Sacrificial gelatin microspheres for tissue engineering applications

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**SACRIFICIAL GELATIN MICROSPHERES FOR TISSUE
ENGINEERING APPLICATIONS**

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SUMMARY

Tissue engineering, or the development of three-dimensional (3D) macroscopic biological tissues *in vitro*, have various important applications: as a medical intervention to replace lost or abnormal cells, tissues and organs through transplantation, as a self-regulating drug delivery vehicle, or as an *in vitro* study model or drug testing platform. The technology is highly anticipated to resolve the severe donor shortage and immune rejection issues of current replacement therapy: allogenic and xenogeneic transplantations. Furthermore, successful development of *in vitro* study and testing models not only decreases the use of animal models which pose deviation from human responses; it also can increase drug safety by providing crucial insights on drug dosing prior to pre- and clinical trials.

However, oxygen diffusion constraints poses a bottleneck in development of macroscopic tissues constructs. We therefore propose to incorporate sacrificial gelatin microspheres in macroscopic hydrogel scaffolds as a versatile tissue engineering platform through two purposes, namely as a porogen for better permeability, and additionally as a cell delivery vehicle for non-anchorage dependent cell (non-ADC) types. Gelatin, being a temperature-responsive hydrogel, dissolves naturally when constructs are cultured for tissue development at physiological temperature; this is a simple and natural one-step strategy of creating cavities in a macroscopic construct.

In the first instance, acellular gelatin microspheres were incorporated into cell-laden hydrogels to increase overall porosity, thereby allowing the development of dense cell populations throughout the construct. Two studies were based on this system: non-anchorage dependent pluripotent stem cells and anchorage-dependent vascular-committed endothelial progenitor outgrowth cells. The former was evaluated for its potential as a three-dimensional culture and differentiation platform for pluripotent stem cells into insulin-producing pancreatic β -cells as a transplantable construct for Type 1 diabetes-inflicted patients; however, although differentiation was partially successful, the 3D platform still holds potential given optimization of the differentiation protocol derived from previously established ones for monolayer differentiation. Using the same system in another study, a network of microvascular structures were self-assembled around the cavities created by the sacrificial microspheres, which was not found in control groups without gelatin microspheres. Both studies highlighted the increased permeability and the resulting high proliferation and development of dense tissue throughout the construct due to sacrificial gelatin microsphere incorporation.

The second instance established sacrificial gelatin microspheres as cell delivery vehicles. A simple, quick and chemical-free fabrication technique based on water-in-oil emulsion was shown to be cell-compatible for at least two types of non-ADCs: hepatocarcinoma cell line HepG2 and primary chondrocytes. This temperature-cured dissolvable gelatin microsphere based cell carrier (tDGMC) platform allowed suspension culture of the non-ADCs into dense tumour

spheroids and cartilage tissue islets respectively, free of any scaffolding material except for the confinement within a macroscopic hydrogel. HepG2 spheroid sizes were shown to be controllable, as their growth in cavities was restricted by the surrounding hydrogel. Its potential as a simple and reproducible, uniform-sized tumour spheroid construct for drug testing and screening platform was also explored with an established chemotherapy drug, Doxorubicin. Finally, primary chondrocytes were encapsulated in tDGMC to be delivered into a cell-laden macroscopic construct, thereby fulfilling two functions: as a porogen to increase permeability as per the first purpose, and as a delivery vehicle for additional cells, thereby greatly accelerating the formation of a cell-dense and functional hyaline cartilage-like tissue free of any material (previously established platform). Both studies highlighted the feasibility and versatility of tDGMC as a cell delivery vehicle into a macroscopic construct for engineering material-free tissue islets.

In summary, the studies in this thesis demonstrated the versatility and simplicity of sacrificial gelatin microspheres in a variety of tissue engineering applications. The temperature-responsiveness of gelatin was exploited to form highly porous macroscopic scaffold systems in a one-step procedure and thereby overcoming diffusion constraints typically faced by conventional systems through three different approaches: increasing the overall porosity, developing a microvascular network through self-assembly, and micro-sized cell carriers as a bottom-up tissue engineering strategy.

