specific monoclonal primary antibody followed by incubation with Alexa fluor tagged secondary antibody. Flow cytometric analysis of the cells revealed 22.3% PDX-1 positive and 27.8% Insulin positive cells **(Figure 3.2(b))**.

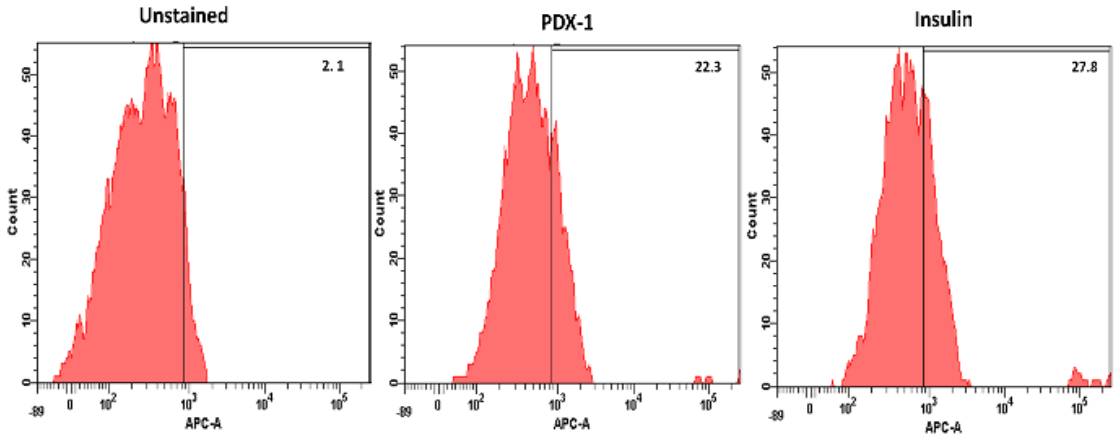


Figure 3.2 (b). Characterization of mice primary pancreatic cells using Flow cytometry. Flow cytometry analysis after four days of culture showed positive expression of PDX-1 (22.3%) and Insulin (27.8%).

3.4.2 **Characterization of the artificial construct with mice pancreatic primary cells**

3.4.2.1 **MTT Assay**

Long term viability of pancreatic cells on the 3D scaffold is an important component of tissue engineering. Cell cytotoxicity on the scaffolds was measured by MTT Assay. Metabolically active cells on the scaffolds converts MTT salt to formazan crystals which gets solubilized in DMSO and gives a purple colour. This colour is quantitated at 570nm using a spectrophotometer. In the current study, the proliferative capacity of the pancreatic cells was studied for a span of 40 days **(Figure 3.3)**. Cells growing on tissue culture plates were used as 2D control. Initially till Day 10, 2D control showed better cell proliferation but with reduced surface area available for further propagation, there was reduction in cell number. On the other hand, AG-CHNp4 scaffold provide ideal growth environment for scaffolds and more surface area owing to the three-dimensional morphology, showed increased growth with each time point. At the end of 40 days, AG-CHNp4 scaffold showed sustained growth of cells as compared to 2D control where the growth reduced with time.

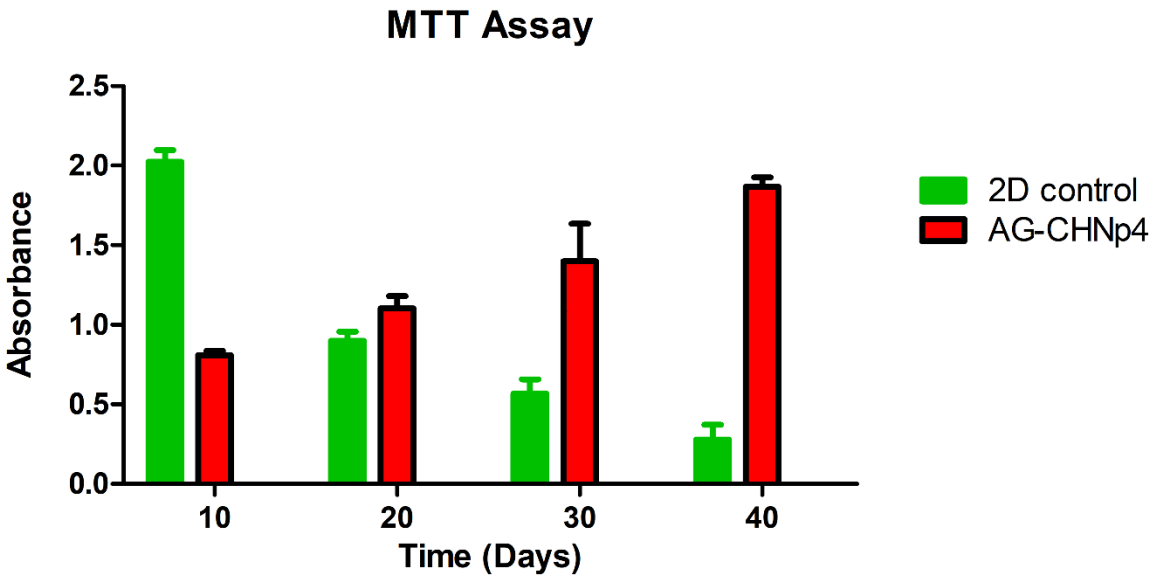


Figure 3.3: Cell viability and proliferation by MTT assay

3.4.2.1 **Microscopic analysis of the construct**

To study the attachment and proliferation capacity of the pancreatic cells on the AG-CHNp4 scaffolds, DAPI staining was performed. DAPI is a nuclear stain. It stained rounded nuclei of the cells and this rounded morphology was seen on the scaffolds after 48 hours of culture **(Figure 3.4)**.

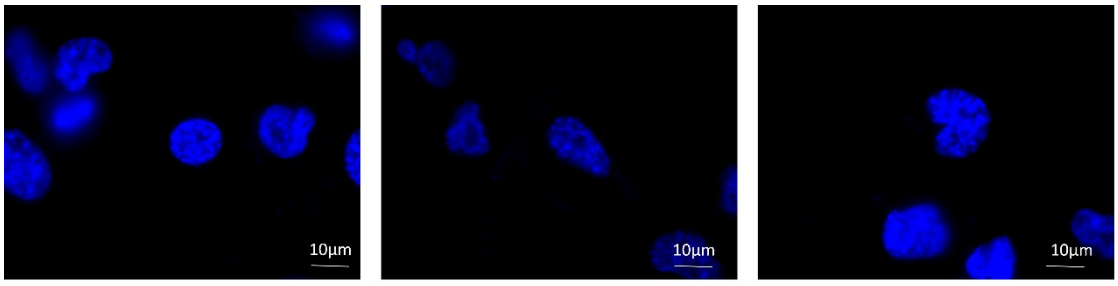


Figure 3.4: DAPI stained sections showing distinct nuclear morphology of the mice pancreatic cells.

3.4.2.2 **Immunofluorescence**

To comprehend whether the pancreatic cells growing on the scaffolds contains insulin producing β cells, expression levels of various pancreatic markers such as pancreatic and duodenal homeobox1 (PDX-1), Insulin and glucagon were explored. PDX-1 is a transcription factor necessary for pancreatic development and β cell maturation. Developing β cells produce PDX-1, whose expression seemingly favours the production of insulin secreting β cells. Thus, increased PDX-1 expression in isolated cells indicates increased survival of β cells. On the other hand, glucagon and insulin are peptide hormones secreted by alpha and beta cells of pancreatic islets respectively. These two hormones work antagonistically to each other thereby, maintaining glucose metabolism in the body. To show the expression of above-mentioned markers in the pancreatic cells seeded on AG-CHNp4 scaffolds, immunofluorescence was performed for PDX-1, Insulin and glucagon using marker specific monoclonal antibody. 30 days post incubation, the scaffolds were paraffinized, and these sections were cut using microtome. The thin sections were then deparaffinized and rehydrated, stained for specific markers and observed under fluorescent microscope. As can be seen in **figure 3.5(a)**, significant levels of PDX-1 were observed inside the nucleus of the cells. Localization of PDX-1 in the nucleus was confirmed by co-localizing DAPI (Blue) and PDX-1 levels (Green) which formed the Cyan colour on merging. In **figure 3.5(b) and (c)**, we observed substantial expression of insulin and glucagon. Cytoplasmic localisation of both insulin and glucagon was confirmed by merging DAPI (blue) and insulin (red) or glucagon (red). Thus, the cells isolated from mice pancreas were surviving and maintaining their functionality even after 30 days in culture and were showing positive expression of PDX-1, insulin and glucagon.

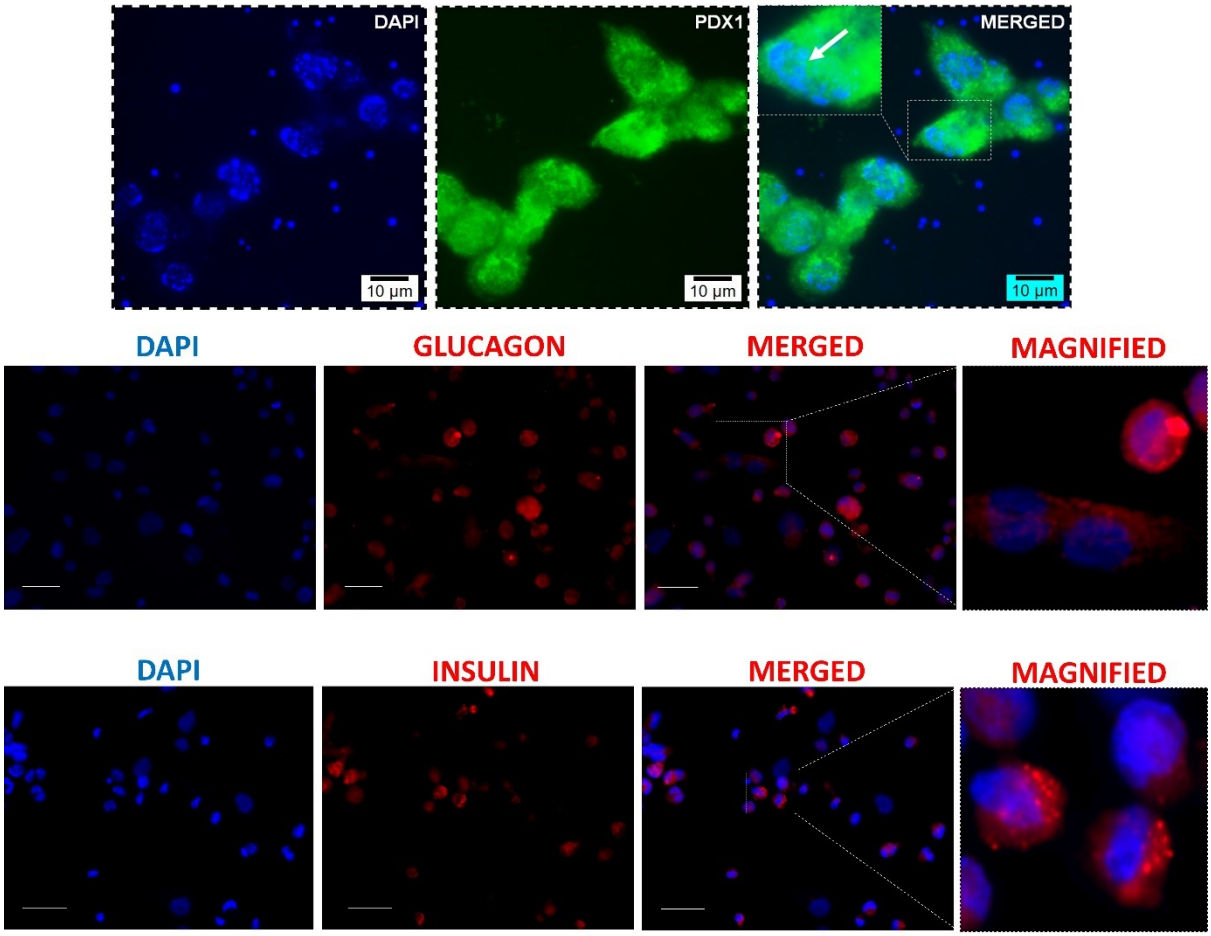


Figure 3.5 Microscopic analysis of mice pancreatic cells growing on AG-CHNp4 scaffold**.** Immunofluorescence staining showed localisation of (a) PDX-1 (scale: 10µm) and positive expression of (b) glucagon (scale: 40µm) and (c) insulin (scale: 40µm) which confirms the presence of islets on Day 30 of pancreatic cell culture.

3.4.2.3 **Qualitative estimation of insulin secretion**

Insulin secretion in human body is maintained at a basal level. To check whether the tissue construct also secretes insulin, western blotting was performed **(Figure 3.6 (a))**. Western blotting analysis showed positive expression of insulin in the spent media at different time points. Complete media used for growing pancreatic cells on the scaffold was used as the control. The bands were quantified using densitometric analysis and were normalized to day 5 time point as the complete media control did not show any expression of the insulin. Coomassie stained gel was used as the loading control.

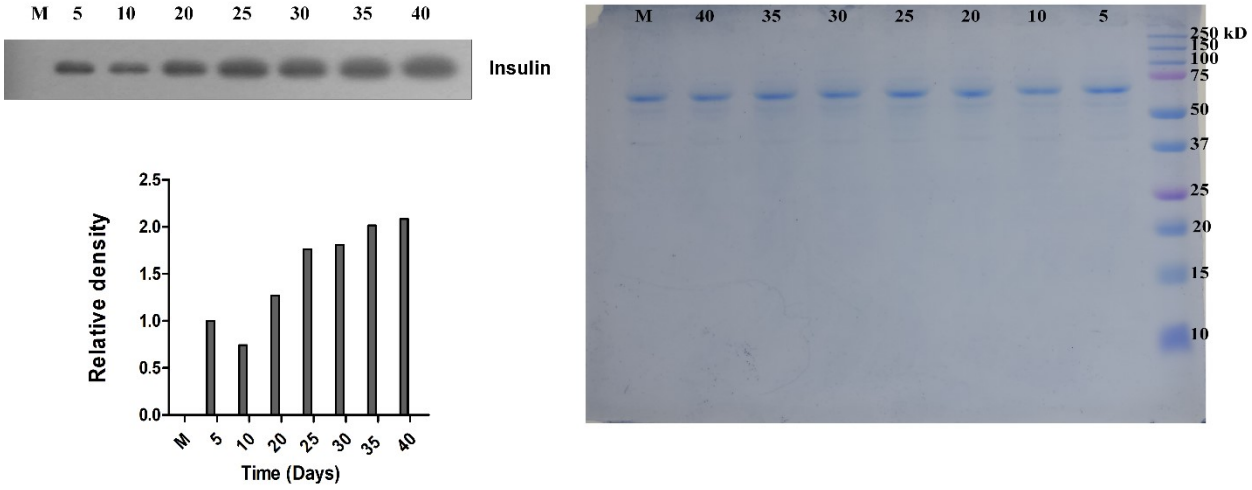


Figure 3.6(a) Qualitative analysis of insulin secretion. Western blot performed on spent media (60µg) showed relative expression of insulin on each day from Day 5 to Day 40. M is the complete media which was used as control. Coomassie stained gel was used as loading control.

3.4.2.4 **Glucose challenge test**

Regulation of insulin secretion under glucose stress is an integral segment of a functional pancreatic construct. Beta cells should be able to adjust to the levels of glucose and secrete adequate concentration of insulin. Insulin secretion upon glucose challenge was qualitatively studied using western blotting. After 45 days of culture, cells were incubated with KRB buffer containing different concentrations of glucose (3.3mM and 17mM). These buffers were run on a 12% SDS PAGE gel and was transferred onto PVDF membrane. Insulin was detected using chemiluminescent reagents. **Figure 3.6 (b)** shows differential levels of insulin in the incubated buffers. Low glucose showed lower intensity band as compared to high glucose concentration. This further confirms long term functionality of beta cells growing on the scaffolds.

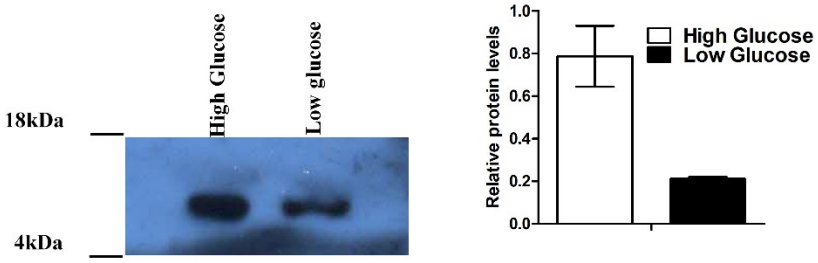


Figure 3.6(b) Qualitative analysis of insulin secretion after glucose challenge test showing its differential expression under different glucose concentrations.

3.5 **Discussion**

Tissue engineering deals with developing renewable resource for organ replacement and regeneration (Sengupta et al, 2014; Khorsandi et al, 2015). One of the important components in tissue engineering, scaffolds play an important part in fabrication of artificial tissue construct. As scaffolds act as the natural environment for cell survival, properties such as biocompatibility, biodegradability becomes paramount (Asghari et al, 2016). Various polymers have been tested for pancreatic tissue engineering, however since all the polymers are not well studied and documented in relation to engineering pancreas, the ideal polymeric scaffold has not yet been identified (Kumar et al, 2018). Moreover, it has been established that islets tend to lose viability post isolation due to lack of extracellular matrix (Jalili et al, 2018). Therefore, appropriate choice of polymer for scaffold design is extremely important. Another setback with pancreatic tissue engineering is the complexity of the organ itself. Pancreas consists of multiple types of cells and every cell type has an important role to play in the overall functioning of the organ (Brereton et al,2015).

In the current study, nanocomposite scaffold made up of agarose and chitosan coated silver nanoparticles were used for application in pancreatic tissue engineering **(Figure 3.7)**.

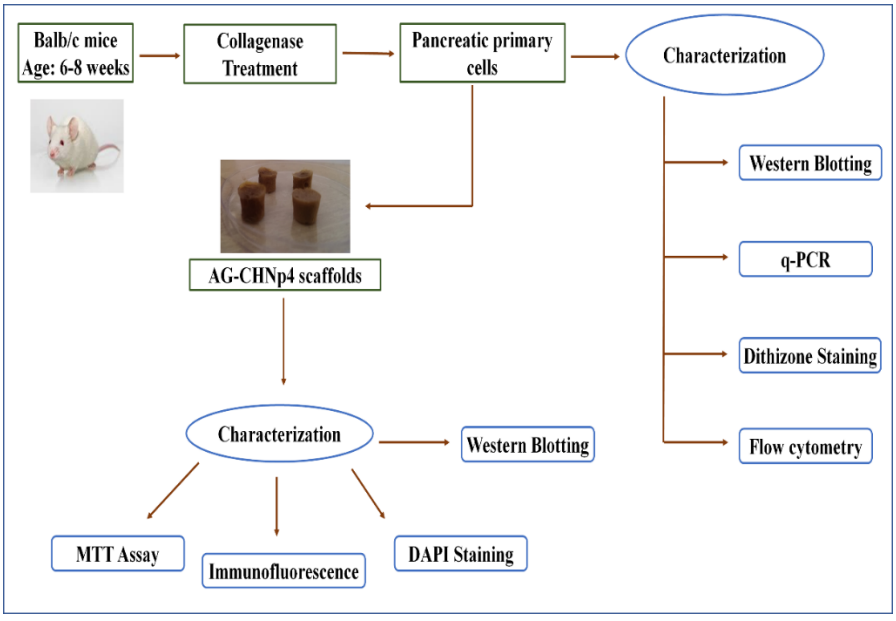


Figure 3.7 Schematic representation showing the application of AG-CHNp4 scaffolds for pancreatic tissue engineering.

AG-CHNp scaffold synthesized using free drying technique, has shown favourable properties such as biocompatibility, biodegradability, good swelling ratio and suitable mechanical strength which has been previously studied by our group (Kumar et al, 2017). In preceding reports, agarose-based scaffolds have been used for tissue engineering applications such as for cartilage, liver, implantation surgery etc (Bhat et al, 2010; Tripathi et al, 2015). For pancreatic tissue engineering, agarose has been primarily studied as an encapsulating agent. Few studies have been conducted using agarose as a 3D scaffold. One group recently showed the effect of co-culturing bone marrow derived mesenchymal stem cells and human islets on agarose gel which proved application of agarose in pancreatic tissue engineering (Kim et al, 2017). But as agarose gel do not support growth of cells, agarose alone might not fit the criteria.

The present report studies the collective effect of all the pancreatic cell types on the AG-CHNp4 scaffold. The pancreatic primary cells were isolated from Balb/c mice and characterized using techniques such as Dithizone staining, q-PCR, western blotting and flow cytometry. Dithizone staining confirmed the presence of islets by staining them crimson red. q-PCR showed variable but positive expression for various pancreatic markers (glucagon, insulin, amylase and PDX-1) suggesting mixed population of cells containing both exocrine and endocrine component. This result was further confirmed by western blotting which showed positive expression for PDX-1, PAX-4, Glucagon and Insulin. The western blotting result is concurrent to the fact that glucagon and insulin are secreted proteins and will show higher levels in comparison to PDX-1 and PAX-4 which are transcription factors associated with differentiated beta cells. Further to investigate the role of AG-CHNp4 scaffold as an artificial ECM for pancreatic tissue engineering, the isolated cells were grown on the scaffold and characterized at various time points. The viability of the cells was established with MTT assay which showed good attachment and proliferation of cells for a period of 40 days in comparison to 2D culture system where the cell number reduced drastically after attaining confluency. This experiment highlights the role of AG-CHNp4 scaffold for long term growth of pancreatic cells. To further confirm the presence of beta cells growing on the scaffold, immunofluorescence was performed for PDX-1. PDX-1 is a transcription factor associated with beta cell maturation and pancreatic development. After 30 days of culture, pancreatic cells showed positive expression of PDX-1, Insulin and glucagon which shows the viability of beta cells in AG-CHNp4 scaffolds. To corroborate the findings at translational level, western blotting was performed on the spent media from the 3D culture system. Western blotting analysis confirmed insulin secretion at all the time points starting from day 5 to day 40 which confirmed sustainable

growth of beta cells for long period of time *in-vitro*. Finally, functionality of beta cells was also confirmed by glucose challenge assay which showed differential intensity peaks for high and low glucose concentration.

In conclusion, agarose-chitosan silver nanoparticle composite shows pronounced compatibility with respect to mice pancreatic primary cells *in-vitro* which was successfully characterized at the transcriptional and translational level. With these current prodigious results, AG-CHNp4 scaffolds looks very promising in the field of pancreatic tissue engineering.

Chapter 4

Differentiation of human umbilical cord derived mesenchymal stem cells into insulin producing cells

4.1 **Introduction**

Since AG-CHNp scaffolds have shown great promise for tissue engineering of pancreas, use of appropriate cell type also becomes extremely important. For pancreatic tissue engineering and implantation, various cell types have been tested previously. These include allogeneic, xenogeneic, and alternative sources {(Embryonic stem cells (ESCs) and Mesenchymal stem cells (MSCs)} (Gazda et al, 2014; Yasuda et al, 2010; Luan et al, 2014; Calafiore et al, 2014). Allogenic and xenogeneic islets are a great source for pancreatic tissue engineering but their limited availability and inferior stability after isolation has circumscribed its application. Generation of pancreatic β cells from embryonic stem cells have shown promising results (Van et al, 2014; Ren et al, 2010; Kahan et al, 2003). However, increased risk of tumor formation, immune rejection, limited sources and ethical concern related to their usage are major shortcomings of this approach (Allahverdi et al, 2015). Hence, owing to the characteristics like multi-potency, high plasticity and fewer ethical concerns regarding their usage, mesenchymal stem cells (MSCs) have been identified as efficient therapeutic alternative for generation of pancreatic β cells (Moshtagh et al, 2013). Moreover, MSCs have demonstrated immunomodulatory effect on the suppression of immune response, thus, contributing to generation of an immunoprivileged site for the transplanted islets (Moshtagh et al, 2013). As a result, MSCs have been identified as better sources for differentiation into pancreatic β cells. Mesenchymal stem cells (MSCs) can be derived from numerous sources including the bone marrow, adipose tissue, umbilical cord, placenta, liver cells, and endometrium (Moshtagh et al, 2013; Gabr et al, 2017). Umbilical cord (UC) MSCs provide an ideal source for differentiation into IPCs due to rapid *in vitro* proliferation, low immunogenicity and ease of transfection (Bai et al, 2013).

The previously synthesized agarose-chitosan coated silver nanoparticle composites (AG-CHNp) scaffolds have shown biocompatibility with cell lines and mice pancreatic cells. Here the AG-CHNp4 scaffold is used for differentiation of human umbilical cord derived mesenchymal stem cells into insulin producing cells. AG-CHNp4 scaffold was used for chemical differentiation of mesenchymal stem cells into insulin producing cells. Most commonly used chemicals for pancreatic differentiation are nicotinamide, activin A, retinoic acid, GLP-1 etc.

4.2 Materials

4.2.1 **Isolation of cells from umbilical cord**

- Phosphate buffer saline, (PBS) (Hi-media, India)
- Minimal essential media (Hi-media, India)
- Fetal bovine serum (Gibco^{TM})
- 100X Penicillin-Streptomycin (Hi-media, India)
- Glutamine solution (Hi-media, India)

4.2.2 **RNA isolation and Real time PCR**

- Trizol (Takara, Japan)
- Chloroform (Fischer Scientific, USA)
- Isopropyl alcohol (Fischer Scientific, USA)
- Ethanol (Hi-media, India)
- Reverse aid cDNA synthesis kit (Thermo Fisher Scientific, USA)
- SYBR® Green PCR master mix (Applied Biosystems, USA)
- Nuclease free water (Takara, Japan)

4.2.3 **Western Blotting**

4.2.3.1 **Lysis buffer for protein isolation (working solution)**

- 1M Tris (Sigma Aldrich chemical Co. (St. Louis, MO))
- 2M Sodium chloride (Sigma Aldrich chemical Co. (St. Louis, MO))
- 0.1M Ethylenediaminetetraacetic acid (EDTA)
- 100 mM Dithiothreitol (DTT) (Sigma Aldrich chemical Co. (St. Louis, MO))
- 1% Triton X100 (Hi-media, India)
- Sodium orthovanadate ($Na_3VO_4 \cdot 2H_2O$)
- 10% glycerol (Hi-media, India)
- Phenylmethane sulfonyl fluoride (PMSF) (Sigma Aldrich chemical Co. (St. Louis, MO))

4.2.3.2 **Lysis buffer for protein isolation (scaffolds)**

- 50mM KCl (Hi-media, India)
- 25mM HEPES (Hi-media, India)
- 1255mM DTT (Sigma Aldrich chemical Co. (St. Louis, MO))
- 1mM PMSF (Sigma Aldrich chemical Co. (St. Louis, MO))
- 1mM Sodium orthovanadate ($Na_3VO_4 \cdot 2H_2O$)
- RIPA buffer (Hi-media, India)

4.2.3.3 **Protein estimation and SDS-Polyacrylamide gel electrophoresis (PAGE)**

- Bradford's reagent (Sigma Aldrich chemical Co. (St. Louis, MO))
- 30% acrylamide and bis-acrylamide solution, 29:1 (Sigma Aldrich chemical Co, USA)
- 10% Sodium Dodecyl Sulfate (SDS) (Sigma Aldrich chemical Co, USA)
- 10% Ammonium persulfate (APS) (Sigma Aldrich chemical Co, USA)
- TEMED (Bio-Rad)
- Stacking gel buffer: 0.5 mol Tris-HCl, pH 6.8 (Sigma Aldrich chemical Co, USA)
- Resolving gel buffer: 1.5 mol Tris-HCl, pH 8.8 (Sigma Aldrich chemical Co, USA)
- Ultra-pure water
- 1X SDS-PAGE Running buffer (Sigma Aldrich chemical Co, USA)
- SDS-PAGE sample loading buffer

4.2.3.4 **Protein transfer and detection**

- 1X western blot transfer buffer (Sigma Aldrich chemical Co, USA)
- Methanol (Thomas Baker)
- Immun-blot PVDF (Bio-rad, USA)
- Membrane blocking buffer: 5% Non-fat milk in 1X PBS
- Membrane washing buffer: 1% Tween20 in 1X PBS

- **Primary antibody**:

  Oct-4 (Sigma Aldrich P0082; 1:2500)

  Nanog (Sigma Aldrich N3038; 1:2500)

  CD-73 (Santacruz biotechnology sc-32299; 1:2500)

  CD-105 (Santacruz biotechnology sc-376381; 1:5000)

  CD-45 (Santacruz biotechnology sc-52490; 1:5000)

  PDX-1 (Santacruz biotechnology sc-390792; 1:2500)

  Insulin (Santacruz biotechnology sc-98941; 1:2500)

  β-actin (Cell signalling 4970; 1:5000)

- **Secondary Antibody**: Goat anti rabbit IgG HRP conjugated (Merck 6.2114E14)

  Anti-mouse IgG HRP linked antibody (Cell signalling 7076)

- Immobilon Western Chemiluminescent HRP Substrate (MERCK, Millipore)

4.3 Methods

4.3.1 **Isolation of primary cells from umbilical cord**

Human placenta samples were collected from nearby maternity hospital, after full term deliveries. A written informed consent was taken before sample collection. All protocols followed ICMR guidelines and were reviewed and approved by the Institutional human ethical committee. All the samples collected from donors who were non-diabetic. The umbilical cord was separated from placenta and was processed within three hours of delivery. All the procedures were reviewed and approved by the institutional ethical committee at Birla Institute of Technology and Sciences, Pilani. Primary cells were isolated from umbilical cord (UC) using direct explant technique. The umbilical cord was washed with sterile phosphate buffered saline to remove blot clots. Small pieces of umbilical cord were placed in tissue culture grade, 6 well plates and supplemented with expansion media (α-MEM + 10% FBS + glutamine + antibiotics). The cells were incubated at 37°C and 5% CO2 in humidified incubator. Media was changed every 3-4 days.

4.3.2 **Characterization of isolated cells**

4.3.2.1 **Actin phalloidin staining**

The cells isolated from umbilical cord (Passage 2) were seeded in the concentration of $1X10^5$ cells per well. Once the cells reach a confluency of 80%, they were washed with PBS and were permeabilized using 0.1% triton X. After permeabilization, the cells were washed with PBS again and were blocked using 1% BSA solution for one hour at room temperature. Rhodamine phalloidin dye was mixed with PBS and was added to the cells which were then incubated for 20 minutes at room temperature. Post incubation, the cells were washed with PBS and counter stained with DAPI and visualized under fluorescence microscope.

4.3.2.2 **Real time PCR**

Total RNA was isolated from mice pancreatic cells using Trizol reagent as per manufacturer's instructions. Following extraction, 1µg of RNA was reverse transcribed to cDNA using Reverse aid cDNA synthesis kit. The synthesized cDNA was used as template to perform Real time PCR (q-PCR) to check the expression of mesenchymal stem cells positive markers (Oct-4, Nanog, CD-73, CD-90, CD-105) and negative (CD-34 and CD-45) using gene specific primers **(Table 4.1)**. 1ul of cDNA was applied in a total reaction mixture of 10 µl containing 5ul of SYBR® Green PCR master mix, 1ul each of forward and reverse primers and 2ul of nuclease free water. This reaction was carried out for 40 cycles in Insta Q96 (Hi-media, India).

Gene	Forward Primer	Reverse Primer
Oct-4	5'-CAGTGCCCGAAACCCACAC-3'	5'-GGAGACCCAGCAGCCTCAAA-3'
Nanog	5'-CAGAAGGCCTCAGCACCTAC-3'	5'-TTGTTCCAGGTCTGGTTGC-3'
CD-73	5'-CTCCTCTCAATCATGCCGCT-3'	5'-TCCCAGGTAATTGTGCCATTGT-3'
CD-105	5'-TGCACTTGGCCTACAATTCCA-3'	5'-AGCTGCCCACTCAAGGATCT-3'
CD-90	5'-ATCGCTCTCCTGCTAACAGTC-3'	5'-CTCGTACTGGATGGGTGAACT-3'
CD-34	5'-TCTGACCTGAAAAAGCTGGGG-3'	5'-GAAGAGTGGTCAGGGTTCCAG-3'
CD-45	5'-ATTACCTGGAATCCCCCTCAAA-3'	5'-TTGTGAAATGACACATTGCAGC-3'
Beta-actin	5'-CGCACCACTGGCATTGTCAT-3'	5'-TTCTCCTTGATGTCACGCAC-3'

Table 4.1: List of primer sequences for human mesenchymal stem cell markers

4.3.2.3 **Western blotting**

Western blotting was performed for mesenchymal stem cells specific antibodies using the protocol mentioned in Chapter 3. Briefly, Cells were harvested by adding 100 µl lysis buffer and stored in -80 ^{0}C overnight. The lysates were thawed on ice, briefly vortexed for 30 secs thrice and centrifuged at 13,000 rpm for 30 mins. After protein estimation with Bradford assay, 30ug was run on 12% SDS-PAGE and transferred onto PVDF membrane for 70 mins at 80 V. The membranes were blocked with 5% skimmed milk for 2 hours at room temperature. After blocking, the membranes were incubated overnight with primary antibody at 4 ^{0}C. Post incubation, the membranes were washed thrice with 1X PBS containing 1% Tween 20 (PBST). The washed membrane was probed with HRP tagged secondary antibody for 2 hours at room temperature. Finally, the membranes, after washing with 1X PBST, were developed on X-ray film using chemiluminescent solvents.

4.3.2.4 **Immunofluorescence**

Immunofluorescence was performed on UC-MSC (Passage 2) cells grown on coverslips. Cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Later, cells were permeabilized with 0.5% saponin and blocked with 5% bovine serum albumin for 1 hour. The cells were incubated with primary antibody overnight. After the incubation, cells were given PBS wash and incubated with FITC tagged secondary antibody for two hours. The coverslips were counterstained with DAPI containing mounting media.

4.3.2.5 **Flow cytometry analysis**

Cells were scrapped out in PBS and centrifuged at 2500 rpm for 5 minutes. PBS was removed completely and add block 0.1% Bovine serum albumin was added for blocking on ice for one hour. Following blocking, the cells were centrifuged at 2500 rpm for 5 minutes at room temperature. Furthermore, the cells were incubated with primary antibody for 30 minutes on ice. Cells were further incubated with fluorophore tagged secondary antibody for 30 minutes on ice. Following incubation, cells were fixed with 4% chilled paraformaldehyde for 1 hour at room temperature. Stained cells were subjected to flow cytometric analysis using BD FACS Canto II cytometer and analysed using FACS Diva software. Multiple gating strategies were used to gate the correct events. Forward scatter (FSC) area vs height measurement was done to remove clumps for single cell analysis, single cells falling along a diagonal were chosen for further analysis. Forward scatter (FSC) and Side scatter (SSC) were used to gate viable and single cells events. Gating was done to exclude debris and dead cells from analysis by categorizing low-FSC events as debris, and events with low FSC and high SSC as dead cells. A compact cell population was thus gated based on size and granularity of the registered events on the scatter plot. Gated CD-105 and CD-73 positive cells were determined as proportionate shift in population.

4.3.3 **Differentiation of mesenchymal stem cells into insulin producing cells**

The UC passage 2 cells were seeded onto AG-CHNp4 scaffolds and tissue culture grade plate (2D control) in the concentration of $1X10^5$ cells per well. The chemical differentiation was carried out in a three-step process (Bai et al,2013; Govindasamy et al,2011)

Step I: DMEM/F12 Ham's + Beta-mercaptoethanol + Insulin-transferrin-serine (ITS) solution (2 days)

Step II: DMEM/F12 Ham's+ Beta-mercaptoethanol + ITS solution + basic FGF + EGF + Nicotinamide + Albumin fraction (7 days)

Step III: DMEM/F12 Ham's + bFGF + Retinoic acid + Nicotinamide + Albumin fraction + B27 solution + N2 solution (7 days)

4.3.4 **Characterization of differentiated cells**

Characterization of differentiated cells was carried out using Reverse-transcriptase PCR, western blotting and immunofluorescence. (Positive markers: CD-73, CD-105, CD-90, Oct-4 and Nanog; Negative markers: CD-45, CD-34; House-keeping marker: Beta-actin)

4.3.4.1 **Reverse-transcriptase PCR**

To determine the expression of pancreatic markers, differentiated cells were harvested. Total RNA was isolated using Trizol reagent as per manufacturer's instructions. Following extraction, 1µg of RNA was reverse transcribed to cDNA using cDNA synthesis kit. The synthesized cDNA was used as template to perform reverse transcriptase PCR (RT-PCR) to check expression of various pancreatic markers (Isl-1, NKX 2.2, PDX-1 and Insulin) using gene specific primers **(Table 4.2)**. All reactions were performed in a total reaction volume of 15 µl and beta-actin was used as the house keeping gene.

Gene	**Forward Primer**	**Reverse Primer**
PDX-1	5'-TTAGACCGAAGGGGAAAACC-3'	5'-TTAGGGAGCCTTCCAATGTG-3'
Isl-1	5'-TCTGTGGGCTGTTCACCAACTGTA-3'	5'-GCCGCAACCAACACATAGGGAAAT-3'
NKX2.2	5'-TCTACGACAGCAGCGACAAC-3'	5'-TTGTCATTGTCCGGTGACTC-3'
Insulin	5'-GCAGCCTTTGTGAACCAAC-3'	5'-GCGGGTCTTGGGTGTGTAG-3'
Beta-actin	5'-CGCACCACTGGCATTGTCAT-3'	5'-TTCTCCTTGATGTCACGCAC-3'

Table 4.2: List of primer sequences for markers associated with differentiated cells.

4.3.4.2 **Western blotting**

Western blot analysis was performed to study the expression of pancreatic marker Insulin in differentiated cells. Cells were harvested after differentiation as described previously. The isolated protein was estimated by Bradford assay and was run on 12% SDS-PAGE with protein ladder and transferred onto PVDF membrane for 70 mins at 80 V. The membranes were blocked with 5% skimmed milk for 2 hours at room temperature. After blocking, the membranes were incubated overnight with primary antibodies at 4^0C. The membranes were then washed thrice with 1X PBST for 5 mins each. The washed membrane was then incubated with HRP tagged secondary antibody for 2 hours at room temperature. The membranes were then washed with 1X PBST for three times 5 min each and developed on X-ray film using chemiluminescent solvents.

4.3.4.3 **Immunofluorescence**

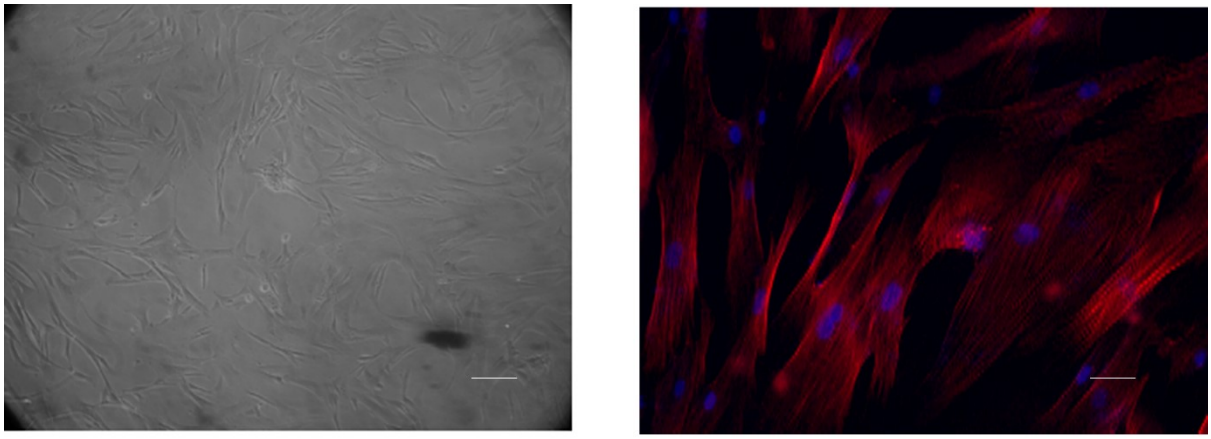
To further confirm the expression of pancreatic markers, immunofluorescence was performed for insulin specific antibody by protocol mentioned above. Briefly, the cells were fixed with 4% paraformaldehyde and were permeabilized with 0.1% triton-X. After PBS wash, the cells were blocked with 1% bovine serum albumin. Furthermore, the cells incubated with monoclonal antibody for insulin overnight. Post PBS wash, cells were incubated with FITC tagged secondary antibody and was later counterstained with DAPI.

4.4 Results

4.4.1 **Characterization of cells isolated from umbilical cord**

Primary cells from umbilical cord was isolated by setting up explant cultures **(Figure 4.1(a))** shows primary cells isolated from umbilical cord (passage 1).

Cytoskeletal organization of the cells were studied using actin phalloidin staining. Cytoskeleton of a cell is primarily made up actin filaments, intermediate filaments, and microtubules. It plays an important role in cellular morphology and growth. **Figure 4.1(b)** shows Actin stained cells showing proper cytoskeletal arrangement.



a b

Figure 4.1: Microscopic analysis of primary cells isolated from umbilical cord. (a) Primary cells from human umbilical cord (scale: 40µm). (b) Actin phalloidin staining of UC passage 1 cells showing cytoskeletal arrangement (Scale: 20 µm).

4.4.1.1 **Gene expression profile**

Isolated primary cells (passage 2) were grown on tissue culture plates for 4 days, harvested, total RNA isolated and c-DNA was synthesized. Expression levels of Oct-4, Nanog, CD73, CD-90, CD-105, CD-34 and CD-45 transcripts were determined using gene specific primers. A 100-fold (approximately) change was observed in mRNA transcript levels of Oct-4, which is one of the most important markers for stem cell characterization **(Figure 4.2)**. Similarly, expression for other mesenchymal stem cell specific markers such as Nanog, CD73, CD-90 and CD-105 were also observed. However, no expression was observed for CD-34 and CD-45 transcripts relative to the reference gene. The transcript levels were normalized with housekeeping gene β-actin and the fold changes were calculated.

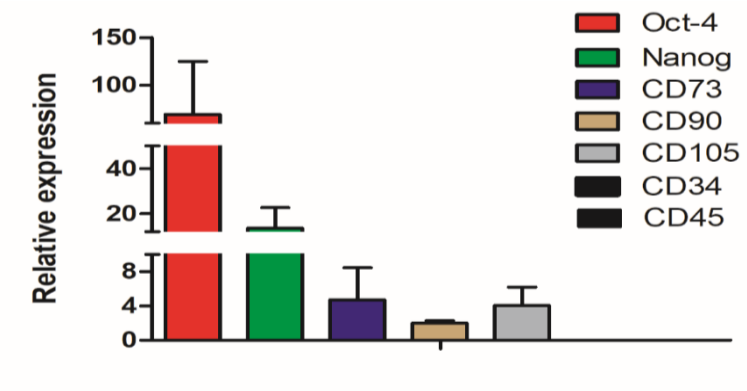


Figure 4.2: Real time PCR analysis showing expression of mesenchymal stem cell markers.

4.4.1.2 **Western blotting**

To confirm the mRNA level expression, western blotting was performed **(Figure 4.3)**. The protein levels of UC-MSC P2 shows the expression of Oct-4, Nanog, CD-105, CD-73, and β-actin (housekeeping control). CD-45 showed negative expression. Western Blotting profile confirms the presence of mesenchymal stem cells in the primary cell pool.

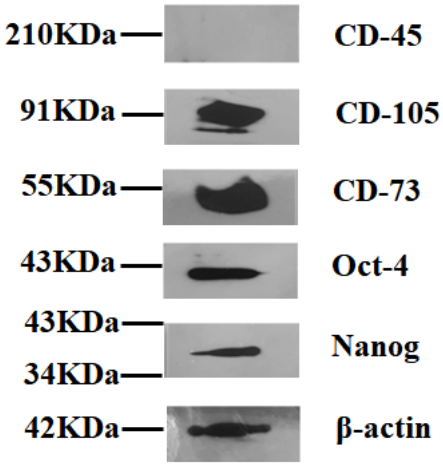


Figure 4.3: Western blotting analysis showing expression of mesenchymal stem cell markers.

4.4.1.3 **Immunofluorescence analysis**

To further confirm the expression for mesenchymal stem cell specific markers, immunofluorescence was performed for primary cells (Passage 2) **(Figure 4.4)**. UC derived primary cells showed positive expression of CD-73, CD-105, Oct-4 and Nanog and they did not show any expression of CD-45. β-actin was used as the housekeeping control.

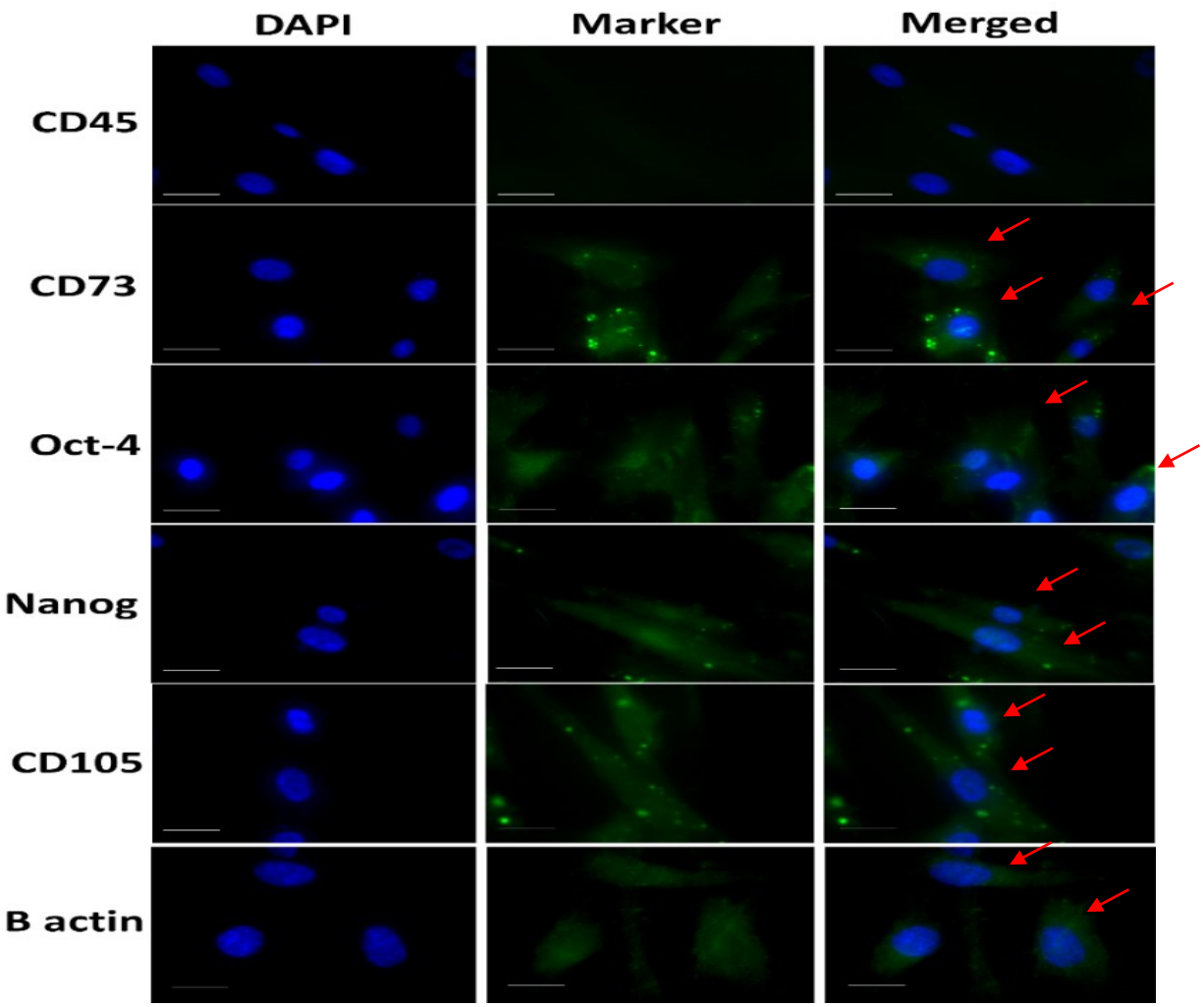


Figure 4.4: Immunofluorescence Images showing presence of CD73, Oct-4, Nanog, CD105 with β actin as housekeeping control and absence of CD45 (scale: 10µm)

4.4.1.4 **Flow cytometry analysis**

The UC derived primary cells (passage 2) was also characterized using flow cytometry to find out the percentage of CD-105 and CD-73. They were fixed and probed with specific monoclonal primary antibody followed by incubation with FITC tagged secondary antibody. Flow cytometric analysis of the cells revealed 81.5% CD-105 positive and 86.1% CD-73 positive cells.