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LIST OF ABBREVIATIONS

2-ME	2-mercapoethanol
3D	Three-dimensional
ADC	Anchorage-dependent cell
ANOVA	Analysis of variance
ARC method	Alginate Recovered Chondrocyte method
b-FGF	Basic fibroblast growth factor, also known as FGF2
blkGEL	blank GEL
Col I	Collagen type I
Col II	Collagen type 2
DAPI	4', 6-diamidino-2-phenylindole
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
EB	Embryoid body
ECM	Extracellular matrix
EF	Edge flourish
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell

FBS	Fetal bovine serum
FDA	Food and Drug Administration, United States
H&E	Hematoxylin & eosin
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
Ins	Insulin
IPN	Interpenetrating network
iPSC	Induced pluripotent stem cell
ITS	Insulin-Transferrin-Selenium
KRB	Krebs Ringer Bicarbonate
LhCG	Living hyaline Cartilage Graft
LIF	Leukemia inhibitory factor
MCG	Micro-cavity gel
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NEAA	Non-essential amino acids
O/W emulsion	Oil-in-water emulsion
PBS	Phosphate buffer saline
PCL	Poly(caprolactone)
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PLGA	Poly(lactic-co-glycolic acid)

Poly(NIPAAm)	Poly(N-isopropylacrylamide)
PTCC	Phase Transfer Cell Culture
qPCR	Quantitative real-time polymerase chain reaction
RA	Retinoic acid
Rpm	Revolutions per minute
RT	Reverse transcription
s.d.	Standard deviation
SDS	Sodium dodecyl sulfate
tDGMC	Temperature-cured dissolvable gelatin microsphere-based cell carrier
TIPS	Thermally induced phase separation
TNF- α	Tumour necrosis factor- α
UCST	Upper critical solution temperature
UV	Ultraviolet light
VEGF	Vascular endothelial growth factor
vWF	Von Willibrand Factor
W/O emulsion	Water-in-oil emulsion

Chapter 1 Introduction

Engineering biological tissues *in vitro* is a promising strategy to counter severe donor shortage and possibly lifetime reliance on immunosuppression drugs if patient-specific tissues are developed. Conventional tissue engineering platforms typically comprise biological cells embedded in a macroscopic scaffold and cultured towards tissue development. However, oxygen diffusion constraint of maximum 200 μm [1] as well as physical constraints usually render the platforms ineffective for long-term culture and proliferation of cells to high densities required – for example, magnitudes of 10^9 to 10^{11} functional cells have been estimated for liver transplantation [2, 3].

In order to overcome oxygen diffusion limits in macroscopic constructs, researchers have devised strategies in three main approaches: increasing the overall porosity through foaming and particulate leaching [4, 5], inducing blood vessel formation throughout the construct via growth factor immobilization [6] and topography cues [7, 8] and using micro-sized constructs as a bottom-up approach towards creating macro-sized constructs by perfusion and sintering [9-12]. However, such strategies are usually complex and specialized, involving at least one resource: that of finance, expertise and time. A simple and inexpensive robust system that can overcome this bottleneck for a variety of engineered tissue will therefore be of tremendous value.

1.1. Hypothesis and objectives

In this thesis, we hypothesize that sacrificial polymeric microspheres can be utilized in all three above-mentioned approaches to overcome diffusion limits in a macroscopic hydrogel scaffold system. We propose the use of gelatin microspheres fabricated from a simple emulsion technique as the sacrificial material. Being a temperature-responsive hydrogel well known for its availability and biocompatibility [13, 14], gelatin naturally dissolves when incubated past its upper critical solution temperature (UCST) between 25 and 35°C. This property makes gelatin an ideal sacrificial material: by simple incubation, or elevation of temperature from below room temperature to physiological temperature (37°C), gelatin gel is automatically reverted into a solution state and removed.

Here, the co-encapsulation of gelatin microspheres in macroscopic cell-laden hydrogel scaffolds at temperatures lower than gelatin's UCST provides a simple method of increasing overall porosity throughout the scaffold. Multiple cavities are automatically formed throughout the hydrogel bulk as gelatin dissolves upon temperature elevation and diffuses into the cell culture media. This macroporous cell-laden scaffold, hereby termed microcavity gel (MCG), is postulated to increase diffusion and space for high cell proliferation and dense tissue development throughout the construct. By altering the hydrogel scaffold's properties, this versatile and simple platform can be used for both anchorage-dependent and non-anchorage dependent cell (ADC and non-ADC) types. These sacrificial microspheres are also hypothesized as a cell carrier for non-ADCs, as a bottom-up development of a macroscopic engineered tissue construct.