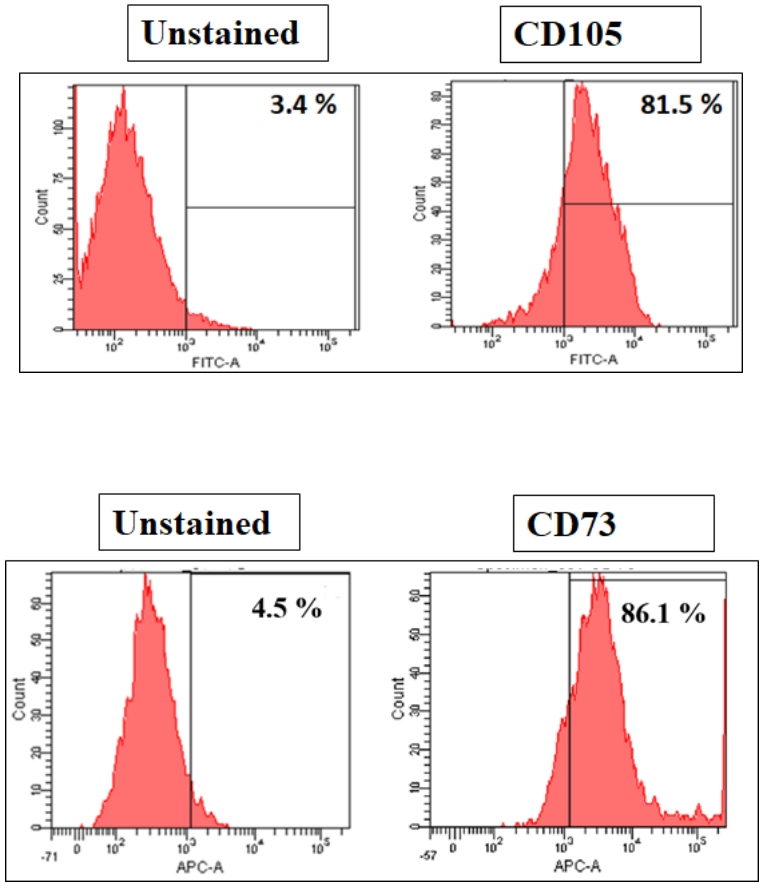


Figure 4.5 Flow cytometry analysis showing percentage of positive cells for CD105 and CD73.

(FITC: Fluorescein isothiocyanate; APC: Allophycocyanin)

4.4.2 **Characterization of differentiated cells**

Differentiated cells were characterized using reverse transcriptase PCR, western blotting and immunofluorescence. Microscopic analysis showed small clusters of cells as seen in **Figure 4.6**

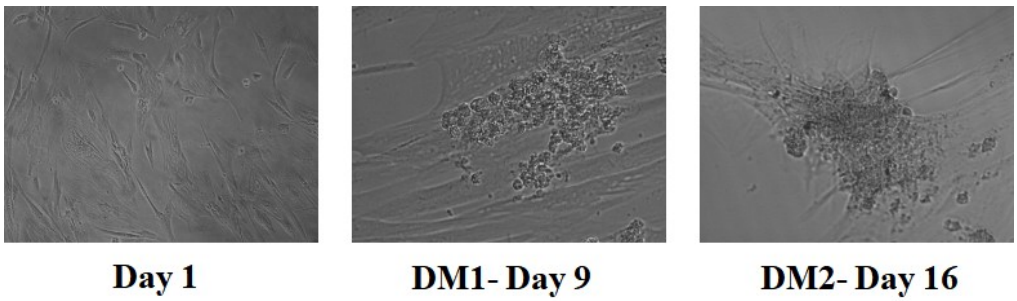


Figure 4.6: Microscopic analysis showing phenotypic changes during differentiation.

4.4.2.1 **Reverse transcriptase PCR (RT-PCR)**

RNA was isolated from 2D differentiated and 3D differentiated cells and c-DNA was synthesized. UC passage 2 cells were used as a control to compare the expression of pancreatic marker in the differentiated and undifferentiated cells. RT-PCR analysis showed positive expression of pancreatic specific markers (Insulin (Ins), PDX-1, Isl-1 and NKX 2.2) in both 2D an 3D differentiated cells. UC passage 2 cells did not show expression of any of the pancreatic genes. Beta-actin was used as the housekeeping control.

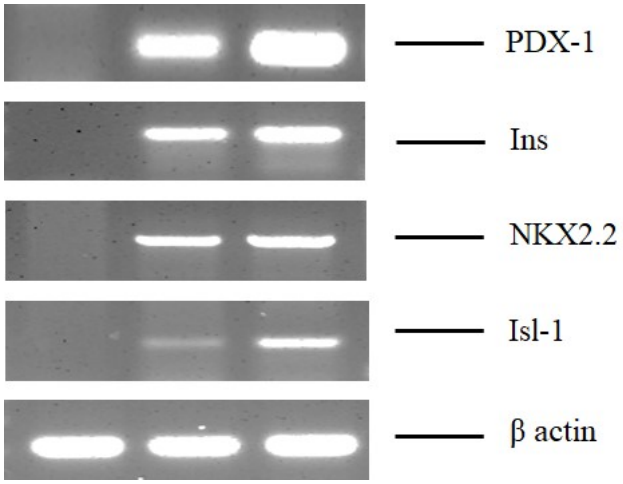


Figure 4.7: Reverse transcriptase PCR showing expression of pancreatic markers in differentiated cells.

4.4.2.2 **Western blotting**

For verifying the mRNA expression, western blotting was performed **(Figure 4.7)**. The protein levels showed the expression of insulin and PDX-1. UC passage 2 cells did not show any expression for either of these. Beta-actin was used as the loading control.

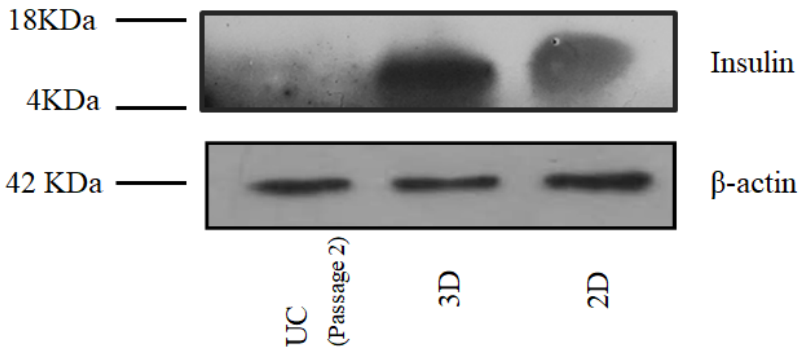


Figure 4.8: Western blotting showing expression of pancreatic marker, Insulin.

4.4.2.3 **Immunofluorescence**

Immunofluorescence analysis further corroborated the transcriptional and translational analysis of the differentiated cells. Immunofluorescence was carried out on the last day of the experiment (17^{th} day). Fluorescence imaging showed positive expression for PDX-1 and insulin **(Figure 4.8)**.

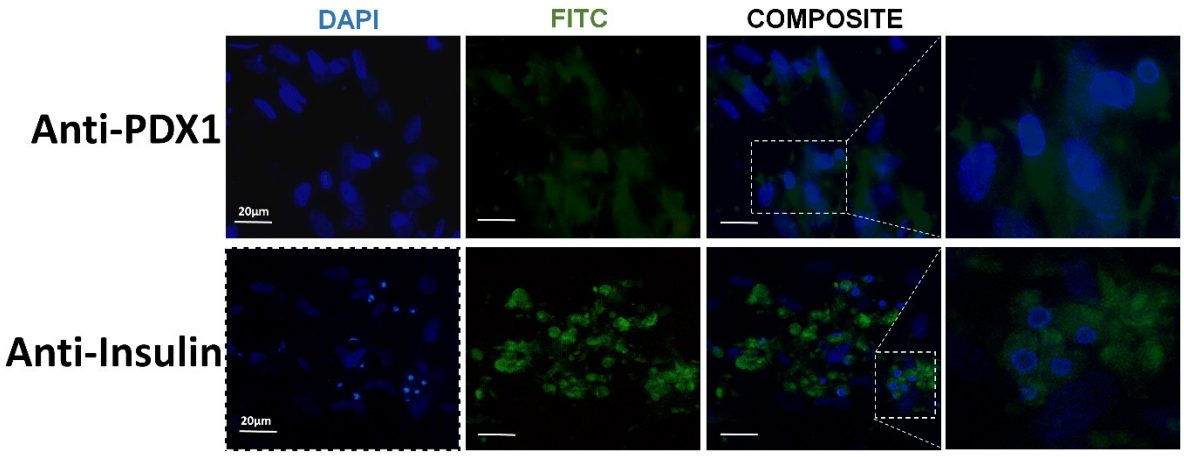


Figure 4.9: Immunofluorescence imagining showing expression of pancreatic markers in differentiated cells.

4.5 **Discussion**

Tissue engineering is dependent upon three major components: scaffolds, cells and bioactive compounds. Cells plays a vital role in making a functional organ construct. For pancreatic tissue engineering, various types of cells have been established. From allogenic to xenogeneic and various types of stem cells have been tested for engineering of pancreas. All these different cell types have their advantages and disadvantages. Mesenchymal stem cells (MSCs) have shown promising results in this field. Scaffolds also plays a pivotal role in tissue engineering of any organ as they act as artificial extracellular matrix for the cells to attach and proliferate. AG-CHNp scaffold has shown long term survival of mice pancreatic cells which was discussed in detail in Chapter 3. This chapter validates the application of AG-CHNp4 scaffold with growth of human cells and its role in differentiation. Mesenchymal stem cells from various sources have shown successful differentiation into insulin producing cells. MSCs from bone marrow (Gabr et al, 2014), dental pulp (Govindasamy et al, 2011), placenta (Şuşman et al, 2015), adipose tissue (Shi et al, 2012), tonsils (Kim et al, 2015) and umbilical cord (Seyedi et al, 2017) have been chemically differentiated to insulin producing cells. Though various sources of cells have been used for isolation of mesenchymal stem cells, the availability of

various sources becomes difficult, hence placenta and umbilical cord becomes viable option for the isolation as they are medical waste and easily available.

Various methods have been employed for differentiation such as chemical differentiation, viral transduction etc. Various chemicals such as nicotinamide, retinoic acid, EGF, FGF, activin A, etc have been used for differentiation of cells. bFGF has been used for differentiation of pluripotent stem cells and have shown improved generation of islet-like clusters (Lumelsky et al., 2001). On the other hand, EGF has shown to enhance the propagation of PDX-1 positive cells (Zhang et al, 2009). Nicotinamide has shown to preserve islets viability and functionality as it is inhibitor for poly (ADP-ribose) polymerase (Yang et al, 2015). In early 90s, studies had shown that improved insulin content in nicotinamide treated human foetal pancreatic cells (Otonkoski et al, 1993).

In the current study, primary cells were isolated from human umbilical cord by setting up explant cultures. The isolated cells were characterized using real time PCR, western blotting, flow cytometry and immunofluorescence. The cells were found to be expressing mesenchymal stem cell positive markers such as CD-73, CD-105, CD-90. They also showed expression for Oct-4 and Nanog which confirmed their stemness. Flow cytometry showed 81.5% CD-105 positive and 86.1% CD-73 positive cells. UC passage 2 cells were used for chemical differentiation. The differentiated cells were characterized on the last day (17^{th} day) of the experiment using reverse transcriptase PCR, western blotting and immunofluorescence. Reverse transcriptase showed the expression of various pancreatic markers such as PDX-1, Insulin, isl-1 and NKX 2.2. UC passage 2 cells did not show any expression of the pancreatic cells. This result was further substantiated by western blotting which showed expression of PDX-1 and insulin in both 2D and 3D culture systems. Immunofluorescence also showed expression of insulin and PDX-1 in the differentiated cells.

In conclusion, results from the study concluded that AG-CHNp4 scaffolds helped in attachment and proliferation of the mesenchymal stem cells and did not hinder in the differentiation of these cells into the pancreatic lineage. These outcomes further validate the use of AG-CHNp4 scaffold for pancreatic tissue engineering. But experiments need to be performed to study role of AG-CHNp4 scaffold in differentiation and long term survival of the differentiated cells.

Chapter 5

Summary and future scope

Summary

Chapter 1

Review of Literature

Diabetes Mellitus (DM) is a metabolic disease, characterized by reduced insulin secretion (type 1) or insulin sensitivity (type 2) or both. There are additional types such as gestational diabetes (GD) and monogenic diabetes. Current treatment includes lifelong insulin therapy which has serious disadvantages and complications associated. Recent approaches have indicated the whole pancreas and islet transplantation as potential alternative treatments of type 1 diabetes. However, these approaches are restricted owing to shortage of donors and complications associated with long-term immunosuppressive therapy. Each of these have been discussed in detail in the study.

To overcome these limitations, research has been focusing on use of tissue engineering to create biological substitutes for replacing damaged tissue and regeneration. The tissue engineering triad comprises of scaffolds, cells and bioactive molecules. Scaffolds are 3-D structures that aim to impersonate the natural microenvironment and provide the necessary structural and mechanical support to the cells. Scaffolding techniques have been thoroughly discussed to obtain an appropriate 3D scaffold. Pancreatic tissue engineering specific advantages and disadvantages of various polymers, both natural and synthetic, have been highlighted in the text. Recent advances in the terms of all the components of the tissue engineering triad have been outlined and gaps in the research were identified.

Chapter 2

Synthesis and characterization of agarose-chitosan coated silver nanoparticle composite scaffold

The property of 3D scaffold to act as a natural microenvironment is central for it to act as a template for regeneration of tissues. In this study, agarose and chitosan coated silver nanocomposites (AG-CHNp) were synthesized using freeze drying technique. Agarose, a natural polysaccharide, has applications in tissue engineering due to its property to form strong gels. However, it lacks the ability of cell attachment. Chitosan, on the other hand, has remarkable properties which includes biodegradability, biocompatibility, non-antigenicity, anti-bacterial properties, etc. Hence, a blend of the two was used to produce a nanocomposite with spongy gel-like properties ideal for tissue engineering of soft tissues.

The synthesized scaffold was characterized for various physical, chemical, mechanical and biological parameters. Shape, size and crystalline nature of the synthesized nanoparticles were determined by TEM studies. The components of the synthesized scaffold and its crystallinity were confirmed using XRD and FTIR. Improved thermal stability was observed for the scaffolds as compared to the controls by Thermo gravimetric analysis. The synthesized scaffold also showed good swelling ratio and strong water retaining capacity. These AG-CHNp scaffolds were shown to have high hemocompatibility and exhibited strong anti- bacterial activity against both gram positive and gram-negative bacteria. Also, biological characterization indicated increased and sustained growth of HeLa, MiaPaCa2 and HEK cells on the synthesized scaffolds as compared to the 2D controls.

Thus, with suitable mechanical strength and biocompatible properties, these scaffolds can be used for tissue engineering of soft tissues such as pancreas.

Chapter 3

Pancreatic tissue engineering using agarose-chitosan coated silver nanocomposite scaffold and mice pancreatic cells

AG-CHNp scaffolds synthesized using freeze drying technique had shown good biocompatibility with cells lines. Hence for confirm its application in pancreatic tissue engineering, AG-CHNp scaffolds were used for testing against mice primary pancreatic cells. Out of the four pre-constructed scaffolds, AG-CHNp4 scaffold exhibited the best results, hence was used for the experiments.

Primary pancreatic cells were isolated from BALB/c mice and were characterized using dithizone staining, real time PCR, western blotting and flow cytometry. The isolated population was found to be positive for glucagon, PDX-1 and Pax-4, while a 200-fold change transcript levels of insulin was observed. Furthermore, the cells were grown on scaffolds and its effectiveness towards insulin secretion was studied using immunofluorescence, MTT assay and western blotting. AG-CHNp4 scaffolds showed pronounced growth of cells for the entire period of 40 days. Western blotting showed insulin secretion at various time points which confirms the functionality of cells growing on the scaffolds. Moreover, glucose challenge test further confirmed the functionality of cells. With the current results, AG-CHNp4 scaffolds showed good advancements with respect to pancreatic tissue engineering.

Chapter 4

Differentiation of human umbilical cord derived mesenchymal stem cells into insulin producing cells

AG-CHNp4 scaffolds were used for differentiation of mesenchymal stem cells which were isolated from human umbilical cord. Before differentiation, the isolated primary cells were characterized for presence of mesenchymal stem cells. Real time PCR and western blotting confirmed the presence of stem cell markers (Oct-4, Nanog, CD-73, CD-90, CD-105). These results were further corroborated by flow cytometry and immunofluorescence. Mesenchymal stem cells were seeded on the scaffolds and tissue culture graded dish (2D control) which was chemically differentiated using nicotinamide and retinoic acid. The differentiated cells were characterized using Reverse transcriptase PCR, western blotting and immunofluorescence. Differentiated cells showed the presence of Insulin and PDX-1. PDX-1 is an important transcription factor essential for development of pancreas and β cell maturation.

This study shows that AG-CHNp4 scaffold not only helps in attachment and proliferation of the mesenchymal stem cells but also aids in the differentiation of these cells into insulin producing cells.

Conclusion and future scope

Based on the results obtained from the thesis, AG-CHNp4 scaffolds have shown great potential as a novel scaffold or an artificial extracellular matrix for tissue engineering of pancreas. Data obtained from *in-vitro* experiments shows that the scaffold promotes long term survival of pancreatic cells on the scaffolds and aids in the chemical differentiation process of mesenchymal stem cells into insulin producing cells. In future, *in-vivo* experiments needs to be done to further corroborate current findings. Transplantation in animals and human beings through clinical trials will validate our claims that the novel scaffolds with cells can be a good source of regulated insulin in type 1 diabetic patients.

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Appendix A

Buffer and Reagent composition

1. Hank's balanced salt solution (for 1L solution)

NaCl (mw: 58.4 g/mol)	8 g	0.14 M
KCl (mw: 74.551 g/mol)	400 mg	0.005 M
Na_2HPO_4 (mw: 177.99 g/mol)	60 mg	0.0003 M
KH_2PO_4 (mw: 136.086 g/mol)	60 mg	0.0004 M
Glucose (mw: 180.156 g/mol)	1 g	0.006 M
$NaHCO_3$ (mw: 84.007 g/mol)	350 mg	0.004 M

2. 30% acrylamide and bis-acrylamide solution (100ml)

Acrylamide	29.2gm
Bisacrylamide	0.8gm
MilliQ water	100ml

3. 1X Running buffer (100ml)

Tris Buffer	303mg
Glycine	1440mg
SDS	100mg

4. 1X Transfer buffer (500ml)

Tris Buffer	1.515gm
Glycine	7.2gm
SDS	150mg
Methanol	100ml

5. Coomassie staining solution (250ml)

Coomassie R_{250}	0.5gm
Methanol	112.5ml
Acetic acid	22.5ml
MilliQ water	115ml

6. Destaining solution (1000ml)

Methanol	500ml
Acetic acid	400ml
MilliQ water	100ml

7. 5X Protein gel loading dye (10ml)

Glycerol	5ml
Bromophenol blue	10mg
10% SDS	1ml
B-mercaptoethanol	500ul
1M Tris (pH 6.8)	3.5ml

8. 50X TAE buffer (500ml)

Tris Buffer	121gm
Glacial acetic acid	28.55ml
EDTA (0.5M)	50ml

9. Whole cell lysis buffer (for scaffolds) (5ml)

Stock Solution	Final concentration	Volume
1M KCl	50mM	250ul
1M HEPES	25mM	125ul
250mM DTT	125mM	2.5ul
100mM PMSF	1mM	50ul
40mM Na_3VO_4	1mM	125ul
RIPA Buffer	--	4.447 ml

10. Krebs-Ringer Buffer (KRB buffer) {pH 7.4}

Components	Required molarity
NaCl (mw: 58.4 g/mol)	115mM
KCL (mw: 74.551 g/mol)	5.9mM
$MgCl_2$ (mw: 95.211 g/mol)	1.2mM
NaH_2PO_4 (mw:119.98 g/mol)	1.2mM
$CaCl_2$ (mw: 110.98 g/mol)	2.5mM
$NaHCO_3$ (mw:84.007 g/mol)	25mM
Glucose	3.3mM and 17mM

Appendix B

List of Publications

B1. List of publications related to the thesis

1. **Kumar, N.**, Desagani, D., Chandran, G., Ghosh, N. N., Karthikeyan, G., Waigaonkar, S., & Ganguly, A. (2018). Biocompatible agarose-chitosan coated silver nanoparticle composite for soft tissue engineering applications. *Artificial cells, nanomedicine, and biotechnology*, *46*(3), 637-649.
2. **Kumar, N.**, Joisher, H., & Ganguly, A. (2018). Polymeric Scaffolds for Pancreatic Tissue Engineering: A Review. *The review of diabetic studies: RDS*, *14*(4), 334-353.
3. **Kumar, N.**, Padhi, A., Sonawane, A., and Ganguly, A. Agarose-chitosan coated silver nanocomposites for sustained long term growth of insulin producing cells.
   (Under review)

B2. Other publications

1. Khandare, D. G., Banerjee, M., Gupta, R., **Kumar, N.**, Ganguly, A., Singh, D., & Chatterjee, A. (2016). Green synthesis of a benzothiazole based 'turn-on'type fluorimetric probe and its use for the selective detection of thiophenols in environmental samples and living cells. *RSC Advances*, *6*(58), 52790-52797.
2. Kumar, V., Chatterjee, A., **Kumar, N.**, Ganguly, A., Chakraborty, I., & Banerjee, M. (2014). d-Glucose derived novel gemini surfactants: synthesis and study of their surface properties, interaction with DNA, and cytotoxicity. *Carbohydrate research*, *397*, 37-45.

B3. Conferences and workshops attended

1. Poster presentation at "National Biomedical Research Competition 2018" held at AIIMS, Rishikesh. (15th October 2018)
2. Poster presentation at "International Conference on Reproductive Physiology and Comparative Endocrinology" (ICRPCE) held at BITS Pilani KK Birla Goa campus. (20th-22nd January 2018)
3. International Conference on Design of Biomaterials @ IISC, Bangalore. (9th-11th December 2012)
4. Fulbright specialist program on "The importance of Engineering-Economics-Entrepreneurship (3-Es) in engineering education and nation building held at BITS-Pilani, KK Birla Goa campus. (28th May-6th July 2018)
5. BIRAC workshop on "Bio-Entrepreneurship, Grant-writing and Intellectual Property Management" held at BITS-Pilani KK Birla Goa campus. (5th February 2018)
6. Workshop on "Biomolecular Interaction" at National Center for Biological Science. (25th – 28th November 2015)
7. Workshop on "Flow Cytometry", ILBS, New Delhi. (18th – 20th August 2015)

B4. Scholarships

1. Awarded "Rudolf Cimdins Scholarship", 2018 by European society of Biomaterials.
2. Awarded Certificate of appreciation (6th rank) in poster presentation in Medical Sciences category at "National Biomedical Research Competition 2018" held at AIIMS, Rishikesh.
3. Awarded Indian Council of Medical Research- Senior research fellowship (ICMR-SRF). (April 2016 – Present)
4. Worked as project fellow on DST-SERB project titled "Tissue engineering of human placental stem cells into functional pancreas". (August 2012- July 2015)

Appendix D

Brief Biography of the Candidate

Nupur Kumar received her M.Tech(Integrated) degree in Bio-technology from Department of Biotechnology and Bioinformatics, Dr. D.Y Patil University, Navi Mumbai, with distinction in 2012. For her master's dissertation, she worked with Dr. Vishal Ranjan on **"**Expression Analysis of FGF-BP *vis-à-vis* Wound Healing."at Actis Biologics Private limited, Mumbai. Nupur has been enrolled in the Ph.D. program of the Department of Biological Sciences, BITS Pilani KK Birla Goa campus. During this time, she worked as a project fellow (August 2012-July 2015) on DST-SERB project titled "Tissue engineering of human placental stem cells into functional pancreas". She later received Senior research fellowship from Indian council of medical research.

Nupur has co-authored 5 international publications and has presented her work at two conferences so far.

Other than science, Nupur likes to travel, listen to music, reading and dancing.

Brief Biography of the Guide

Anasuya Ganguly is an Associate Professor in the Department of Biological Sciences, BITS Pilani KK Birla Goa campus since 2005. Dr. Ganguly received her Master of Science degree in Zoology with a specialization in Advanced Cytology and Genetics from the University of Calcutta in 1996. She received her Ph.D. degree from Jadavpur University in 2003. Her Ph.D. thesis entitled" Regulation of Cell Division in Entamoeba" was carried out under the supervision of Prof. Anuradha Lohia, Bose Institute, Calcutta, India. Dr. Ganguly was subsequently employed as a post-doctoral fellow at the University of Texas Health Science Center, Department of Cellular and Structural Biology, Texas, USA for three years where she worked extensively with cell and tissue culture techniques with an emphasis on cellular stress and aging. Dr. Ganguly has diverse research interests ranging from stem cells and tissue engineering to environmental biotechnology and biodiversity studies.

Dr. Ganguly has been the Principal Investigator in three projects and co-investigator for 4 extramural projects. She has co-authored 23 publications so far in her career. Dr. Ganguly is also the co-Director of the company" Bactreat Environmental Solutions LLP" along with Prof. Srikanth Mutnuri since April 2015.

Other than science, Dr. Ganguly is interested in everything other than politics and economics.

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Biocompatible agarose-chitosan coated silver nanoparticle composite for soft tissue engineering applications

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ABSTRACT

With increasing gap in the demand and supply of vital organs for transplantation there is a pressing need to bridge the gap with substitutes. One way to make substitutes is by tissue engineering which involves combining several types of synthetic or biomaterials, cells and growth factors cross-linked together to synthesize a functional scaffold for repair or replacement of non-functional organs. Nanoparticle based composites are gaining importance in tissue engineering due to their ability to enhance cell attachment and proliferation. The current study focuses on synthesizing agarose composites embedded with chitosan-coated silver nanoparticles using glutaraldehyde as the cross-linker. The synthesis of chitosan coated silver nanoparticles within the scaffold was confirmed with UV-visible spectroscopy. Physical and chemical characterization of the synthesized nanoparticles were done by XRD, FTIR, TGA and SEM. DMA showed higher mechanical strength of the scaffolds. The scaffolds showed degradation of ~37% within a span of four weeks. The higher physical support provided by the synthesized scaffolds was shown by *in-vitro* cell viability assay. Broad spectrum anti-bacterial activity and superior hemocompatibility further showed the advantage it offered for growing cells. Thus a biopolymer based nanocomposite was synthesized, with intended widespread use as scaffold for engineering of soft tissues due to its enhanced biocompatibility and greater surface area for cell growth.

Abbreviations: XRD: X-ray diffraction; FTIR: Fourier transformation Infrared spectroscopy; TGA: Thermogravimetric analysis; DMA: Dynamic mechanical analysis; SEM: Scanning electron microscopy; DAPI: 4',6-diamidino-2-phenylindole; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's modified eagle medium; FBS: Fetal bovine serum; MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NADP: Nicotinamide adenine dinucleotide phosphate; FCC: Face centered cubic; ECM: Extracellular matrix

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Agarose; chitosan; silver nanoparticles; tissue engineering; scaffolds

Introduction

Every single day thousands of people undergo surgery or surgical procedures to replace or repair damaged tissues. Tissue damage due to disease, trauma or injury mostly involves transplantation of tissue from one site from same person to another or from another person. While there has been a revolution in the field of transplantation, it has its shortfall of being expensive and unavailability of donors. Despite constant awareness about organ donation and transplantation, the gap between supply and demand is constantly increasing. As per the united network for organ donation (UNOS) report, till February 2015, there were 123,204 people waiting for organ transplant and each day around 22 people die waiting for the transplant [1]. This gap between organ supply and demand has raised several ethical, moral and societal issues regarding voluntary donors. Moreover, there are various other problems associated with organ transplantation such as low availability, high cost, graft rejection and surgical complications because of which live organ transplantation might be unsuccessful [2].

These continuing necessities of organs have made scientists to search for alternatives which have paved way for the field of tissue engineering. Tissue engineering aims to create biological substitutes that can replace defective or damaged tissues which will help in restoring or maintaining tissue function [3]. The developing field aims to regenerate damaged tissues by growing cells on the scaffold, which acts as a template for tissue regeneration. Tissue engineering is an interdisciplinary field which applies the principles of engineering and cell science together for developing substitutes for damaged organs.

Scaffolds along with cells and bioactive molecules or growth factors form the tissue engineering triad [4]. Correct combination of these three components aids in development of a substitute for growing damaged tissues. Tissue engineering relies heavily on synthesizing a 3D scaffold which could act as a template for regeneration of tissues. In order for the cells to assemble and function properly it is important that the 3D scaffold acts as a natural microenvironment. Numerous scaffolds have been synthesized using different

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Supplemental data for this article can be accessed here.

biomaterials, but regardless of the tissue to be grown on it number of key factors determines the suitability of the scaffolds for application [3]. The most important of them being biocompatibility of the scaffold, allowing the cells to adhere, grow and migrate. Secondly the scaffold should possess time dependent degradation thereby replacing the defective tissue over time [5]. Furthermore, the scaffolds should possess architecture with interconnected pores and with high porosity allowing diffusion of nutrients and wastes [6].

As scaffolds are vital elements of tissue engineering, a variety of fabrication techniques have been employed for designing them. These techniques include freeze drying, solvent casting, gas foaming, electrospining, micromolding, etc. [7]. Freeze drying or lyophilization is one of the most commonly used fabrication technique. This procedure works on the principle of sublimation where the polymer is mixed with a solvent and frozen. Later this solvent is removed by the process of lyophilization. Lyophilization reduces the pressure of the system which will allow the water in the frozen polymer solution to sublimate directly from solid to gas phase thereby giving porous and interconnected structures [8,9]. An ideal scaffold should have certain favourable properties such as biocompatibility, biodegradability, mechanical properties such that they provide support till the time new ECM is formed, surface that promotes cell growth and attachment, porosity, etc. [10].

The basis of any scaffold lies in the polymer that is being used to construct the same. Polymers are of two types: natural polymers and synthetic polymers [11]. Agarose is a naturally occurring polysaccharide isolated from red purple sea weed. It has been extensively used for fabrication of scaffolds because of properties like biodegradability, soft tissue like mechanical property, porosity which helps in cell spreading and proliferation [11]. Agarose forms strong gels even at low concentrations due to its specific chemical structure. Agarose has been reported to have application in gene delivery, as drug delivery systems, hydrogel for maxillofacial and oral surgeries, etc. [11]. One of the major disadvantages associated with agarose is the fact that it lacks the ability of cell attachment, which limits its role in this field [12]. To overcome this, various blends of agarose and other polymers including gelatin, chitosan, fibrin, etc., have been used for tissue engineering applications [12,13]. Also in combination with other polysaccharides, agarose gel provides moist environment and enhance the stability of the system.

Chitosan is a positively charged linear polysaccharide composed of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine. It is the most commonly used polymer in the field of tissue engineering because of its remarkable properties which includes biodegradability, biocompatibility, non-antigenicity, anti-bacterial properties, etc. [14–17]. Chitosan forms secondary interactions (via hydrogen bond) with other polymers as it has polar groups present in its chemical structures. Therefore, chitosan has found application in drug delivery systems, nutrition supplement and in wound healing. Chitosan blended with other polymers have been used in regeneration of almost all the organs including liver, kidney, pancreatic islets, heart, bone, etc. [18–24].

In recent years, research has shifted towards understanding the biology behind the extracellular matrix that provides the cells with necessary foundation to grow and survive. Different organs in the body have different compositions of extracellular matrix, but most of the ECM has been shown to consist of various nanostructures. These nanostructures not only provide strong frame work for the cells but also help in cell attachment and subsequent proliferation due to increased surface area [25,26]. Therefore, including nanoparticles within the scaffolds not only helps in replicating the natural ECM, it would also aid in cell attachment and proliferation.

The current study, taking into consideration the complications in using synthetic polymers has incorporated chitosan coated silver nanoparticles into Agarose scaffolds. The biogenic polymers provide the advantage of reduced toxicity and increased surface area for cell attachment and proliferation. Though the usage of nanoparticles is not new to tissue engineering, but the combination of using Agarose as the scaffold along with chitosan nano-formulation as the framework for cell attachment with glutaraldehyde as the crosslinking agent is for the first time. Various cell lines has been shown to attach and grow on the scaffold.

Materials and methods

Bacterial strains and reagents

Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Klebsiella pneumonia were grown in Luria-Bertani (LB) broth at 37 °C on a shaker at 180 rpm. Chitosan, Silver nitrate and DAPI mounting media (Fluoroshield™) were purchased from Sigma Aldrich chemical Co. (St. Louis, MO). Agarose (Ultrapure™) was purchased from Invitrogen. Glutaraldehyde solution (25%, for synthesis) was purchased from MERCK. Sodium hydroxide, acetic acid and DMSO were acquired from Fischer Scientific. Dulbecco's modified eagle's media (DMEM), foetal bovine serum (FBS), 0.25% trypsin, phosphate buffer saline, 3-(4,5, di methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Hi-media, India. HeLa cell line (human cervical carcinoma cell line), MiaPaCa2 (human pancreatic epithelial carcinoma cell line) and HEK (human embryonic kidney cell line) were procured from National Centre for Cell Sciences (Pune, India). All the other chemicals used in this study were of analytical grade.

Synthesis of chitosan coated silver nanoparticles

Chitosan-coated silver nanoparticles were synthesized using a protocol mentioned by Jena et al. [27]. Chitosan was prepared at a concentration of 1 mg/ml at 45 °C with constant stirring. 0.1 N sodium hydroxide followed by 100 mM silver nitrate was added to the mixture on a continuous basis. Immediate colour change to dark yellow suggested the formation of chitosan coated silver nanoparticles. The suspension was then allowed to settle and later centrifuged at 2000 rpm. The pellet was washed with distilled water twice, dried and dissolved in 0.1% acetic acid.

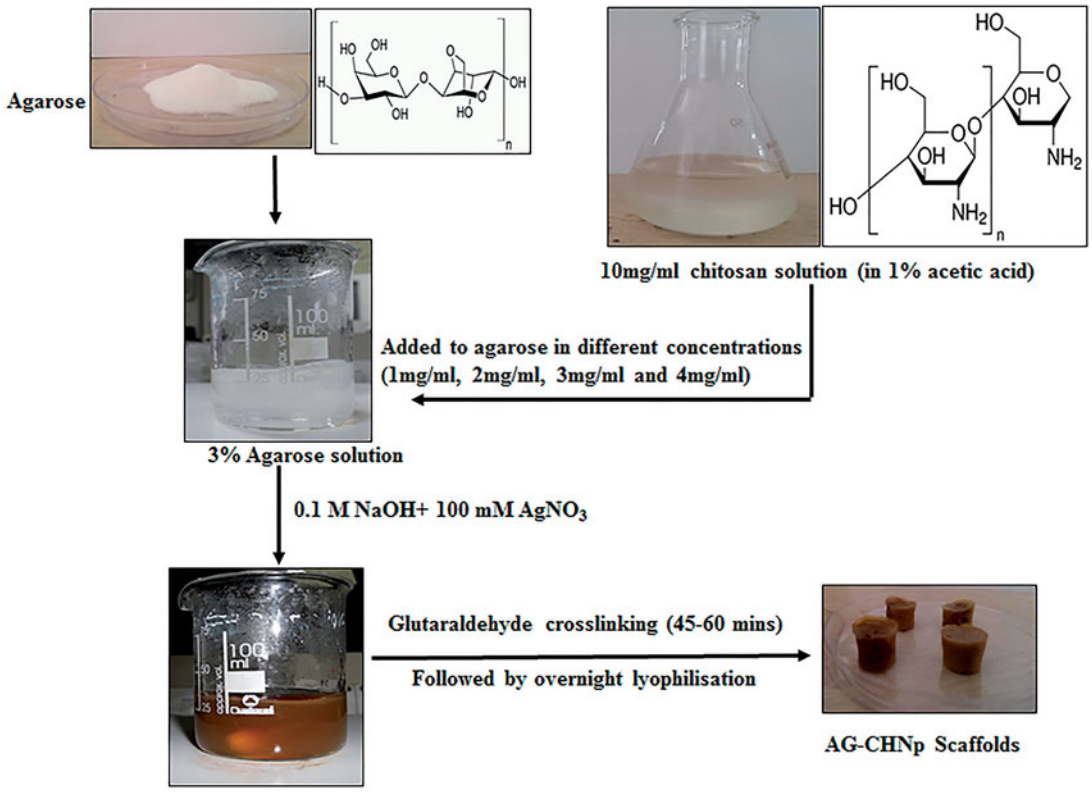

Figure 1. Preparation of Agarose-chitosan-coated silver nanoparticles (AG-CHNp) scaffolds. 3% agarose solution was prepared in deionised water. Different concentrations of chitosan (1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml) were added to it from the stock solution (10 mg/ml) followed by sodium hydroxide and silver nitrate to a final concentration of 0.1 M and 100 mM, respectively, to give chitosan-coated silver nanoparticles embedded in the agarose solution. The AG-CHNp scaffolds were prepared by cross linking the gel using glutaraldehyde (final concentration -1%) followed by overnight lyophilization.

Nanoparticle characterization

In conjunction with the colour change, various other parameters were used to characterize chitosan-coated silver nanoparticles. Synthesized nanoparticles were characterized by UV spectroscopy using Shimadzu UV-2450 spectrophotometer. For transmission electron microscopy (TEM), a drop of aqueous solution of chitosan coated silver nanoparticles was placed on the carbon-coated copper grids. The samples were dried and kept overnight under a desiccator before loading them onto a specimen holder. TEM measurements were performed on JEM-2100, HRTEM, JEOL, JAPAN operating at 200 kV. The size distribution and zeta potential of nanoparticles were determined by Dynamic Light Scattering (DLS) using Zeta sizer nano-ZS Malvern Instruments, UK at Room Temperature. 1 ml of sample was mixed well by vortexing, poured into disposable sizing cuvette for size distribution analysis. Three different peak readings, size versus percentage intensity were plotted. Three such readings were taken and average size distribution was determined. Similarly, zeta potential was analyzed with help of clear disposable zeta cell. Average of three readings was taken to determine zeta potential distribution

Synthesis of Agarose scaffolds impregnated with chitosan coated silver nanoparticles

Agarose was dissolved in water to prepare a 3 wt% polymeric solution. The chitosan coated silver nanoparticles were synthesized with the Agarose solution. Different amounts of chitosan dissolved in acetic-acid aqueous solution was added to Agarose and allowed to mix for 15 min under constant stirring. The nanoparticles were synthesized as described above, leading to an immediate colour change into brownish yellow. Finally, the scaffold was prepared by using a cross linker, i.e. glutaraldehyde (1%) for one hour (Figure 1). The synthesized scaffolds would be denoted as AG-CHNp1, AG-CHNp2, AG-CHNp3 and AG-CHNp4 according to the increasing concentration of chitosan used. The synthesized scaffolds were frozen at $-80\,^{\circ}C$ for 24 h, followed by lyophilization for 16–18 h. Freeze-drying will cause sublimation of ice crystals directly into vapour phase which will in turn cause formation of porous structures.

Physical characterization of scaffolds

The synthesized scaffold were characterized by several physio-chemical methods. UV-visible spectroscopic analysis of the scaffold just before gelation was performed to confirm the synthesis of embedded nanoparticles. The crystalline structure of the scaffolds were studied using X-ray diffraction. The XRD patterns were generated using Rigaku MiniFlex II at room temperature operating at a voltage of 30 kV. The readings were taken at 2θ angle range from 5 to 50°. For analyzing the chemical bonding and functional groups present within the scaffold, FTIR analysis was performed using Shimadzu IR Affinity-I with the help of an attenuated total reflectance (ATR) accessory. The FTIR spectrum was analyzed from 400-4000 cm^{-1}. To examine the thermal stability of the

scaffolds, TGA was performed using Shimadzu DTG-60. The samples were heated from 30 to 600°C with a heating rate of 10°C/minute. The storage and loss modulus of the scaffolds was examined by dynamic mechanical analyser using Universal VA.5 A TA instruments.

Determination of degree of swelling of scaffolds

The degree of swelling for a scaffold was studied using phosphate buffer saline (pH = 7.4) as described previously by Srinivasan et al. [28]. The dry weight of scaffolds was measured before immersing in PBS solution for various time intervals (days 1, 3, and 7). On the respective day, the scaffolds were removed and excess solution was drained out by blotting onto a filter paper. Following which, the wet weight of the scaffolds were measured and swelling ratio was calculated as follows

$$\text{Swelling Ratio} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Dry Weight}}$$

In vitro *degradation studies*

In vitro degradation of the scaffolds was studied as previously described by Srinivasan et al. [28]. Initial weight of the scaffolds was noted down (Wr_i) and then were incubated in phosphate buffer saline containing lysozyme (10,000 units/ml) at 37 °C for different time periods (7, 14, 21, 28 days). Scaffolds were respectively removed, washed with deionized water and lyophilized. The final weight of the scaffolds was noted (W_f).

Percentage degradation was calculated using the following formula:

$$\text{Rate of degradation} = \frac{W_i - W_f}{W_i} \times 100$$

Estimation of hemocompatibility

Estimation of hemocompatibility was done using protocol given by Pal et al. [29]. Fresh human blood was collected in 15 ml centrifuge tube containing sodium citrate (10:1). The blood was then diluted with normal saline (8 ml blood +10 ml saline). To study hemolysis, scaffolds were cut into 5mmX5mm size and placed in a tube containing normal saline solution and incubated for 30 min at 37°C. Diluted blood was added to the tube and incubated for 60 min at 37°C. The positive control had diluted blood added to sodium carbonate solution which caused hemolysis and the negative solution had blood in normal saline solution. Following the incubation, all the tubes were centrifuged for 5 min at 3000 rpm. The supernatant was transferred to the cuvette and readings were taken at 545 nm.

Percentage hemolysis was calculated with the following formula:

$$\frac{\text{OD (test)} - \text{OD (Negative Control)}}{\text{OD (Positive Control)} - \text{OD (Negative control)}} \times 100$$

Assessment of anti-bacterial activity

Scaffolds were also analyzed for their anti-bacterial activity against both Gram positive and Gram negative bacteria by resazurin assay. The scaffolds were casted in a 96 well plate (approximately 150 µl) and different concentration of bacterial cells (10^4, 10^5 and 10^6 cfu of bacteria) were added. The plate was incubated at 37°C overnight. Following day, resazurin dye was added to all the wells and incubated for 3-4 h. Resazurin levels were quantified spectrophotometrically at 570 nm with a reference wavelength of 600 nm [30].

MTT assay

To assess the biocompatibility of the scaffolds, MTT assay was performed. AG-CHNp scaffolds were analyzed for their cytotoxicity with the help of HeLa cell line as previously described by Bhat et al. [31]. Scaffolds were placed in an uncoated 24 well plate and sterilized using increasing concentration of ethanol followed by UV radiation for 20 min. The scaffolds were then equilibrated with complete media to facilitate gaseous exchange for 2–4 h. Following this, HeLa cells were seeded at a concentration of $1X10^5$ cells per well and incubated for a period of 16 days at 37°C with 5% CO_2. For control, same cell density was seeded in poly-lysine coated 24 well plates. Media was changed every alternate day. Media was removed from the well and the scaffolds were given a gentle PBS wash before addition of MTT at 0.5 mg/ml for 3–4 h. After incubation, media was aspirated out and the MTT crystals were dissolved with DMSO and incubated for 15–20 min for colour development. Absorbance was measured at 570 nm to calculate number of viable cells.

Microscopic analysis (DAPI staining)

To visualize the attachment and proliferation of cells on the scaffolds, two cell lines (MiaPaCa2 and HEK) were seeded on the scaffolds and incubated at 37°C with 5% CO_2 for a period of five days. On the day of experiment, the media was aspirated out and the scaffolds were washed with PBS. Later the cells were fixed with 2.5% glutaraldehyde and dehydrated using ethanol gradient. Various sections of the fixed scaffold were cut and the best obtained sections were stained with DAPI containing mounting media for 15-20 min. Following incubation, sections were observed under fluorescence microscope using excitation (405 nm) and emission wavelength (450 nm) of DAPI.

Scanning electron microscopy (SEM) analysis

The microstructures of the prepared scaffolds were examined by SEM (FEI Nova Nano FESEM 450; FEI, Hillsboro, OR). Sample preparation is same as above. All the samples were gold coated and scanning was carried at voltage of 10 kV (for visualizing the surface of the scaffolds) and 5 kV (for visualizing MiaPaCa and HEK cells on the scaffolds).

Statistical analysis

Statistical analysis was performed with GraphPad Prism v 5.0 (GraphPad Software, La Jolla, CA, (http://www.graphpad.com). Significance was referred as *** for $p < .0001$, ** for $p < .001$, * for $p < .05$, ns for non-significant.

Results

Synthesis and characterization of chitosan coated silver nanoparticles

Ultraviolet visible spectroscopy showed a prominent and characteristic peak at 420 nm, suggesting the formation of chitosan-coated silver nanoparticles (Supplementary Figure S(a)). Shape, size and crystalline nature of the synthesized nanoparticles were determined by TEM studies. TEM micrographs showed monodispersed and spherical nanoparticles [32] (Supplementary Figure S(b)). Size distribution profile of the synthesized nanoparticle were determined by dynamic light scattering. Three peaks corresponding to 23.12, 353.1 and 4841 nm were obtained suggesting that most of the particles synthesized were below 500 nm (Supplementary Figure S(c)). But a fine peak at 4800 nm suggested agglomeration of a small proportion of nanoparticles. The degree of stability of the colloidal dispersion and degree of surface potential as measured by zeta potential was found be positive (Supplementary Figure S(d)).

Synthesis and characterization of AG-CHNp scaffolds

Using a 3% polymeric solution of Agarose and varying concentration of chitosan coated silver nanoparticles as the base four scaffolds were synthesized. The chitosan nanoparticles were synthesized inside the scaffolds to obtain a uniform and porous nanoparticle base. The synthesis was confirmed by UV spectroscopy and visible colour change (Figure 2(a)). Glutaraldehyde, shown to be an effective crosslinking agent for stabilization of several kinds of biomaterials was used to obtain a cross-linked Agarose scaffold.