The specific aims are listed and elaborated on as follows:

1. **Investigate the potential of a macroporous hydrogel using sacrificial microspheres (MCG) for development of non-anchorage dependent tissues**
 - Establish a convenient and robust platform for pluripotent stem cell proliferation and embryoid body (EB) formation;
 - Evaluate and characterize the efficacy of stepwise differentiation of EBs towards pancreatic β -cell lineage; and
 - Evaluate the functionality of β -like cell islets derived from pluripotent stem cells in insulin production and glucose-stimulated response.
2. **Evaluate the potential of a MCG system for development of anchorage-dependent microvascular network**
 - Synthesize and characterize gelatin-methacrylate to impart photocrosslinking properties to gelatin, a cell-adhesive hydrogel;
 - Establish a photo-crosslinkable MCG system for vascular-committed progenitor cell encapsulation and proliferation; and
 - Evaluate the effect of the MCG system in microvascular network formation in contrast to conventional systems without sacrificial microspheres.
3. **Develop a simple and chemical-free microsphere fabrication technique for delivery of non-anchorage dependent cells**

- Establish a simple water-in-oil emulsion technique for formation of cell-laden gelatin microspheres (temperature-dissolvable gelatin microsphere-based cell carrier, tDGMC);
- Characterize the distribution and cytotoxicity of the fabrication technique;
- Evaluate the feasibility of developing tumor spheroids of controlled sizes by confinement in an alginate hydrogel; and
- Evaluate the effect of additional primary chondrocyte delivery through tDGMC into a cell-laden alginate hydrogel for accelerated development of a material-free hyaline cartilage graft.

1.2. Organization of this thesis

This thesis is organized into 7 chapters.

Chapter 1 (this chapter) provides an introduction to this thesis, stating the hypothesis and objectives of this thesis as well as the structure of this thesis.

Chapter 2 provides a review of current research on tissue engineering approaches.

An overview on two cell type categories – ADCs and non-ADCs – is firstly provided with a focus on pluripotent stem cells and stepwise differentiation *in vitro*, followed by hydrogels as scaffolding systems. Lastly, current research strategies on overcoming constraints faced by conventional macroscopic hydrogel constructs in tissue engineering applications are discussed.

The next four chapters are investigative reports. Chapters 3 and 4 explore the use of sacrificial acellular microspheres in cell-laden hydrogels to create MCG systems. In contrast, chapters 5 and 6 explore the use of sacrificial cell-laden microspheres as cell delivery vehicles into a hydrogel.

Chapter 3 investigates the feasibility of non-cell adhesive MCG system for pluripotent stem cell culture and differentiation towards pancreatic β -cell lineage (**Specific Aim 1**). This study evaluates the efficacy of stepwise differentiation of respectively encapsulated embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) towards macro-sized constructs of β -cell islets as a tissue replacement strategy for patients inflicted with Type 1 diabetes.

Chapter 4 then investigates the feasibility of a cell-adhesive MCG system as a strategy for vascularization (**Specific Aim 2**). This study evaluates the self-assembly of a microvascular network using endothelial progenitor outgrowth cells (EPOCs) throughout a cell-adhesive gelatin-methacrylate MCG system.

Chapters 5 and 6 establish a cell delivery system using sacrificial microspheres for non-ADCs (**Specific Aim 3**). A simple, quick and chemical-free protocol is developed to fabricate cell-laden gelatin microspheres, named temperature-cured dissolvable gelatin microcarrier-based cell carrier (tDGMC). In Chapter 5, this platform is used to deliver hepatocarcinoma HepG2 cells into cavities in a macroscopic alginate construct, and provides a suspension culture-like environment for spheroid development. The alginate bulk serves to constrain the spheroid size within the cavity, providing user control of spheroid size by

preselection of tDGMC size. It then evaluates the potential of the spheroid-laden construct as a chemotherapy drug testing platform.

Chapter 6 extends the same microsphere technology to encapsulate primary chondrocytes, and its feasibility as a cell delivery vehicle and porogen for cartilage tissue engineering is evaluated in a previously established platform for material-free hyaline cartilage graft.

Finally, Chapter 7 provides an overall summary and conclusion on the findings before suggesting future possible work.