To identify the components of the synthesized scaffold and its crystallinity, powder XRD was performed. Obtained peaks were analyzed to identify individual components of the synthesized scaffold. A peak at $2\theta = 20^{\circ}$ was identified to be of chitosan, which corresponds to previous literature and the single peak indicates its semi-crystalline nature (Figure 2(b)) [33]. Pure agarose also exhibits a single peak at $2\theta = 20^{\circ}$. This can be due to the amorphous nature of agarose. XRD pattern for silver nitrate shows distinct diffraction peaks at around 25°, 37°, 45°, 70° and 76° which are characteristics of (110), (111), (200), (220) and (311) planes of Face centred cubic (FCC) silver, respectively [18,34]. Presence of peak at $2\theta = 20^{\circ}$

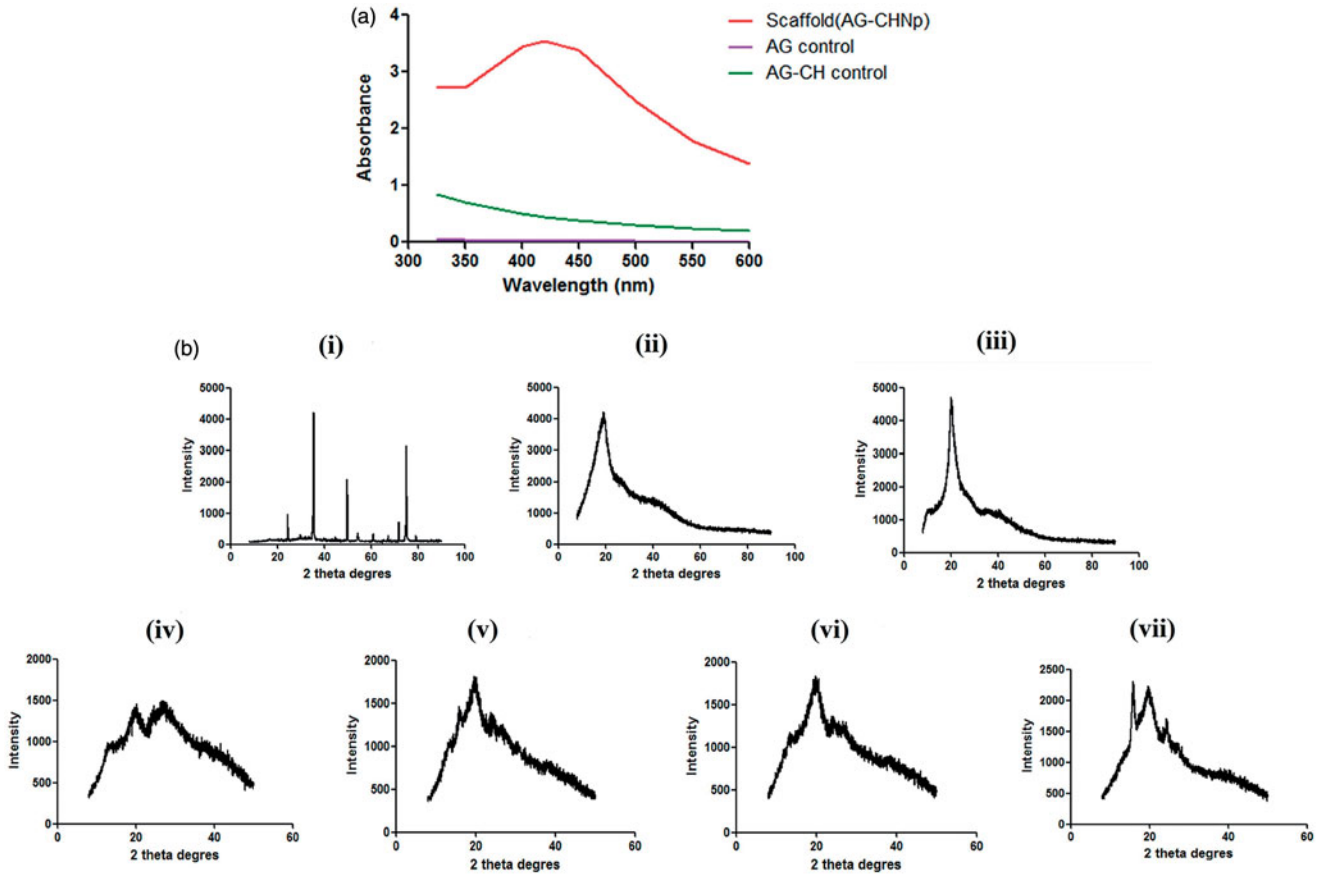

Figure 2. (a) UV-visible absorption spectrum of AG-CHNp scaffolds. AG-CHNp scaffolds showed a prominent peak at 420 nm (characteristic of silver nanoparticles) as compared to agarose control (AG control) and agarose-chitosan control (AG-CH control). (b) X-ray diffraction analysis of the AG-CHNp scaffolds and controls. The diffraction pattern of all the four scaffold (AG-CHNp1 (iv), AG-CHNp2 (v), AG-CHNp3 (vi), AG-CHNp1 (vii)) shows characteristic peaks of the polymers, i.e. agarose and chitosan at $2\theta = 20^{\circ}$. It also showed peaks for silver nitrate suggesting that all the polymers and silver nitrate were present in the scaffolds. (i), (ii), (iii) shows diffraction profile for the silver nitrate, chitosan and agarose respectively.

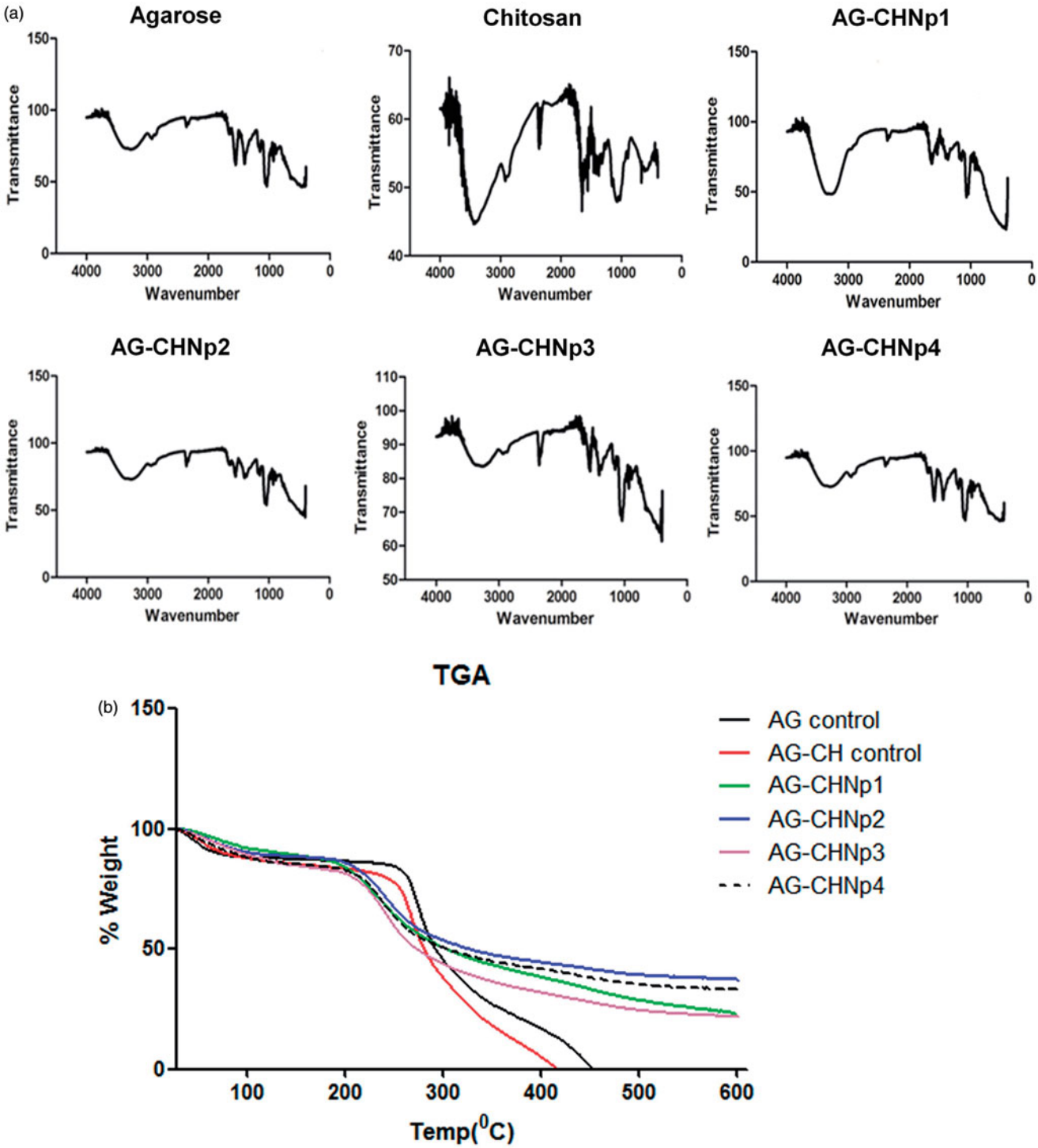

Figure 3. (a) FTIR spectrum of all the four AG-CHNp scaffolds and controls (agarose, chitosan and silver nanoparticles). Typical peaks for –OH stretching, –CH axial deformation, –C–O axial deformation and 3,6-anhydrogalactose were visible in the agarose spectrum. Chitosan spectrum showed specific peaks for –OH group and $–NH_2$ group and O–C–O group. All the AG-CHNp scaffolds showed peaks from both the polymers and presence of peak at 1645 cm^{-1} attributed to presence of imine group (C=N) confirms bond formation between aldehyde group of glutaraldehyde and amino group of chitosan. (b) TGA profile of AG-CHNp scaffolds and agarose control. Thermal profile for scaffolds showed two transition temperatures First at 75–100 °C due to moisture vaporization and second 200–250 °C due to polymer degradation. As compared to control, AG-CHNp scaffolds showed improved thermal stability.

and silver nitrate peak in the AG-CHNp scaffolds confirms the presence of chitosan coated silver nanoparticles. To study the conformational changes in the functional groups of the polymers present in the scaffolds, FTIR analysis was performed. The FTIR spectrum for all the scaffolds obtained from ATR-FTIR spectrometry is depicted in Figure 3(a). Agarose showed characteristic peaks at around 3300 cm^{-1} attributed to –OH stretching, 2913 cm^{-1} attributed to –CH axial deformation, 1083 cm^{-1} for –C-O axial deformation and 946 cm^{-1} for 3,6 anhydrogalactose [12,35]. Chitosan showed its characteristic peaks at around 3400 cm^{-1} (–OH and $–NH_2$ groups), 1553 cm^{-1} and 1370 cm^{-1} (amino groups) and 1080 cm^{-1}

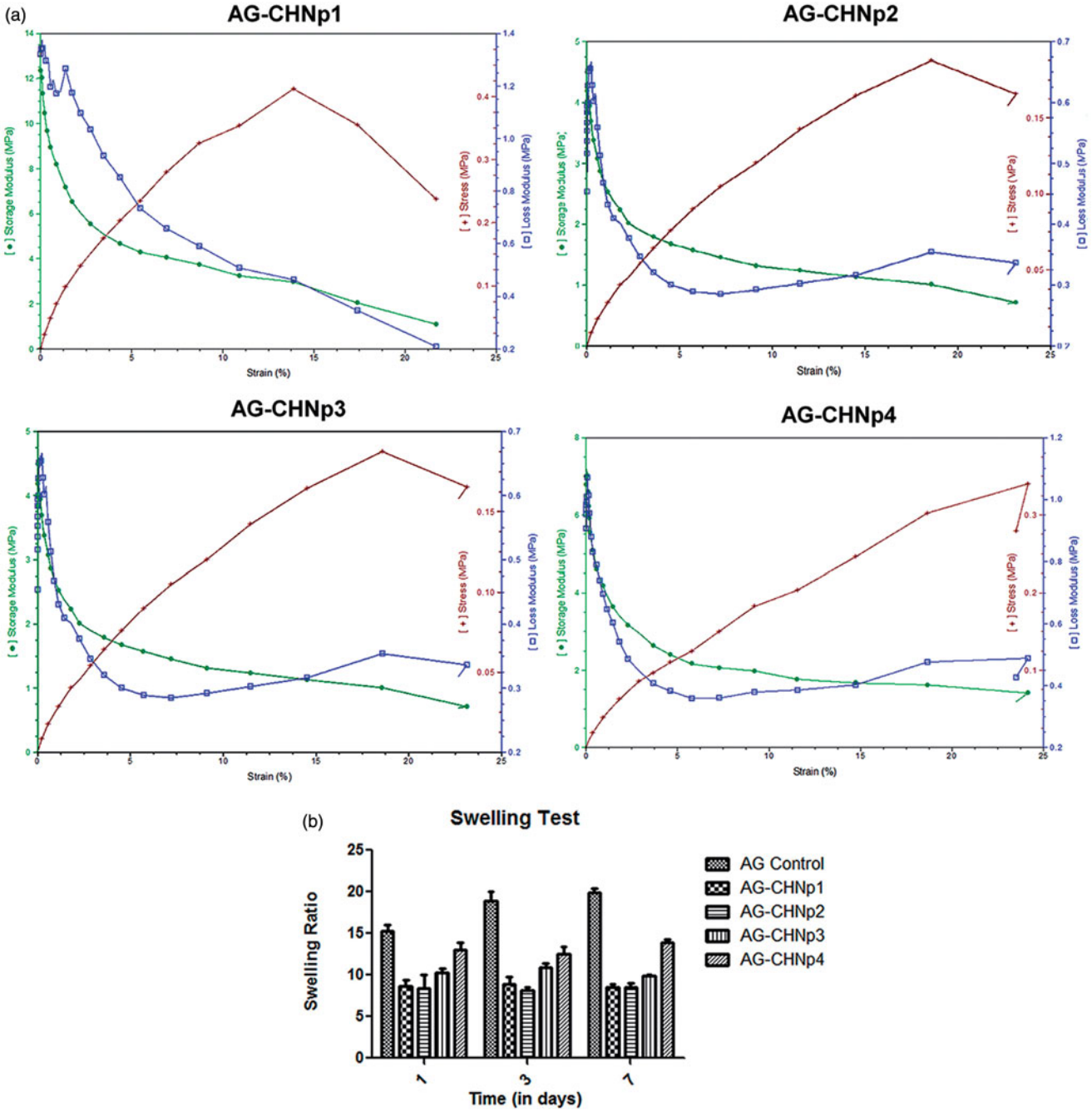

Figure 4. (a) Dynamic mechanical analysis of the AG-CHNp scaffolds. The mechanical strength of the scaffolds was found to be in the range of 5–8 MPa which shows its application in soft tissue engineering. (b) Swelling profile of Agarose control and AG-CHNp scaffolds. All the AG-CHNp scaffolds showed increase in swelling till the final time point. As compared to the control, scaffolds showed lower swelling ratio possibly because of additional cross-linking resulting in strong interaction between the polymer and nanoparticle thereby restricting entry of water.

(O–C–O group) [36]. The presence of combination of functional groups from both the polymers is clearly visible in spectrum of all the four AG-CHNp scaffolds. The presence of peak at around 1645 cm^{-1} (C=N) can be attributed to glutaraldehyde crosslinking within the composite [18,35].

Thermal stability of the compounds was assessed by Thermogravimetric analysis, with the profile showing two prominent stages (Figure 3(b)). First transition occurs between 75 and 100°C, which can be attributed to moisture vaporization causing the weight loss. The second transition occurs between 200 and 250°C. This can be due to decomposition of both the polysaccharide polymers, i.e. agarose and chitosan. The profile also suggests improved thermal stability of the scaffolds as compared to the controls as there is a significant difference in the complete degradation rates which can be attributed to the presence of chitosan-coated silver nanoparticles.

Mechanical strength and elasticity of the AG-CHNp scaffolds

When a specific amount of force is applied to a scaffold, it tends to deform which can cause changes in its storage

modulus and loss modulus. A scaffold should be able to provide adequate mechanical strength so that it can sustain the wear and tear in the body. For soft tissues, mechanical strength in the range of 0.4–350 MPa has been found to be suitable [37]. The results were shown in the form of graph plotted between storage modulus, loss modulus and percentage strain against applied force. The value of all the four AG-CHNp scaffolds was found be in the range of 5–8 MPa (Figure 4(a)). A decrease in elastic deformation (indicated by storage modulus) and viscous response of the scaffold (as indicated by loss modulus) is evident. The strength of the scaffold was found to increase with strain, thereby showing the strain hardening effect. With the excellent mechanical strength and spongy-like property, AG-CHNp scaffolds show additional advantage in tissue engineering of soft tissues such as pancreas, liver, heart, etc.

Swelling test

The ability of the scaffolds to retain liquid or water is an important property for tissue engineering applications. The swelling studies conducted on the scaffold are presented in Figure 4(b). Dry weight of the scaffolds was noted and then soaked in PBS for a period of 7 days. Readings were taken at three different time intervals and swelling ratio was calculated. All the samples showed increase in swelling till the final time point. The scaffolds showed lesser swelling ratio as compared to the control. This can be due to strong interaction between agarose and chitosan-coated silver nanoparticles. This strong interaction can result in the formation of additional cross links which can restrict the entry of water. Our results were in accordance with what has been previously observed [18,38]. The strong water retaining capacity of the synthesized scaffolds could be attributed to their hydrophilicity and a 3-D architecture. Understanding the degree of swelling is very critical for tissue engineering as swelling will cause increase in pore size thereby helping in movement of oxygen and nutrient in and out of the tissue [34].

In vitro *biodegradability*

The *in vitro* degradation profile of all the AG-CHNp scaffolds is shown in Figure 5. Rate of degradation was measured using phosphate buffer saline (pH 7.4) containing lysozyme for a period of 28 days. The degradation rate was measured in terms of change in dry weight of the scaffolds at a given time interval. All the scaffolds showed a gradual rate of degradation with time and were about 35–39% after 4 weeks of incubation. This result is in accordance with the previously published reports by Rad et al. and Frydrch et al. where the scaffolds showed a degradation rate of 36.85% (7 days) and 55–56% (31 days) respectively [39,40]. The degradation can be primarily because of lysozyme action on the macromolecules and degrading it to smaller chains. When the scaffold is implanted in the body, cells grow on its surface and simultaneously secretes its own extra cellular matrix, i.e. rate of degradation should match the rate of tissue formation.

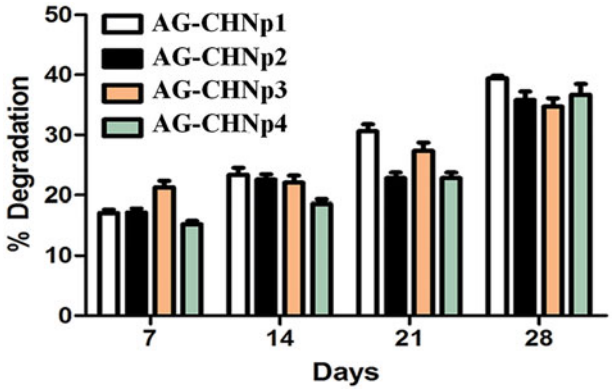

Figure 5. *In-vitro* degradation profile of all AG-CHNp scaffolds. The degradation profile for all four scaffolds showed optimum rate of 35–39%. This profile shows sustained and continuous degradation of the scaffolds which is also important for the cells to secrete their own ECM.

Table 1. Hemocompatibility assay of AG-CHNp scaffolds.

Sample	O.D at 545nm	Percentage hemolysis	Remarks
Positive control	0.785		
Negative control	0.0125		
AG-CHNp1	0.0255	1.68	Highly hemocompatible
AG-CHNp2	0.031	2.39	Highly hemocompatible
AG-CHNp3	0.020	0.97	Highly hemocompatible
AG-CHNp4	0.020	0.97	Highly hemocompatible

Hemocompatibility for all the four scaffolds was tested using fresh human blood. Positive control used was blood diluted with sodium carbonate causing haemolysis and negative control had blood diluted with normal saline. Samples after equilibration were incubated with blood and then absorbance was taken. Percentage haemolysis was calculated using absorbance of the controls and samples (AG-CHNp scaffolds). For all the four AG-CHNp scaffolds, the percentage haemolysis was found to be less than 5%. Therefore, the scaffolds are highly hemocompatible.

Therefore, it is important that the scaffolds degrade with time and degradation should be optimum. The above result suggests that these formulated scaffolds are biodegradable and have an optimum rate of degradation which can allow cells to secrete their own extracellular matrix.

Acceptable hemolysis of the scaffolds

The degree of hemocompatibility of the biomaterials refers to the degree of mutual interaction between the components of the scaffolds and blood. Though the scaffolds were found to be porous, it is important that the scaffolds should not alter the integrity of blood when they come in contact with it. To check the degree of haemolysis of the synthesized scaffolds, the scaffolds along with appropriate controls were incubated with equal amount of diluted blood. Hence, hemocompatiblity of AG-CHNp scaffolds was analyzed and is shown in Table 1. Hemolysis is calculated in terms of percentage and can be placed in one of the following categories [29]:

1. Highly hemocompatible (<5% haemolysis)
2. Hemocompatible (5–10% haemolysis)
3. Non-hemocompatible (>20%)

The percentage hemocompatiblity of the synthesized scaffold was found to be less than 5% suggesting that it lies within the acceptable range and is highly compatible.

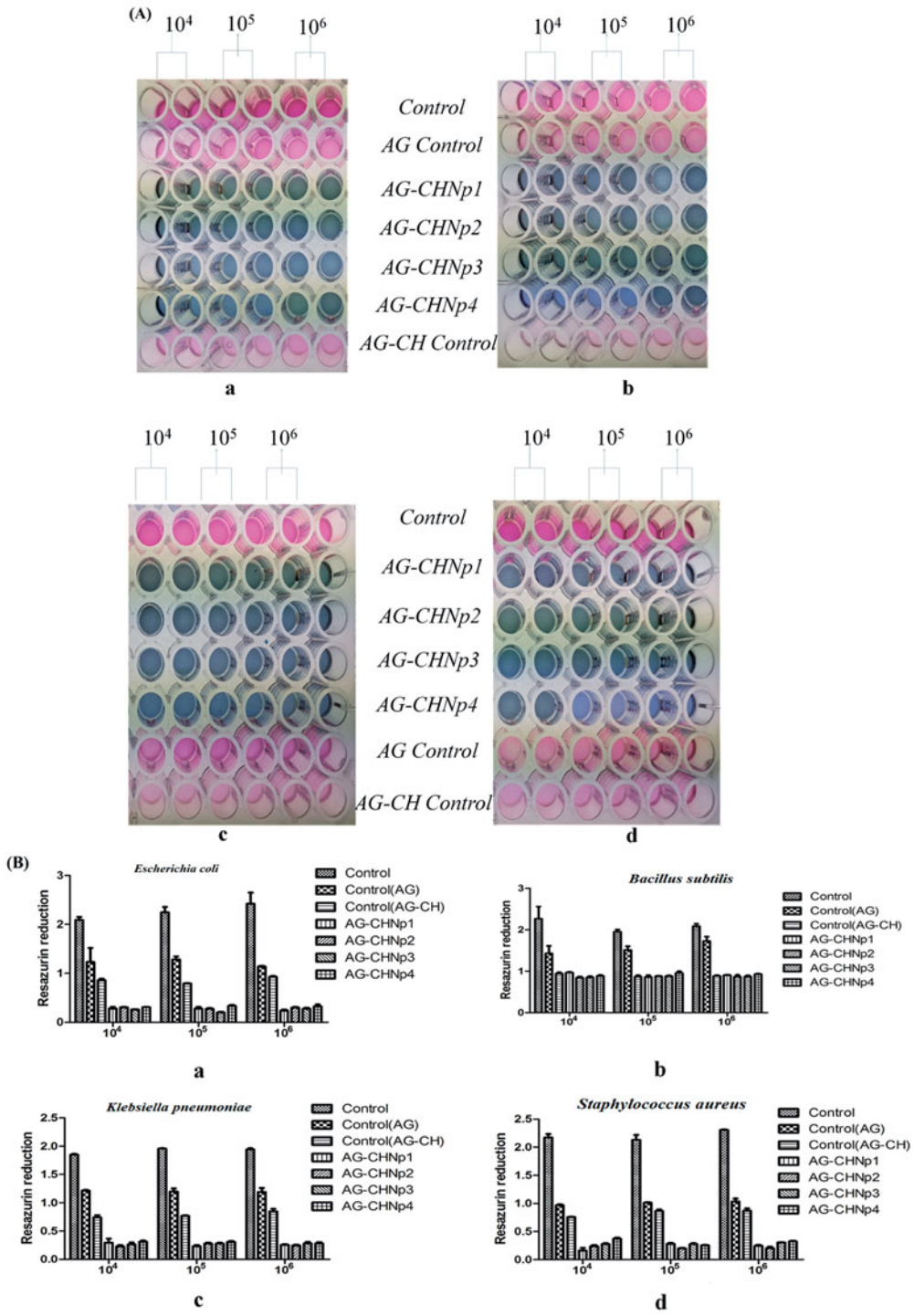

Figure 6. Estimation of anti-bacterial activity of AG-CHNp scaffolds using resazurin assay. (A) Scaffolds were tested for their anti-bacterial activity using resazurin dye. All the four scaffolds showed no change in resazurin as compared to the control. (a) *Escherichia coli2345*, (b) *Bacillus subtilis*, (c) *Klebsiella pneumoniae* and (d) *Staphylococcus aureus737*. (B) Graph showing the resazurin reduction for all the bacterial cultures; (a) *Escherichia coli2345*, (b) *Bacillus subtilis*, (c) *Klebsiella pneumoniae* and (d) *Staphylococcus aureus737*.

This indicates that the scaffold is safe for tissue engineering applications.

Effect of scaffolds on bacterial growth

Certain biological scaffolds have been surprisingly resistant to bacterial infections [41]. Moreover, scaffolds made of natural polymers showed resistance to even deliberate infections in clinical set ups. Thus, the antibacterial activity of the scaffolds was determined using resazurin assay. Different concentrations of bacteria were treated with scaffolds along with necessary controls and resazurin was added to observe colour change (Figure 6(A)). Live bacterial cells convert resazurin dye into resorufin, which is pink in colour. If bacterial growth is not there, resazurin does not get converted and hence will remain blue in colour. Evident colour change in case of controls as compared to the scaffolds indicated at significant antibacterial activity. The absorbance values were compared

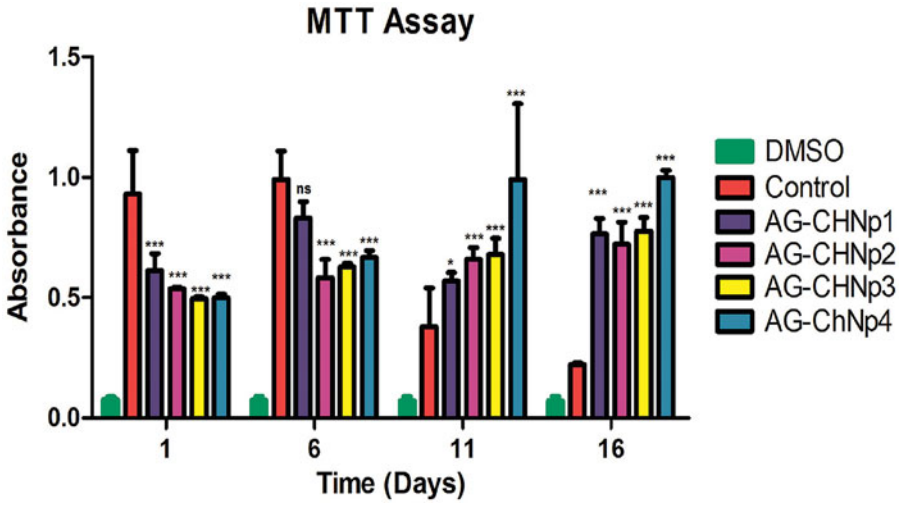

Figure 7. Cell viability and proliferation of HeLa cells in AG-CHNp scaffolds evaluated by MTT assay. All the four scaffolds showed pronounced cell growth with time as compared to 2D control where cell death was seen after day 6. Out of the four scaffolds AG-CHNp4 showed best cell proliferation capability, possibly due to higher chitosan content.

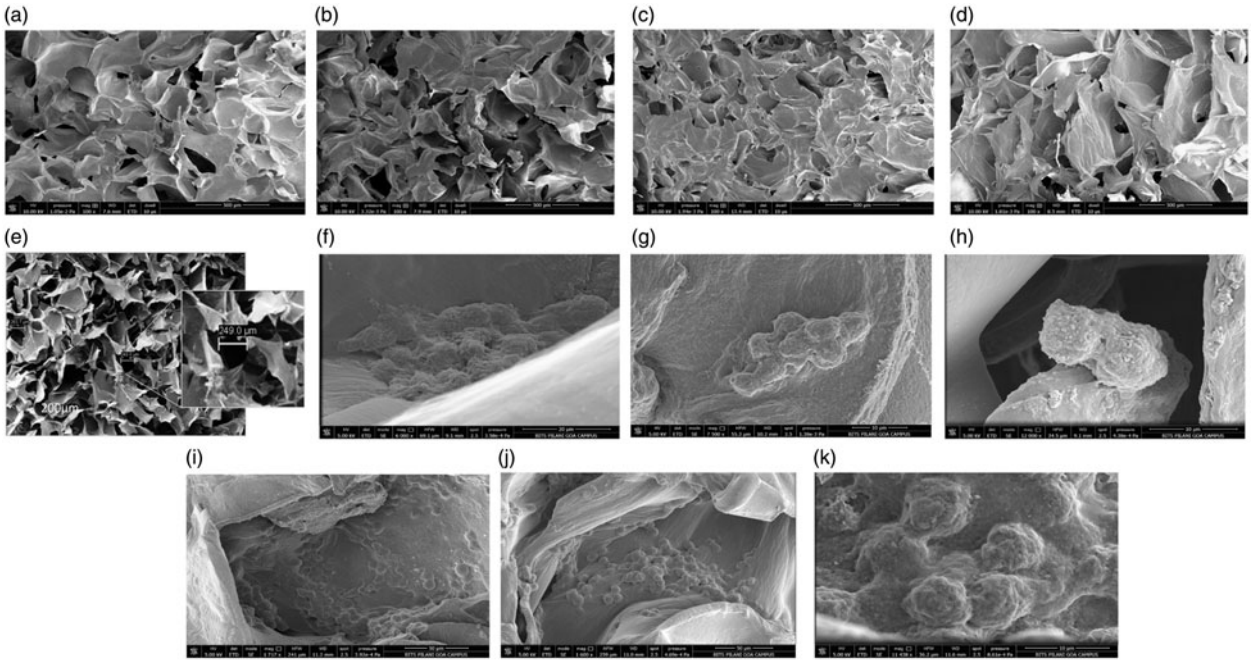

Figure 8. Scanning electron micrographs of AG-CHNp scaffolds. SEM micrographs of all the four scaffolds showed interconnected pores and an average pore size of 175–300 μM (a) AG-CHNp1, (b) AG-CHNp2, (c) AG-CHNp3, (d) AG-CHNp4, (e) SEM image of AG-CHNp scaffold showing the pore, (f),(g),(h) Image showing HEK cells growing the on the scaffold, (i),(j),(k) Image showing MiaPaCa2 cells growing on the scaffold.

with blank wells containing resazurin reagent without cells. Six hundred nanometres absorbance readings were subtracted from average absorbance at 570 nm of experiment wells. Finally, 570–600 nm or resazurin reduction readings were plotted against bacterial number (Figure 6(B)). As shown in the graphs, all the scaffolds showed significant reduction in the bacterial number as compared to the controls. This suggests strong anti-bacterial activity of all the four scaffolds against both Gram positive and Gram negative bacteria.

Biocompatibility of AG-CHNp scaffolds

The viability of cells was assessed by MTT assay using HeLa cell line shown in Figure 7. Metabolically active cells contain dehydrogenase enzymes which reduces yellow coloured MTT salt (3-(4, 5-dimethylthiazolyl-2)–2, 5-diphenyltetrazolium bromide) to NADP and NADPH. The resulting purple formazan crystals are solubilized in DMSO and quantified spectrophotometrically at 570 nm. Thus, an increase in optical density helps to assess cell's proliferative capacity within the scaffold. The viability of HeLa cells was studied for a period of 16 days. As compared to the 2D control, all the four scaffolds showed increased and sustained growth of cells for a longer period. In the 2D controls, HeLa cells grew and reached confluence by day 6. With no more surface area available, there is immediate reduction in the cell number owing to cell death. On the other hand, as the scaffolds have 3D morphology and provide better surface area for growth,

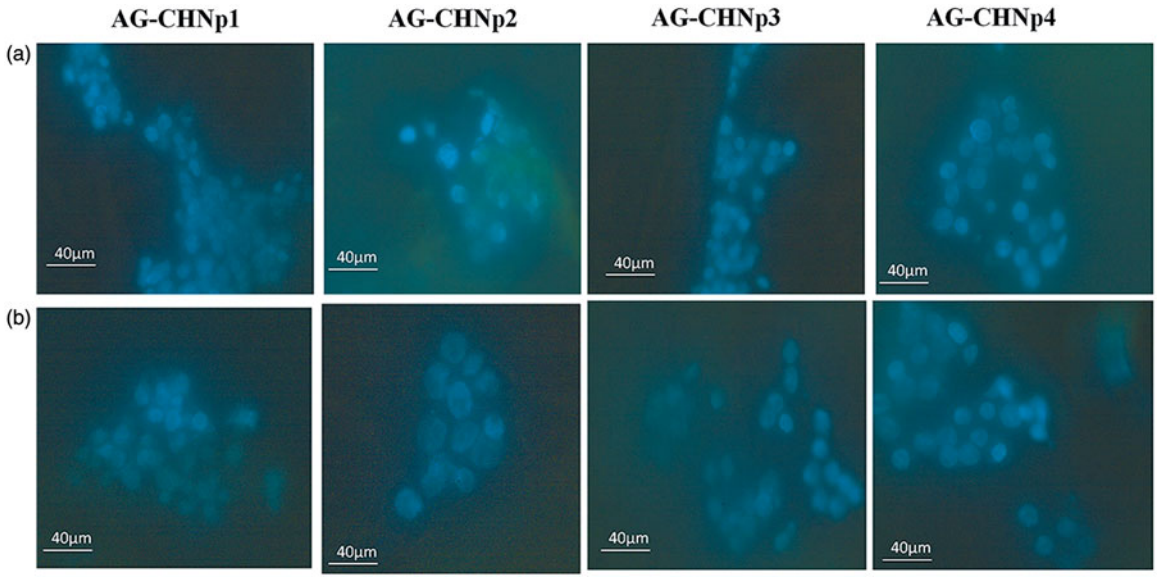

Figure 9. Fluorescent images of DAPI staining for AG-CHNp scaffolds showing MiaPaCa2 and HEK cells. Sections of scaffolds with MiaPaCa2 (a) and HEK (b) cells were stained with DAPI and visualized under fluorescence microscope. The images showed proper cell attachment and rounded morphology of the cells on the scaffolds.

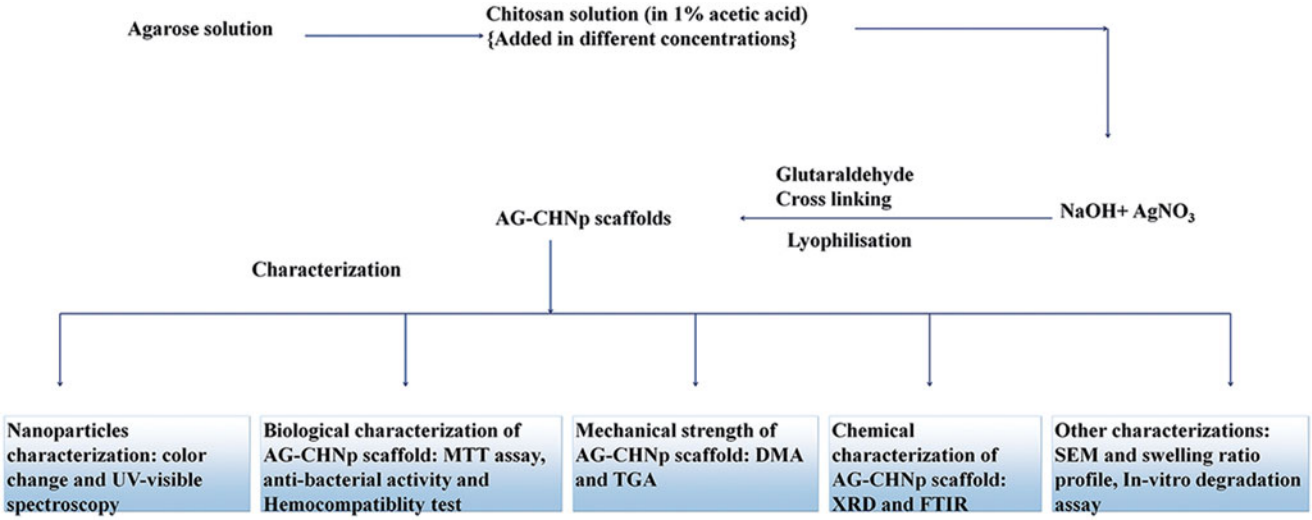

Figure 10. Schematic representation of fabrication and characterization of AG-CHNp scaffolds. AG-CHNp scaffolds were synthesized using the polymers agarose and chitosan by freeze drying technique. Chitosan-coated silver nanoparticles were synthesized within the scaffolds. All the four scaffolds were then characterized on various parameters (physical, chemical, mechanical, biological and morphological).

there is a visible sustained growth during all the time points. Furthermore, AG-CHNp4 showed better growth of cells as compared to all the other scaffolds. This could be attributed to higher concentration of chitosan in AG-CHNp4. The above-mentioned results show excellent biocompatibility of all the four scaffolds.

Microstructure analysis and cell attachment

The scaffolds were designed in such a manner to accommodate cell attachment, growth and migration. The porous structure allows for diffusion of nutrients and removal of toxic compounds. To ascertain the gross morphology of the 3D structure and to determine the pore size SEM analysis was performed. The scaffolds were found to have an average pore size of 175–300 μM. Pore size between 100 and 500 μM is important for cell attachment, nutrient and oxygen diffusion [42]. Scaffolds with similar pore size have been used before for tissue engineering of pancreas, liver, etc. [43,44] (Figure 8). The cell adhesion and attachment property was analyzed using DAPI staining of MiaPaCa2 and HEK cell lines. As DAPI is a nuclear stain, rounded morphology of cell's nucleus was visible. Furthermore, post-attachment cells were found to grow in an optimal and sustained manner (Figure 9).

Discussion

Most of the tissue engineering applications demand the need of biodegradable materials with potential to serve multiple purposes. The most common approach for tissue engineering is seeding cells onto a biomaterial matrix. The design of the scaffold prior to cell application is of prime importance. The

scaffold used must provide a surface for the attachment and growth of cells, also providing tissue growth. Thus the choice of material is of great importance. The material should be biocompatible and biodegradable, and should also have the required mechanical strength to offer a structural support. Moreover it must be able to allow smooth diffusion of nutrients and also discharge of toxic compounds released. To achieve all of this, most designed scaffold deliberately mimic the structure of the naturally occurring extracellular matrix.

Chitosan, derivative of chitin is one such biomaterial and has been extensively used to prepare porous scaffolds allowing the growth of several tissues. Agarose a saccharide polymer originating from sea weed possesses significant mechanical strength in aqueous solution. It forms a transparent colourless gel, forming a porous three-dimensional structure. Nanoparticles, for their unique structural aspects provide the connecting link between bottom up synthetic methods and top-down fabrication methods. Hydrogel-based nanocomposite scaffolds have found application in multiple tissue engineering work along with drug delivery as they allow controlled release of drugs, and other growth factors required for cell growth [25,45]. This wide application in tissue engineering of nanoparticles is due to the structural advantage it offers as well as the mechanical support to grow cells. They also act as porogens in forming a porous scaffold.

In the current study, a blend of agarose and chitosan coated silver nanoparticles was optimized using freeze drying technique to produce a nanocomposite with spongy gel-like properties ideal for tissue engineering of soft tissues. Previously, agarose-chitosan ionogel nanocomposites have been synthesized using sol-gel technique have shown promising results and suggests application in various biomedical and biotechnological fields [18]. Similarly, agarose-chitosan-gelatin blend prepared using cryogelation technique have been used for cartilage tissue engineering [31]. A recent study also suggested use of agarose-chitosan blend prepared at sub-zero temperatures for 3D liver tissue engineering [35]. Though nanostructures are an important component of the extracellular matrix of any organ, use of nanoparticles for preparation of composites has been sparsely studied. Therefore, to our best knowledge, agarose-chitosan-coated silver nanoparticles composites cross linked by glutaraldehyde are being reported for the first time. A simple and efficient preparation protocol was optimized which has shown to give soft, elastic nanocomposite using freeze drying. The scaffold was characterized for various physical, chemical, mechanical and biological parameters (Figure 10). The scaffolds showed good swelling ratio, excellent hemocompatibility, appreciable anti-bacterial activity against both Gram positive and Gram negative bacteria. The scaffolds also showed good biocompatibility with HeLa, MiaPaCa2 and HEK cells and have showed their sustained growth. With suitable mechanical strength, these scaffolds can be used for tissue engineering of soft tissues such as pancreas, kidney heart, liver, etc.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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REVIEW

Polymeric Scaffolds for Pancreatic Tissue Engineering: A Review

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■ Abstract

In recent years, there has been an alarming increase in the incidence of diabetes, with one in every eleven individuals worldwide suffering from this debilitating disease. As the available treatment options fail to reduce disease progression, novel avenues such as the bioartificial pancreas are being given serious consideration. In the past decade, the research focus has shifted towards the field of tissue engineering, which helps to design biological substitutes for repair and replacement of non-functional or damaged organs. Scaffolds constitute an integral part of tissue engineering; they have been shown to mimic the native extracellular matrix, thereby supporting cell viability and proliferation. This review offers a novel compilation of the recent advances in polymeric scaffolds, which are used for pancreatic tissue engineering. Furthermore, in this article, the design strategies for bioartificial pancreatic constructs and their future applications in cell-based therapy are discussed.

Keywords: diabetes · transplant · scaffold · polymer · pancreas · islets · tissue engineering

1. Introduction

With changing economic development patterns, the world has experienced a steep increase in the number of patients with lifestyle diseases. Diseases associated with lifestyle imbalance include diabetes, hypertension, cardiovascular diseases, and certain types of cancers. Such diseases are associated with a lack of physical activity, unfavorable occupational habits, and increased obesity. Diet and lifestyle play an important role in maintaining physical and mental health [1]. For centuries, infectious diseases have been considered as the main killer around the world. But with non-communicable diseases (NCDs) taking the front seat, it is estimated that by the year 2020 NCDs will cause seven out of ten deaths in developing nations [2]. Diabetes, one of the four priority NCDs, is currently the eighth leading cause of death in both sexes [3, 4]. Initially labeled as a disease of rich countries, diabetes has shown a tremendous increase in the past few years, even in middle income nations. According to the WHO's global diabetes report 2016, a total of 422 million people across the world are currently suffering from diabetes [5]. Its global prevalence increased from 4.7% in 1980 to 8.5% in 2014 (**Figure 1**) [5]. As per the International Diabetes Federation (IDF) report (2015), China ranks first in relation to the number of diabetic patients (between the age of 20 and 79 years) followed by India and the USA (**Figure 2**) [6].

Diabetes mellitus is classified as one of the metabolic disorders characterized by a chronic hyperglycemic condition. This state is mainly attributed to defects in insulin secretion or to the action of insulin in cells or both. Most cases of diabetes are one of two types: type 1 diabetes (T1D) and type 2 diabetes (T2D). There are additional types such as gestational diabetes (GD) and maturity onset diabetes of the young (MODY). T1D, also known as insulin-dependent diabetes mellitus

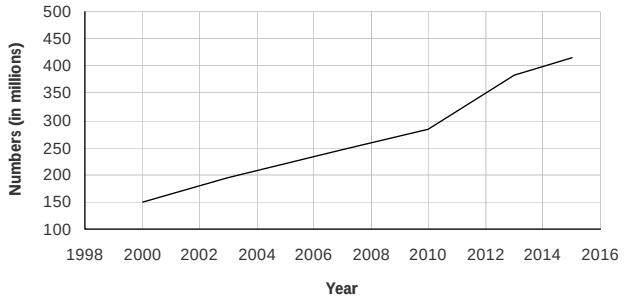

Figure 1. Prevalence of diabetes worldwide. Worldwide increase in the occurrence of diabetes in the past two decades. Source: IDF Diabetes Atlas; http://www.diabetesatlas.org.

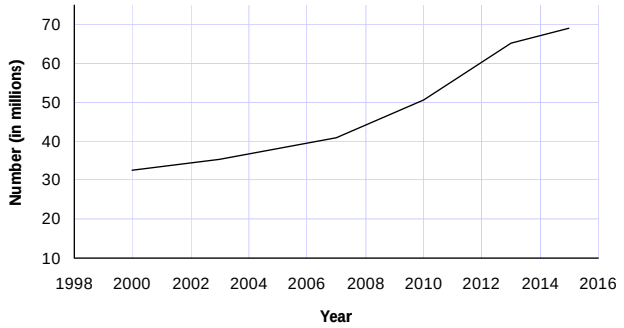

Figure 2. Prevalence of diabetes in India. Increasing similar to worldwide trend. Source: IDF Diabetes Atlas, http://www.diabetesatlas.org.

(IDDM), is an autoimmune condition where the body attacks its own β-cells, destroying them, and rendering them unfit to produce insulin, thereby increasing blood glucose levels [7, 8].

Currently, treatments for diabetes include insulin therapy, drugs such as biguanides, sulfonylureas, megalitinides, thiazolidinediones, alfa gluocosidase inhibitors, and others, whole pancreas transplantation, islet transplantation, and bariatric surgery. Even after the award-winning discovery of insulin therapy in 1921, diabetes treatment has not come a long way [9]. Over the years, significant research has focused on the development of substitute routes for insulin administration like nasal, rectal, and oral [10]. Several insulin release devices such as insulin pumps, pen injectors, and inhalation patches have been engineered to enhance patient amenability [11]. For controlled and tunable release of insulin, carriers made of hydrogels, microspheres, and nanoparticles have been formulated [12].

Although multiple modifications in the source, structure, and delivery mode of insulin have been made to improve the management of diabetics, millions of patients still continue to inject themselves with insulin several times a day. Apart from numerous injections, prolonged usage of insulin can cause diabetic retinopathy, ketoacidosis, weight gain, etc. All these treatment options have serious disadvantages which have led to a search for better options. In recent years, tissue engineering has shown promise in the treatment of various conditions.

Abbreviations:

ANCOVA	analysis of covariance
2D	two-dimensional
3D	three-dimensional
AG-CHNP	agarose chitosan-based nanocomposite
BM-MSC	bone marrow-derived mesenchymal stem cell
ECM	extracellular matrix
EC	endothelial cell
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EGF	epidermal growth factor
FDA	US Food and Drug Administration
FGF	fibroblast growth factor
GD	gestational diabetes
GLP-1	glucagon-like factor 1
HEK	human embryonic kidney
HeLa	cervical cancer cell line (Henrietta Lacks)
IDDM	insulin-dependent diabetes mellitus
IDE1	inducer of definitive endoderm 1
IDF	International Diabetes Federation
IKVAV	isoleucine-lysine-valine-alanine-valine
INS-1	insulinoma cell line
IPN	interpenetrating polymer network
iPSC	induced pluripotent stem cell
Mia PaCa-2	human pancreatic carcinoma cell line
MODY	maturity onset diabetes of the young
MSC	mesenchymal stem cell
NCD	non-communicable diseases
NHS	N-hydroxysuccinimide
NKX2.2	NK2 homeobox protein 2
PAA	polyacrylic acid
PCL	polycaprolactone
PDMS	polydimethylsiloxane
PDX1	pancreatic and duodenal homeobox 1
PEG	polyethylene glycol
PGA	polyglycolic acid
PLA	polylactic acid
PLG	polylactide-co-glycolide
PLGA	polylactic-co-glycolic acid
PU	polyurethane
PVP	polyvinylpyrrolidone
RGD	arginylglycylaspartic acid
RIN-5	rat insulinoma cell
ROS	reactive oxygen species
SC β-cell	stem cell-derived β-cell
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
VEGF	vascular endothelial growth factor
WHO	World Health Organization
YIGSR	tyrosine-isoleucine-glycine-serine-arginine

2. Introduction of tissue engineering

Tissue engineering comprises several disciplinary fields including engineering, material science, and life sciences; it aims to produce biologically viable substitutes for tissue and organ regeneration [13]. Tissue engineering provides a means to synthesize substitutes for repair or replacement of tissues or organs damaged due to pathology, trauma, or injury. It provides an alternative to bridge the ever-growing gap between demand and supply of organs for transplantation [14].

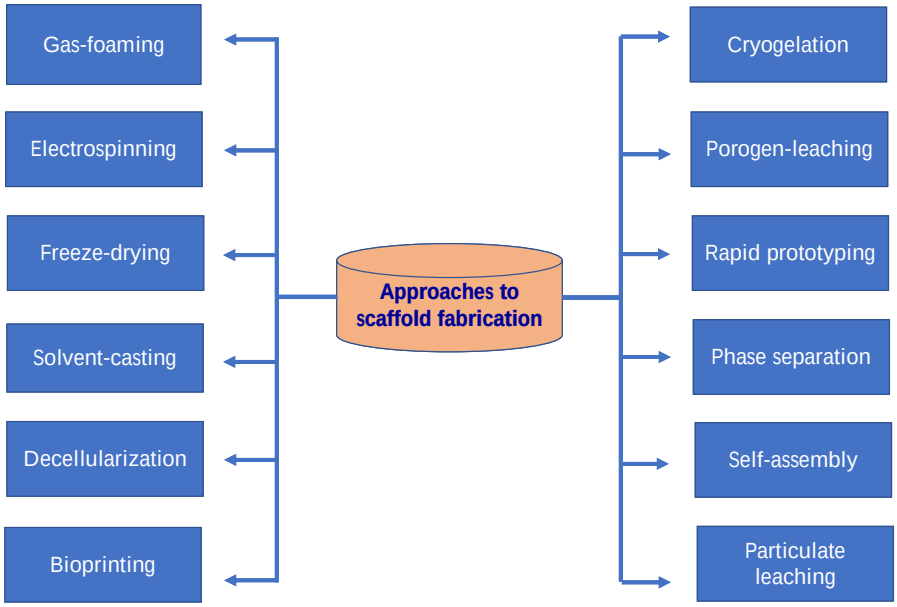

Figure 3. Fabrication techniques for scaffold preparation. Over the last two decades, various approaches have been implemented in the fabrication of scaffolds for tissue engineering, including electro-spinning, phase-separation, solvent casting, freeze-drying, and self-assembly. The selection of the appropriate scaffolding approach depends on the scaffold requirements and tissue-specific considerations.

Major research efforts have focused on an *in situ* tissue engineering approach, with the aim of leveraging the innate regenerative potential of the human body to enable tissue regeneration at the site of injury using bioactive molecule-based cues [15]. The tissue engineering triad comprises a combination of cells, scaffolds, and biologically active molecules or growth factors [16]. Scaffolds are three-dimensional constructs with the prime function of being able to mimic the physico-chemical properties of the natural extracellular matrix (ECM) [16]. For successful application in the field of tissue engineering, scaffolds need to be able to provide structural and mechanical support to the cells and to promote regeneration by effectual delivery of therapeutic molecules [17].

Polymeric scaffolds have been used widely for tissue engineering applications. Biodegradable polymers provide the advantages of enhanced inflammatory tolerance, high biocompatibility, and nontoxic enzymatic degradation *in vivo* [16]. Numerous approaches have been implemented for the fabrication of these biomimetic scaffolds, including electro-spinning, phase-separation, solvent casting, freeze-drying, and self-assembly (**Figure 3**) [18]. Electro-spun nanofibers from biomaterials form structures analogous to the fibrous native ECM, and possess beneficial mechanical properties and enhanced cellular infiltration [19, 20]. Pre-vascularized tissue constructs possessing enhanced cell proliferation have been developed by pre-seeding endothelial cells and fibroblasts on hydrogels [13].

An alternative is provided by scaffold-less tissue engineering. It has been developed by virtue of 3D bioprinting using self-assembling multicellular units as bioink particles, and has been used to realize self-organizing vascular constructs [21, 22]. The latest strategy of 4D bioprinting, which involves time as the additional dimension, has enabled the development of smart biomaterials which can evolve their shapes as a function of time in response to exogenous cues like pH and temperature [23]. Organ decellularization is another recent avenue where organs, decellularized by detergents, retain ECM and vascularization, and hence can be transplanted after *in vitro* recellularization [24]. Hydrogels made from natural and synthetic polymers have been used for encapsulation and to protect the transplanted cells from the host immune system. The permeability of the encapsulating matrix is fine-tuned to block the passage of antibodies and T cells, and at the same

Table 1. Advantages and disadvantages of natural polymers for tissue engineering applications

Polymer	Advantages	Disadvantage
Collagen	Biocompatible and biodegradable [37]	Low mechanical strength [43]
Gelatin	Biocompatible [45] Biodegradable [46] Non-antigenic and non-immunogenic [47-48]	Low mechanical strength [49]
Fibrin	Self-assembly [53] Soft elasticity [53] Low toxicity to cells [54] Good attachment, proliferation and migration properties [54, 55]	Immunogenicity [53]
Agarose	Biodegradability [61] Soft tissue-like mechanical properties [60] Rapid gelling capacity [60]	Low cell attachment and proliferation [14]
Alginate	Non-toxic approach to encapsulate cells [70] Excellent gelling properties [70]	Limited cell adhesion [70]
Silk	Excellent mechanical strength [83] Good biocompatibility [83] Water-based processing [83-84] Simplicity of chemical modification [83] Biodegradability [83]	Stimulates host immune response [92]

time, to allow the inflow and outflow of bioactive signaling molecules, thus aiming to avoid the usage and eventual side-effects of immunosuppressive agents [25, 26].

3. Pancreatic tissue engineering

A surgical cure for diabetes has been proposed by pancreatic transplantation, which is accompanied by long-term immunosuppressive therapy [27]. To reduce the extent of surgical intervention and the risk involved in pancreatic transplantation, new strategies have been developed for islet transplantation [28]. It has been found that immunoisolation of islets, using tissue engineering techniques like encapsulation and coating with semi-permeable and biocompatible biomaterial membranes, minimizes the need for long-term immunosuppression [29].

Cell-based (HEK293) microencapsulation of islets has also been tested which showed sustained release of insulin [30]. Although this technique requires further improvement, islet surface modifications with growth factors such as vascular endothelial growth factor (VEGF) and peptides such as arginylglycylaspartic acid (RGD), isoleucine-lysine-valine-alanine-valine (IKVAV), and tyrosine-isoleucine-glycine-serine-arginine (YIGSR) have already been shown to enhance islet engraftment and reduce immunogenicity in pancreatic islet transplantation [11].

To increase donor tissue sources, transplantation of β-cells derived from stem cells differentiated *in vitro* has become a new focus in diabetes research [31]. Hydrogels and microspheres made of polymers like alginate, polyethylene glycol (PEG), agarose, and chitosan-gelatin among others have been used for β-cell encapsulation. These encapsulated β-cells have been shown to have enhanced viability, cell survival, and insulin-secretory potential. Major efforts have been directed to mimic the islet niche and native interactions in the capsules to improve the efficacy of islet transplantation [11]. Natural and synthetic polymers have been widely vetted as a means of transplantation to enhance the efficacy of islet survival [32].

4. Polymers

Polymers can be categorized as natural or synthetic polymers depending on their origin. Naturally occurring polymers like polysaccharides (chitosan, alginate, hyaluronic acid), inorganic polymers (hydroxyapatite), and natural proteins (collagen, fibrin, silk) exhibit several benefits such as low toxicity, biocompatibility, and enzymatic degradation [16, 33]. Natural polymers also contain bioactive motifs, which help to establish cell-scaffold interactions, thus enhancing tissue functionality [34]. The downsides associated with natural polymers include temperature sensitivity, immunogenicity, and source-dependent heterogeneity (**Table 1**) [17].

The second family of polymers, synthetic polymers, includes alpha-hydroxy acids such as polylactic acid (PLA), polyglycolic acid (PGA), polylac-

Table 2. Advantages and disadvantages of synthetic polymers for tissue engineering applications

Polymer	Advantages	Disadvantages
PGA	Biocompatible [94] Tunable degradation rate [33] Stable three-dimensional structures [33]	Increased release of acidic degradation products [33] Inflammatory response [33] Rapid *in vivo* absorption [33]
PLA	Biocompatible and biodegradable [101] Long half-life [101] Tailorable physico-chemical properties [103]	Hydrophobic nature with low biomimetic and cell adhesion properties [102]
PLGA	High biocompatibility [108] Non-toxic biodegradation [108] Tunable mechanical strength [109] Biodegradation rate [109]	Poor protein absorbance, cell affinity, and surface characters like hydrophilicity [110]
PCL	Biodegradable [16] Low melting point [118] Remarkable blend compatibility, versatile mechanical properties, and viscoelastic properties [118]	Hydrophobic nature, limited bio-regulatory activity, and susceptible to bacteria-mediated degradation [33]
PDMS	High biocompatibility [125] Excellent oxygen solubility [125] Ideal for slow release of compounds [126]	Hydrophobic surface, low cell adhesion [126]
PEG	Low immunogenicity, tissue-like elasticity, well-defined chemistry [130]	Biologically inert, does not support cell growth [129]

Legend: PCL - polycaprolactone, PDMS - polydimethylsiloxane, PEG - polyethylene glycol, PGA - polyglycolic acid, PLA - polylactic acid, PLGA - polylactic-co-glycolic acid.

tic-co-glycolic acid (PLGA) copolymers, and polycaprolactone (PCL) [33, 35]. Synthetic polymers are widely applied in the field of tissue engineering because of their tunable physico-chemical properties. The polyester family of synthetic polymers provides controllable and reproducible material properties, including elasticity and degradability, which are very useful in tailoring matrices with desired functions (**Table 2**) [34]. The lower possibility of infections and risk of immunogenicity give synthetic polymers an edge over natural ones [36].

Using the advantages of both classes of materials, recent work has focused on synthesizing hybrid scaffolds with both natural and synthetic components. Although there have been several prior studies focusing on the selection of the ideal polymer for encapsulation of islets, there has been no review to date which deals comprehensively with the optimal choice of scaffold materials for islets or pancreatic tissue engineering. The present review offers such a comprehensive insight into the application of natural and synthetic polymer-based scaffolds. This review also analyzes critically the problems associated with the construction of the bioartificial pancreas and discusses different design strategies.

5. Natural polymers

5.1 Collagen

Collagen is a structural basement membrane protein and a widely used biomaterial for cell adhesion and proliferation [37]. Being a part of the extracellular matrix, collagen has found wide application in tissue engineering. It has been used for engineering heart valves [38], lung [39], bone [40], and other organs. Several reports have indicated the use of collagen for pancreatic tissue engineering. Jalili *et al*. incorporated fibroblast in type-1 collagen gels. Before solidification, islets were also embedded in the collagen gel. Collagen provides the ECM for islet growth, and fibroblasts maintain matrix integrity. This scaffold showed improved cell survival and insulin secretion. Importantly, incorporation of fibroblasts reduced the number of islets required to reverse diabetes through transplantation [41]. In another study, basement membrane proteins (laminin and heparin sulfate proteoglycan) were combined along with collagen to form gels in which islets were embedded. These cells showed better proliferation, attributed to the reduced caspase-3 expression, and improved cell survival [42].

To carry neonatal porcine islets Ellise *et al*. used scaffolds containing the following constituents:

- Rat tail collagen cross linked with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) N-hydroxysuccinimide (NHS)
- A combination of chondroitin-6-sulfate, chitosan, and mouse laminin

The islets survived up to 28 days indicated by positive insulin and glucagon staining. Also, the matrix did not show any signs of inflammation, and the scaffold could maintain its shape and size for over 28 days [43]. Another study illustrated early restoration of euglycemia post-transplantation (from 17 to 3 days) relative to controls using PLG scaffolds coated with collagen-IV, laminin, and fibronectin [44]. The collagen IV-modified scaffolds showed improved islet survival, enhanced islet metabolism, and better glucose-induced insulin secretion.

Collagen alone does not provide the mechanical strength required for pancreatic tissue architecture. Hence, a combination of other polymers such as chitosan, chondroitin-6-sulfate, and laminin or crosslinking has been used to improve the scaffold [43]. Almost all the studies mentioned above showed that incorporating collagen with other basement membrane proteins tended to improve islet survival and function.

5.2 Gelatin

Gelatin, a natural product generated from hydrolysis of collagen, has offered great potential as scaffolding material [45]. Being a natural polymer and having the beneficial properties of biocompatibility, biodegradability, and lack of antigenicity and immunogenicity, gelatin-based scaffolds have shown promising results for tissue engineering of cartilage [46], bone [47], skin [48], and other tissues. Various research groups have shown effective application of gelatin and its blends for engineering islets. Collagen is a component of the basement membrane of ECM in the adult human pancreas, thereby providing gelatin with an advantage over other polymers. One of the major properties required for pancreatic tissue engineering is good mechanical strength. Gelatin alone does not fulfill this criterion. Therefore, various blends of gelatin with other polymers have been used.

Gelatin has been used for encapsulating rat pancreatic islets grown on polyglycolic acid scaffolds. These engineered islets were transplanted into streptozotocin-induced (STZ-induced) diabetic nude mice. The diabetic mice maintained normal glycemia until 120 days of transplantation, with the islets showing potential to secrete exogenous insulin [49]. Muthyala *et al*. used gelatin to synthesize 3D porous interpenetrating polymer network (IPN) scaffolds along with polyvinylpyrrolidone (PVP) using the cross-linkers glutaraldehyde and EDC hydrochloride by freeze-drying [50]. IPN scaffolds displayed ideal properties for tissue engineering with good mechanical strength. Out of the many scaffolds synthesized, one of them (gelatin-PVP-semi-IPN) showed good growth of viable β-cells even up to 30 days [50]. Moreover, the authors showed that a combination approach, consisting of mouse islets grown on the gelatin-PVP semi-IPN scaffold encapsulated in a PU-PVP semi-IPN microcapsule, (a capsule made up of polyurethane extrusion grade Tecoflex 60D (TFPU) and PVP coated with semi-IPN solution), reversed diabetes in rat models for up to 90 days [51].

Previous studies have used gelatin in combination with dextran to produce three scaffolds (DEXGEL). Sodium meta-periodate was used to incorporate the aldehyde group in dextran which could link with the amine group of gelatin, thereby negating the use of additional cross-linkers. DEXGEL served as a platform for differentiation of adipose stem cells into islet-like clusters. These islets provided higher levels of insulin secretion than 2D culture systems [52].

5.3 Fibrin

Fibrin is a protein involved in blood clotting. It has been used widely for tissue engineering applications because of properties such as self-assembly and soft elasticity [53]. Fibrin hydrogel has shown various impressive properties such as low toxicity to cells, good cell anchorage, proliferation, and migration [54, 55]. Fibrin gels have been used to differentiate chemically human endometrial stem cells into pancreatic β-cells using activin A, nicotinamide, fibroblast growth factor (FGF) and epidermal growth factor (EGF) [56]. Insulin secretion was found to be higher in 3D fibrin gels enclosed with differentiated cells than in their 2D counterparts. Khorsandi *et al*. have shown differentiation of bone marrow-derived mesenchymal stem cells (BM-MSCs) into insulin-producing cells using 3D culture and fibrin glue [57]. Previously, long-term proliferation of a rat insulinoma cell line (INS-1) on fibrin gel had shown increased insulin secretion in response to glucose stimulation [58].

Fibrin has also been shown to significantly improve insulin secretion in diabetic mice which were transplanted with fibrin-cultured islets. These mice had shown highly vascularized islets along with improved viability [59]. This shows the importance of fibrin in maintaining islet cell viability and angiogenesis. Although the use of fibrin for islet proliferation has brought about much improvement, one of the major drawbacks associated with it is the risk of immune response *in vivo* [53]. Furthermore, the potential application and risks associated with fibrin for islets proliferation and transplantation are not yet fully elucidated.

5.4 Agarose

Agarose, a naturally occurring polysaccharide, is one of the most widely used polymers in the field of tissue engineering. Its favorable properties include biodegradability, soft tissue-like mechanical abilities, and strong and rapid gelling capacity, and make it an ideal candidate for soft tissue engineering [60]. Agarose gel has been used as gene delivery vehicle [60], scaffold for implantation surgery [61], cartilage tissue engineering [62], liver tissue engineering [63], and other applications.

Recently, our group formulated an agarose chitosan-based nanocomposite (AG-CHNP) using a freeze-drying technique. Our scaffold showed good biocompatibility with various cell lines, including HEK, Mia PaCa-2, and HeLa, hemocompatibility, and antibacterial activity. These scaffolds showed continuous increased growth of HeLa for a period of 16 days [14]. We further used AG-CHNP scaffolds for chemical differentiation of BM-MSCs into insulin-producing cells. The differentiated cells showed positive results for the pancreatic markers PDX1 and NKX2.2. The differentiated cells secreted insulin confirmed by western blot (unpublished data). These preliminary results suggest that such agarose-based scaffolds are suitable for pancreatic tissue engineering.

For islet engineering, agarose-agarose islet macrobeads were used to encapsulate porcine islets. These macrobeads were xenotransplanted into pancreatectomized dogs. This, along with anti-inflammatory pravastatin therapy, showed prolonged functionality and biocompatibility of the islets [64]. Luan *et al*. transplanted islets into a prevascularized subcutaneous space. Induction of blood vessels was performed using freeze-dried agarose rods comprising basic FGF (bFGF) and heparin. 1500 islets were transplanted into the prevascularized subcutaneous tissue without using any immunosuppressive regimen. This therapy reverted hyperglycemia, and showed long-term allogeneic islet graft survival and function [65].

One report used agarose microwells made up of polydimethylsiloxane (PDMS) molds for the formation of primary islet aggregates, which are pseudoislets with pre-defined proportions [66]. Dissociated islets, when aggregated in a controlled environment, led to a change in the core mantle arrangement of α- and β-cells, which underwent modification after implantation under the kidney capsule. After transplantation, these islets behaved almost like native islets. This observation demonstrated the importance of cell-to-matrix interaction, and the necessity for the islets to have the appropriate size and shape for the maintenance of their structure and function *in vivo* [66].

Apart from this finding, Ichihara *et al*. used size-controlled pseudoislets from rat pancreas on agarose gel-based microwells. The micromolds were synthesized using soft lithography of different diameters (100, 300, 500 μm). These small islet aggregates showed better insulin secretion and cell survival than medium-sized and large aggregates. Also, native tissue-like cell organization was observed in both small- and medium-sized islet aggregates [67]. This study highlighted the role of size in islet transplantation. Recently, a novel approach of combining agarose gel scaffolding with BM-MSCs showed improved insulin secretion compared to controls where islets were grown on agarose gel only. This study highlighted the role of BM-MSCs and agarose gel in improving the overall functionality of islets. It is suggested that BM-MSCs provide growth factors and paracrine signaling, and agarose gel allows cells to absorb nutrients in an unlimited manner which improves islet function [68].

5.5 Alginate

As mentioned above, microencapsulation is a process of entrapping cells or tissues within a polymeric membrane that acts as immunosuppressive barrier [69]. Currently, much research has been done in microencapsulating β-cell grafts to allow easy transplantation, immunoprotection, and the use of non-human islets [70]. A commonly used polymer for microencapsulation is alginate, a polysaccharide isolated from brown sea weed [71]. It has gained tremendous popularity after Lim *et al*. used islet-encapsulated alginate beads as artificial pancreas [72]. From then on, alginate has been widely used.

Alginate hydrogels have found application as beads, delayed gelation systems, macroporous scaf-

folds, 3D printed scaffolds, etc. [73]. Although alginate beads enable a rapid and non-toxic encapsulation of cells, their property of limited cell adhesion is a huge disadvantage for their wide application in tissue engineering. Therefore, in the field of islet tissue engineering, alginate has been mainly used for microencapsulation only. A breakthrough in alginate-based encapsulation techniques was made in 2010 when Opara *et al*. suggested a multi-layer model of bioartificial pancreas containing two alginate layers separated by a semi-permeable membrane made up of poly-l ornithine. The inner layer was used to encapsulate the islets, and the outer layer for the adjunct of angiogenic proteins. These microcapsules were implanted into the omental pouch of rats. The authors reported that the use of such alginate beads enabled controlled delivery of growth factors and initiation of blood vessel formation, thereby improving graft viability and function [74].

Gelation of alginate takes place in the presence of ions (Ca^{2+} or Ba^{2+}). But an ionically bound alginate hydrogel of this kind may not be able to withstand the mechanical stress associated with implantation. Therefore, alginate was modified by incorporating a carboxylic group into alginate backbone, and covalent linking to modified PEG (phosphine group at the end) using Staudinger ligation. This hydrogel had better stability and cell attachment than the alginate controls [75]. The microencapsulation system proposed by Opara *et al*. was improved with a thick and cross-linked outer alginate layer. This procedure helped to maintain the stability of the system for a longer period; the microcapsule remained intact even after 90 days of transplantation. This work also suggested the omental pouch as a potential implantation site for islet transplantation [76].

Richardson *et al*. demonstrated a stage-wise directed differentiation of alginate-encapsulated human embryonic stem cells into islet-like cells. Clear viable colonies were evident after differentiation and maturation. Encapsulated cell differentiation resulted in strong maturation marker expression and improved hormone secretion as compared to their 2D counterparts [77]. Additionally, 3D bioplotting has been used to formulate alginate-gelatin porous scaffolds which can be used as extrahepatic islet-delivery systems (bioplotting is a technique that causes extrusion polymers to create custom-engineered scaffolds). When islets were removed from the hydrogel, they showed full functionality [78]. This study is one of the most recent reports on the use of 3D bioplotting for islet engineering.

Another recent study proved the benefits of a modified form of alginate, triazole-thiomorpholine dioxide (TMTD) alginate, for islet implantation [79]. Emphasizing that the size of the microspheres affects the immunological response to the implants, human embryonic stem cell-derived β-cells (SC β-cells) were encapsulated in 1.5 mm TMTD alginate spheres, which showed better glycemic control than the conventionally used 500 µm alginate spheres. This was the first study to report long-term glycemic control in immune-competent mice containing SC β-cells [79]. This report highlights the role of alginate and its derivatives as an immuno-isolatory device in a xenotransplantation setting.

Alginate-encapsulated islets have also been used for clinical applications in patients with type 1 diabetes by various groups. They have shown long-term stability of the capsule *in vivo* with continuous reduction of exogenous insulin [70]. However, a perfect site of implantation, which overcomes all the disadvantages, is yet to be found [69].

5.6 Silk

Silk protein is commonly used in the textile industry; it is produced by silk worms and spiders. The fibrous protein in it native form consists of a component (sericin) which can elicit an inflammatory response [80]. However, this component can be removed by the process of alkali- or enzyme-based “degumming”.

Apart from textiles, silk is also applied in tissue engineering and drug delivery. It offers various outstanding properties which amplify its role as a biomaterial. One of these beneficial properties for tissue engineering is its excellent mechanical strength, which is higher than that of Kevlar, a synthetic fiber used as a reference point in fiber technology [81]. Apart from this advantage, silk has further beneficial properties that simplify handling, including good biocompatibility, water-based processing, chemical modifiability, and biodegradability [81, 82]. Silk can be molded into any form such as films, electro-spun fibers, hydrogels, scaffolds, and particles.

In the field of tissue engineering, silk (alone and in combination with other polymers and nanostructured fibers) has been used for wound healing [83] as well as the regeneration and reconstruction of bones [84], tendons and ligaments [85], urethra [86], cartilage [81, 87], and other tissues. Silk has also been widely used for pancreatic tissue engineering.

Silk hydrogels have been used to encapsulate mice islets. These hydrogels provided a 3D environment in which the islets could maintain their viability and functionality. In the normal pancreas, islets are surrounded by ECM-containing collagen, laminin, and fibronectin which help in cell adhesion and proliferation. To mimic a similar environment, extracellular proteins and secondary stromal cells were incorporated in silk hydrogel which showed enhanced islet function [88]. Do *et al*. showed that oral ingestion of silk fibroin hydrolysates helps in maintaining pancreatic β-cell integrity, and improves insulin secretion by increasing β-cell mass in hyperglycemic mice [89].

Co-encapsulation of β-cells and mesenchymal stem cells (MSCs) using silk hydrogels has also been explored. Though silk is a magnificent biomaterial, it may still stimulate host inflammatory responses which harm islet growth. However, the presence of MSCs reduces this effect because of their immunomodulatory and angiogenic properties. This multi-dimensional approach has proved successful in terms of graft functionality and revascularization, with an undesirable drawback of bone differentiation [90]. Recently, Kumar *et al*. microencapsulated silk scaffold with alginate and agarose. This scaffold showed sustained growth for rat insulinoma cells (RIN-5). Rat β-cells also showed better growth on the 3D scaffold as compared to its 2D counterpart which was confirmed by expression of primary pancreatic genes [91].

6. Synthetic polymers

6.1 Polyglycolic acid

Polyglycolic acid (PGA) is a biocompatible polymer approved by the US Food and Drug Administration (FDA). It is obtained by ring cleavage polymerization of glycolide. PGA hydrolyses *in vivo* to give glycolic acid, which is a metabolite in the citric acid cycle, thus resulting in low toxicity [92, 93]. PGA has a wide range of applications in the field of tissue engineering due to its tunable degradation rate and intrinsic tendency to form stable 3D structures [33]. However, PGA undergoes rapid absorption *in vivo*, causing failure of the scaffold. Also, inflammatory responses are provoked because of increased release of acidic degradation products. Combination of PGA with several copolymers such as PLGA or PEG has been shown to enhance its beneficial physical and mechanical properties [33]. PGA has been widely used to make bioresorbable sutures and cartilage regeneration [16].

A hybrid scaffold of collagen and PGA with basic fibroblast growth factor has been developed to promote wound healing in type 2 diabetic mice. This hybrid matrix has enhanced compression strength, thus suppressing wound contraction, while also inducing angiogenesis and granulation tissue formation [94]. A study by Chun *et al*. showed that the islet cells grown on PGA scaffolds functionalized with a layer of poly-l-lysine enhanced the surface activity and adhesion capacity of PGA scaffold, and promoted cell proliferation. The PGA scaffold was also shown to provide superior nutrient absorption and metabolite excretion to the cultured islets, providing an appropriate microenvironment for their growth and survival. The cultured islets exhibited enhanced viability, improved morphology, and increased glucose-stimulated insulin secretion [93].

The viability of PGA islet grafts transplanted into the leg muscles of rats with STZ-induced diabetes has also been investigated. This scaffold provided a compatible 3D microenvironment with visible adhesive growth of islets on the scaffold and an adequate supply of blood and nutrients. The results showed increased insulin secretion and significantly decreased blood glucose concentration in rats transplanted with PGA islet grafts as compared to controls [95].

Recently Li *et al*. used PGA scaffolds for increasing the efficacy of islet coating by endothelial cells (ECs). Coating islets with ECs has been shown to improve revascularization and to reduce initial inflammatory response. Due to the presence of PGA scaffolds, enhanced coating efficiency of ECs on the islets was observed. Islet functionality was also improved with enhanced glucose-stimulated insulin release. The authors thus recommended the use of PGA scaffolds in pre-transplant culturing of islet cells and ECs [96].

6.2 Polylactic acid

Polylactic acid (PLA) is also approved by the FDA. It is an aliphatic polymer widely applied in the field of biomedical devices and tissue engineering [97]. PLA hydrolyses *in vivo* to release lactic acid, which becomes incorporated into the citric acid cycle and is naturally excreted, thus making PLA biocompatible and biodegradable in nature [98]. However, numerous surface treatments need to be implemented to hydrophobic PLA to impart enhanced biomimetic and cell adhesion properties [99]. PLA has tunable and versatile physical and chemical properties, and can be molded to take on a myriad of shapes, including microspheres, scaf-

folds, sutures, and nanoparticles [100]. Taking advantage of its long half-life, PLA has been extensively used in fabrication of long-term implantable devices for therapeutic applications [98]. PLA and its copolymers are extensively applied in tissue engineering, including skin grafting and the regeneration and reconstruction of bone, spinal cord and nerve tissue [100].

In the field of pancreatic tissue engineering, the potential therapeutic application of PLA microspheres has been studied in the treatment of diabetic periodontitis. The microspheres, loaded with 25-hydroxyvitamin D3, were shown to prevent inflammatory responses and bone loss in rats with diabetic periodontitis [101].

PLA-PEG-based nanoparticles have also been used as a means for subcutaneous delivery of insulin. Nanoparticles, loaded with 50 IU of insulin per kg, were shown to control blood glucose levels, thereby restoring normoglycemia in diabetic rats. These biodegradable nanoparticles proved to be non-toxic in nature; they are thus qualified as potential candidates for parenteral insulin therapy [102].

Kasujo *et al*. described the application of PLA-based porous capsules to obtain a vascularized microenvironment for extrahepatic islet transplantation. The bioartificial cavity showed numerous vessels and guided infiltration of the host's connective tissue cells and vascular endothelial cells with no significant infiltration by inflammatory cells, providing a favorable microenvironment for islet transplantation [103]. A 3D delivery system has been developed which can be used for encapsulation and implantation of pancreatic cells. The PLA-based nanogland provided support to islet-like aggregates derived from differentiation of human MSCs, enhancing their viability and maintaining their function *in vitro*. The nanogland provided steady secretion of insulin, demonstrating potential benefits for diabetic cell therapy [98]. Recently, a 3D printed encapsulation system has been formulated using polylactic acid for subcutaneous implantation of pancreatic islets. After surface treatment was employed to functionalize the system, it was implanted with VEGF-enriched platelet gel to enhance vascularization. This system enabled transcutaneous refillability and potential retrievability of the graft [99].

6.3 Polylactic-co-glycolic acid

Polylactic-co-glycolic acid (PLGA) is an FDA approved copolymer obtained by ring-opening copolymerization of lactide and glycolide [104]. PLGA has been widely used in varied forms such as films, porous scaffolds, hydrogels, and microspheres for biomedical tissue engineering and drug delivery purposes due to its high biocompatibility and non-toxic biodegradation [36]. An additional advantage of the physico-chemical properties of PLGA is the tunable mechanical strength and biodegradation rate achievable by altering the PLA:PGA ratio [35]. However, PLGA has adverse surface characters such as hydrophilicity, protein absorbance, and poor cell affinity [105]. Numerous surface modulation strategies like surface immobilization, physical adsorption of bioactive molecules, plasma treatment, and incorporation of other biocompatible materials into the PLGA matrix have been tested to make the interface between PLGA and its environment more biomimetic which improved cell affinity [36].

Recently, biocompatible PLGA scaffolds have been produced using 3D printing for use in tissue engineering [106]. Electrospun PLGA-based hybrid nano-fibrous membranes and scaffolds have been widely used for skin, bone, nerve, and soft tissue engineering applications [105].

In the field of pancreatic tissue engineering, micro-porous, biodegradable PLGA has been successfully utilized as a platform for islet transplantation in mouse models [107]. Salvay *et al*. explored the effects of PLGA scaffolds with adsorbed ECM components on the survival of transplanted islets. It appeared that adsorption of these proteins by the scaffold enhanced the efficacy of islet grafts and significantly decreased the time needed for the reversal of diabetes in mice [108]. The effects of integrated ECM components on long-term maintenance of human pancreatic islets cultured in a micro-fabricated PLGA scaffolds have also been investigated *in vitro*. The PLGA scaffold provided a viable niche, with the *in-vitro*-cultured islets displaying insulin release profiles characteristic of native islets [109].

Kheradamand *et al*. demonstrated the use of PLGA scaffolds as an extra-hepatic site for islet transplantation [110]. The addition of ethylcarbodiiminde-fixed (ECDI-fixed) donor splenocyte infusions to the PLGA scaffolds enhanced the efficacy of tolerance induction *in vivo*, and indefinite normoglycemia was maintained in diabetic mice models [110]. Bioresorbable PLGA microspheres have been designed for encapsulation and sustained administration of β-cell-proliferative compounds to intact mouse islets in culture [111]. The improved bioavailability of the mitogen to β-cells *in vivo* may lead to increased β-cell proliferation, and may thus be regarded as a therapeutic appli-

cation in the restoration of normoglycemia in diabetic patients [111].

Recently, Liu *et al*. investigated the fabrication of artificial islet tissues using a fibroblast-modified PLGA membrane for differentiating pancreatic stem cells into insulin-producing cells. This construct secreted insulin and was shown to reduce blood glucose levels in diabetic nude mice. The modified PLGA membrane showed higher compatibility, improved proliferation, and increased viability of pancreatic stem cells compared with the unmodified membrane. Also, it had an enhanced histocompatibility with nude mice [112].

6.4 Polycaprolactone

Polycaprolactone (PCL) is a hydrophobic, biodegradable FDA-approved polymer prepared by ring-opening polymerization of ε-caprolactone in the presence of SnO_2 and heat [16]. PCL has gained an edge in the field of biomedical research because of its low melting point, remarkable blend compatibility, and viscoelastic properties. PCL has been widely used in drug delivery systems as surgical sutures and scaffolding material for tissue engineering because of its tunable degradation rates and beneficial mechanical properties [113]. Drawbacks associated with PCL include hydrophobicity, limited bio-regulatory activity, and susceptibility to bacteria-mediated degradation [33]. To enhance favorable cellular responses, various functional groups have been incorporated into the polymer, making it more hydrophilic and biocompatible [113]. PCL and its copolymers such as PCL-PEG and PCL-PLA have various applications in cartilage, bone, and peripheral nerve regeneration [16].

Nano-fibrous PCL scaffolds have been used for differentiation of human induced pluripotent stem cells (iPSCs) into definitive endoderm cells using inducer for definitive endoderm 1 (IDE1). Electrospun PCL scaffolds exhibited more pores, decreased toxicity, and reduced thickness of the nanofibers, enabling more surface space for cellular proliferation and attachment [114]. A composite hydrogel made from polycaprolactone (PCL) and polyacrylic acid (PAA) is applied in oral delivery of the drug gliclazide, which is used in the treatment of type 2 diabetes. The balance of hydrophobic PCL with hydrophilic PAA provided the property of the hydrogel that controls swelling. The PCL/PAA hydrogel offered a controlled release of the drug and was shown to enhance its bioavailability, resulting in reduced glucose levels [115].

PCL is also applied in diabetic wound healing. Gholipour-Kanani *et al*. blended PCL with chitosan to avoid the use of chemical cross-linkers and achieve a nano-fibrous scaffold with sustainable integrity in aqueous media. This poly(caprolactone)-chitosan-poly(vinyl alcohol) (PCL:Cs:PVA) scaffold was found to promote diabetic wound healing because of its biocompatibility and structural similarity to native ECM [116]. Ranjbu-Mohammadi *et al*. showed the application of curcumin-loaded poly(ε-caprolactone) (PCL)/gum tragacanth (GT) (PCL/GT/Cur) nanofibers in the field of wound healing. The antibacterial nanofibrous membranes enhanced the healing process by simulation of native ECM, presence of curcumin and GT, and improved mechanical stability of the scaffolds because of the presence of PCL.

Tissue-engineered scaffolds were also shown to decrease blood glucose levels in rat models [117]. A current finding highlighted the use of heparanized ring-shaped PCL scaffolds functionalized with VEGF for carrying islets in an alginate core. Vascularization was successfully induced throughout the scaffold by the presence of immobilized VEGF. The embedded islets were shown to maintain their viability and functionality, responding normally to glucose stimulations, and at the same time, possessing obvious immune protection properties. The scaffold demonstrated improved revascularization; it may thus be used as potential vessel for subcutaneous islet transplantation [118].

Recently, Smink *et al*. demonstrated the use of a modified PCL, poly (D,L-lactide-co-ε-caprolactone) (PDLLCL) to create a scaffold which acted as an artificial and retrievable subcutaneous transplantation site for pancreatic islets. PDLLCL was shown to be compatible with islet viability and functionality. Also, islets cultured on PDLLCL exhibited comparatively more insulin granules and lower release of immune system-provoking double-stranded DNA, suggesting PDLLCL as a suitable scaffold with potential for application in the treatment of type 1 diabetes [119].

6.5 Polydimethylsiloxane

Polydimethylsiloxane (PDMS), a silicon based organic polymeric compound, has been commonly used as surfactant, stamp resin for soft photolithography, and other applications. Its superior properties make it a better choice for tissue engineering than other synthetic equivalents. PDMS has high biocompatibility, biostability, and oxygen solubility, which makes it a perfect candidate for implantation [120]. PDMS was used to construct a macroporous scaffold via solvent casting and a particulate leaching method. PDMS has a hydro-

phobic surface which is ideal for slow release of compound, but does not support cell adhesion. Therefore, fibronectin was added to the surface of PDMS scaffolds to make it hydrophilic.

Islets loaded onto PDMS scaffolds and implanted into the omental pouch showed good islet retention and long-term normoglycemia. Interestingly, islets on the scaffold showed enhanced viability and function under low oxygen tension compared to 2D controls [121]. Another study group seeded a fibrin platelet-derived growth factor hydrogel loaded with islets onto the PDMS scaffold and transplanted it into mice. This system helped to reduce the time required for attaining normoglycemia, and enabled increased vessel branching [122].

Recently, PDMS scaffolds have been used for delivery of anti-inflammatory agents such as dexamethasone and fingolimod [123, 124]. Dexamethasone was added to PDMS scaffolds in various quantities. Low concentration of dexamethasone showed improved islets engraftment, but higher concentrations were found to be detrimental as they alter glucose-induced insulin secretion by suppressed activation of the PLC/protein kinase C signaling system [123, 124]. Fingolimod exhibited persistent release, but at a very low concentration (0.1% w/w), which does not have any significant effect on the islets [125].

6.6 Polyethylene glycol

Polyethylene glycol (PEG) is one of the most popular synthetic polymers used for tissue engineering applications. PEG is a low-immunogenic material, has tissue-like elasticity, and its chemistry is well-defined, which provides an advantage over other polymers for islets engineering [126]. PEG has been used in the form of scaffolds and encapsulating agents for islet transplantation. As PEG is biologically inert, it does not support any form of cell growth. Therefore, for use as a scaffold, it must be augmented with another co-polymer.

Mason *et al*. used collagen fibrils in PEG hydrogels, and studied their effect on encapsulated embryonic pancreatic precursor cells. The cells on these scaffolds showed high glucose responsiveness, and had an improved level of insulin gene expression [127]. PEG scaffolds have also been supplemented with fibrin ribbons, which were used for co-culturing endothelial cells and islets [128]. Endothelial cells were encapsulated within the fibrin ribbons, and islets were implemented in the PEG hydrogel. The results suggested an optimum growth for both cell types, penetration of endothelial cells into the hydrogel, and improved vascularization.

A major problem associated with islet transplantation is the requirement for large numbers of islets. To overcome this problem, surface modifications of islets are currently being tested. The main objective of this technique is to reduce the number of islets required for transplantation [126]. Glucagon-like peptide 1 (GLP-1) is produced by the L-cells of the distal ileum, and is an insulinotropic ligand. Kizilel *et al*. directly immobilized GLP-1 on the surface of islets by layer-by-layer assembly of biotin-PEG-NHS, streptavidin, and biotin-PEG-GLP-1. Coated islets showed better insulin secretion in response to high glucose than control islets, which proved the efficiency of this technique. This study also addressed the issue of donor shortage as it required lower numbers of transplanted islets to achieve normoglycemia [129]. Another study using PEG as an encapsulating agent developed a device which had rat islets growing on an acellular scaffold and encapsulated in a PEG/VA semi-permeable membrane. This device was implanted in diabetic rats, and showed a reduction in insulin requirement for at least 2 weeks, restoring partial insulin secretion. To achieve complete euglycemia, the question of the optimal islet number to be transplanted needs further investigation [130]. PEG-based hydrogel microwells have also been developed using photolithography. MIN6 β-cells were seeded on the microwells, and maintained for 5 days preceding retrieval and encapsulation. This PEG-based microwell consistently demonstrated successful formation of MIN6 aggregates. Also, the encapsulated MIN6 aggregates showed better insulin secretion and positive expression of the intracellular binding protein E-cadherin as compared to single cell encapsulations [131].

One of the major causes of islet loss after transplantation is hypoxia which affects the longevity of the implant. Therefore, to facilitate short oxygen supply to the islets, PEG-stabilized hemoglobin had been used as an artificial oxygen carrier [132]. But this system does not support long-term use because of continuous conversion of hemoglobin to methemoglobin by autoxidation and free radical damage, which is deleterious to the cells. Therefore, PEG-based hemoglobin conjugates cross-linked with antioxidant enzymes (superoxide dismutase and catalase) have been used [133], and demonstrated excellent protection against free radicals and oxygen-induced stress in RINm5F cell line. The viability of RINm5F cells was higher and the generation of reactive oxygen species (ROS) was reduced for cells treated with the conjugates.

The results also showed sustained or increased insulin release from the treated islets under partial oxygen pressure situations. This study provided insight into the value of PEG-based conjugates for preventing hypoxia-induced graft failure. Another attempt to prevent post-transplantation islet loss was made by Golab *et al*., where islets were immunoprotected by coating them with Treg cells conjugated with biotin-PEG-SVA (succinimidyl valeric acid ester). This approach was found to be favorable compared to that using biotin-PEG-NHS for coating pancreatic cells with Treg cells, and showed slightly improved insulin secretion [134].

PEG hydrogels have also been used for encapsulation of islets in combination with BM-MSCs, GLP-1, and ECM-based cell adhesion ligands [135]. Insulin secretion could be increased 7-fold by the synergistic effects compared with islets alone, islet functionality and viability could be improved, and MSCs have shown immunomodulatory effects. As mentioned previously, angiogenesis plays a major part in maintaining islets functionality. Pancreatic islets comprise only 1-2% of the total pancreatic cell population, but require as much as 15-20% of the total pancreatic blood supply [136]. Therefore, maintaining similar angiogenic effects post-transplantation is absolutely required. Phelps *et al*. created PEG hydrogels with mild maleimide-thiol cross-linking. These scaffolds were further modified by the addition of RGD motif for cell adhesion and VEGF for vascularization [137]. This study highlighted the use of mesentery as a transplantation site which is far less invasive than hepatic portal transplantation. This scaffold and the delivery strategy showed beneficial outcomes in terms of vascular invasion and insulin secretion. This research also negated injection of islets into the blood stream which can cause immediate inflammatory reaction. Recently, a blend of synthetic PDMS and natural PEG polymer was used for transplantation of islets into epididymal fat pad [138]. Islets were mixed with PEG and then included in photo-linked PDMS molds. As the islets were encapsulated in PEG and PDMS, they were protected from immediate inflammatory attack by the immune system. An interesting finding of this study was that glucose tolerance test revealed normoglycemia within 90 minutes of transplantation.

7. Generating a functional pancreatic construct

Diabetes is one of the leading causes of death in the world with an increasing global prevalence. The available treatment options have their own set of serious implications. Therefore, the focus of current research has shifted to the search for new and safe options which include the bioartificial pancreas. Research associated with the development of the artificial pancreas has seen numerous changes over the years. Various approaches include the use of polymer-based scaffolds, organ decellularization, scaffoldless tissue engineering, bioprinting, and encapsulation.

Polymeric tissue engineering involves the synthesis of an artificial scaffold using natural and/or synthetic polymers and a cross-linker. Various scaffolding approaches are available, including freeze drying, electrospinning, solvent casting, and others [18]. Both natural and synthetic polymers have their own set of advantages and disadvantages.

One of the major problems associated with pancreatic tissue engineering is the complexity of the organ itself. The pancreas contains both an exocrine section (including ductal and acinar cells) and an endocrine section (including the islets of Langerhans) [139]. Though β-cells are the major players in glucose metabolism, other small members of the islet cell family also play a critical part in the overall islet functioning [140]. Therefore, multiple cells types need to be considered in the process of islet graft engineering, which further complicates the work.

Another important factor is angiogenesis. Since islets require a much greater blood supply than any other pancreatic cell compartment [137], the choice of transplantation site becomes important to allow the transplanted construct receiving sufficient blood supply for survival. Various sites for transplantation have been examined, including the peritoneal cavity [122], hepatic portal vein [118], subcutaneous space [65], and subcapsular space of the kidney [41, 49]. Some sites such as omental pouch [76] and mesentery [137] have shown promising results, but the problem of hypoxia and shortage of blood supply is yet to be resolved.

Finally, the islets themselves are a cause of concern for pancreatic engineering since they have poor viability and stability *in vitro* [141]. The limited supply of islets intensifies this problem.

7. Conclusions

The construction of a bio-artificial pancreas is subject to a number of difficulties that need to be overcome. These difficulties include:

- Choice of cell type

- Culture environment
- Site of implantation
- Scaffolding approach
- Requirement of encapsulation

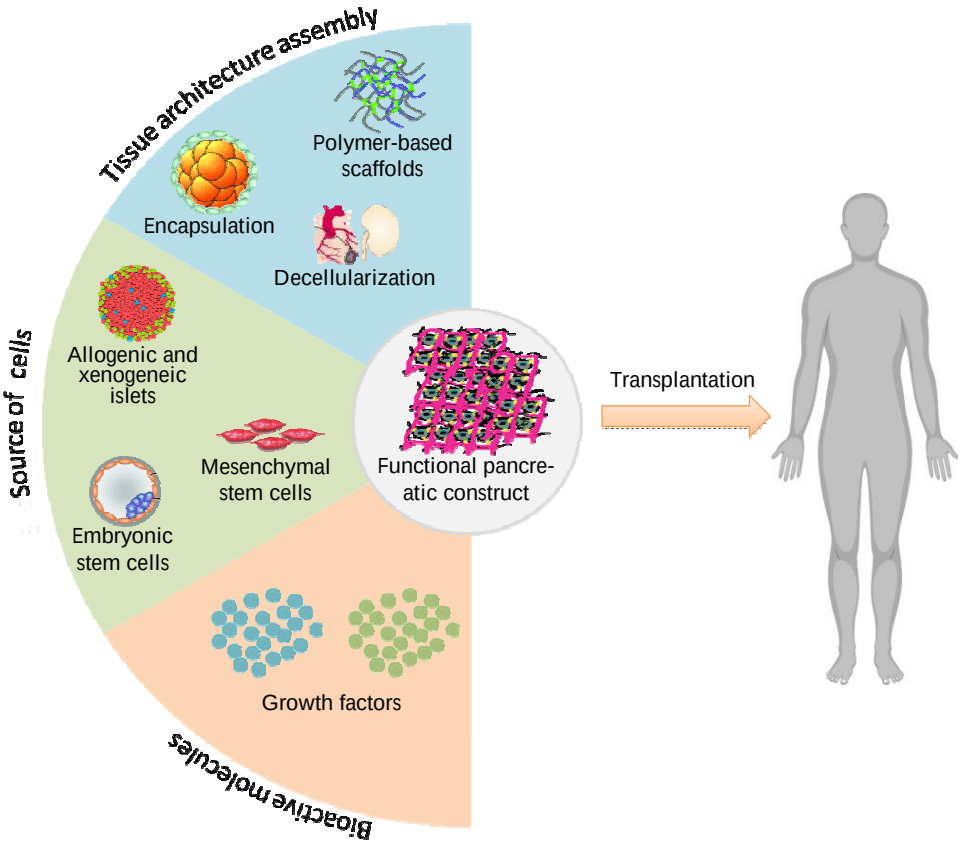

Figure 4. Advances in pancreatic tissue engineering. Various cell sources have been used for pancreatic tissue engineering, including allogeneic and xenogeneic islets (porcine and murine), mesenchymal and embryonic stem cells. Different growth factors have contributed to enhance the stability and proliferation of islet transplantation. Several scaffolding approaches have been employed to mimic the native microenvironment. Micro- and macro-encapsulation of transplanted islets has improved their overall viability and functionality.

Various categories of cell type have been tested for pancreatic tissue engineering and implantation, including allogeneic, xenogeneic, and alternative sources (embryonic stem cells, MSCs) [57, 64, 65, 79]. Although allogeneic and xenogeneic islets have shown promising results, limited availability and poor stability post-isolation have restricted their usability. Therefore, stem cells have received much attention in current research as they have shown promising differentiation potential [31]. While embryonic stem cells are banned in various countries because of the ethical issues, MSCs isolated from different sources have been widely studied [52, 57].

After selection of the cell type, it is important to choose the appropriate culture environment, i.e. whether to culture pancreatic cells individually or co-culture them with other cell types. Transplantation of β-cells alone has shown limited success. Co-culture with other cell types such as fibroblasts or MSCs have shown improved viability, functionality, and insulin secretion [41, 68, 90]. However, the major problem associated with islet transplantation is still substantial cell loss post-isolation and again post-transplantation due to hypoxia-induced apoptosis, loss of suitable microenvironment, and immune response [142-144]. Therefore, current research has been actively focusing on the use of encapsulation that is able to prevent the transplanted cells from direct crossfire from the host's immune system. Moreover, growth factors and angiogenic factors encapsulated within the construct may help to maintain the viability and functionality of the islets [74].

There are many approaches that are under consideration for assembly of a functional bio-artificial pancreas **(Figure 4)**. Considering the organ complexity and factor-dependent stability of the construct, research in the field of pancreatic tissue engineering has come a long way. Advances in tissue engineering and nanotechnology have provided tremendous insight into how we can improve the current approaches for creating a functional bio-artificial pancreas.

We are still awaiting the creation of an appropriate scaffold that can act as a perfect environment for the growth of cells. Various polymers

have been tried and tested for their application in scaffold designs. However, both natural and synthetic polymers fail to address all major requirements of an optimal scaffold for pancreatic tissue engineering. As the scaffold acts as a natural environment for the growth of cells, characteristics such as biocompatibility, biodegradability, vascularization, toxicity, and immunogenicity are critical [16]. One obstacle to finding the perfect polymer for pancreatic tissue engineering is that no single polymer has been studied intensively enough to learn whether it meets all the above-mentioned requirements.

As a result of our review, we found silk to be one of the most appropriate polymers for scaffold synthesis. Silk is well studied in the context of pancreatic tissue engineering, and found to be biocompatible and biodegradable [81, 82]. Apart from these advantages, the simplicity of chemical modification and its superior mechanical strength give it a small advantage over other natural polymers [81]. Silk hydrogels have been shown to provide a suitable environment for islets, and enable good islet viability and functionality [88]. Despite these favorable properties, its major drawbacks are its immunogenicity [90] and lack of evidence that silk scaffolds alone can induce angiogenesis. Several attempts have been made to overcome these shortcomings. While reduced immunogenicity and preliminary angiogenesis were observed when MSCs were co-cultured with β-cells, this approach led to unfavorable osteogenesis and chondrogenesis [90]. Additional work may focus on avoiding this colateral differentiation. A recent attempt to suppress immunogenicity was the macroencapsulation of silk scaffolds using alginate and agarose. This study showed positive results with respect to reduced immunogenicity [91]. Future research is necessary to improve the immunomodulatory effect of encapsulated silk scaffolds and to improve the overall viability and functionality of the transplanted islets.

As mentioned above, silk fulfills most of the criteria for application as a scaffold in engineering the bio-artificial pancreas. Since it is well studied and documented, silk may be a forerunner for scaffold design. However, future research on other polymers is also necessary since most have not been studied intensively and their complete capabilities have not been determined. It is thus impossible today to determine the optimal candidate. Future studies aimed at fully characterizing the available polymers and overcoming the existent limitations associated with scaffold fabrication may provide new avenues in the construction of the bio-artificial pancreas to prepare it for routine clinical application.

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