Chapter 2 Background

The concept of utilizing cells as a therapeutic technique has significantly evolved, particularly the paradigm shift from monolayer culture to biomimetic three-dimensional (3D) platforms for cell culture, since it has been well-recognized that prolonged monolayer culture results in dedifferentiation [15, 16]. Tissue engineering, or the development of 3D macroscopic biological tissues *in vitro*, have various important applications: as a medical intervention to replace lost or abnormal cells, tissues and organs through transplantation, as a self-regulating drug delivery vehicle, or as an *in vitro* study and drug testing model. The technology is highly anticipated to resolve the severe donor shortage and immune rejection issues of current replacement therapy: allogenic and xenogenic transplantations. Furthermore, successful development of *in vitro* study and testing models can not only decrease the use of animal models which pose deviation from human responses, it can also increase drug safety by providing crucial insights on drug dosing prior to pre- and clinical trials.

In order to create biomimetic engineered tissue, biological cells and scaffold system, alongside the appropriate biochemical cues, must work in tandem. However, conventional systems that simply embed cells in a macroscopic scaffold system face the bottleneck issue of diffusion constraints, especially that of oxygen which is estimated to be 200 μm [1]. In other words, cells must be no

more than 200 μm from a nutrient and oxygen source. Therefore, strategies to overcome these in a tissue engineered construct have been widely studied.

This chapter is broken down into three main parts to discuss: firstly, biological cell types with focus on pluripotent stem cells; secondly, available scaffold systems with emphasis on polymeric hydrogels and material-free tissue constructs; and lastly, strategies to overcome diffusion constraints in conventional cell-laden hydrogel constructs are reviewed.

2.1. Biological cells and biochemical signals

Therapeutic biological cells can be broadly categorized into two types: anchorage-dependent or non-anchorage-dependent cells. ADCs in their native environment require extensive adhesion to the extracellular matrix (ECM) and exhibit a stretched or spreading morphology. Examples include osteoblasts, fibroblasts, neural, epithelial, endothelial and smooth muscle cells [17, 18]. On the other hand, non-ADCs exhibit a rounded morphology in their native environment and do not require extensive cell adhesion to the surrounding ECM. These cells include blood cells, chondrocytes, hepatocytes, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

The survival of ADCs depend on a homeostatic mechanism whereby a specific apoptotic pathway is activated when the cell is unable to adhere to the surrounding ECM [17]. This apoptotic process, coined as *anoikis* and meaning “homelessness” in Greek, functions to prevent detached cells from being able to survive and proliferate dysplastically in non-native locations, which is especially

important for preventing tumourigenesis [17]. This attachment-dependent survival has been widely documented in various cell types, including epithelial and endothelial cells.

In *anoikis*, cells that are not attached to their native ECM sense the loss of integrin (comprising α and β subunits) contact and translate these mechanical cues into intracellular signalling cascades, leading to apoptosis (Figure 4.1) [19, 20]. There are namely two pathways – intrinsic and extrinsic – that both ultimately effect a caspase cascade leading to endonuclease activation and DNA fragmentation [21]. When cells are properly attached to their appropriate ECM, anti-apoptotic signals Bcl-2 and Bcl-XL are expressed to maintain mitochondrial membrane's integrity by binding to pro-apoptotic Bad and Bax and sequestering pro-apoptotic Bim [22, 23]. In the intrinsic pathway, when cells fail to attach to the ECM, integrin disengagement increases the amount of Bim in the cytoplasm by allowing its release from cytoskeleton whilst preventing its degradation (inhibition of ERK and PI3K/Akt-mediated phosphorylation of Bim) [21]. Apoptosis activators Bim and Bid as well as apoptosis sensitizers Bad, Puma, Bik, Noxa, Hrk and Bmf are activated [21, 24]. Apoptosis sensitizers on the cell membrane act as competitive inhibitors of apoptosis activators for Bcl-2 [25, 26]. Apoptosis activators, which are usually repressed by anti-apoptotic Bcl-2 and Bcl-XL, then assemble Bax and Bak into Bax-Bak oligomers in the outer mitochondrial membrane (OMM) [27]. The OMM is hence permeabilized and cytochrome c is released from the mitochondria into the cytoplasm [20]. Subsequently, cytochrome c binds with caspase-9 and apoptosis protease activating factor (APAF) to form an

apoptosome which activates caspase-3, resulting in a caspase signalling cascade concluding in apoptosis [28, 29].

There are two branches of the extrinsic pathway, both of which begins with the binding of extracellular death ligands Fas ligands (FasL) and tumour necrosis factor- α (TNF- α) to the cells' corresponding transmembrane receptors Fas and TNF- α receptor TNFR, which are upregulated upon disengagement from ECM [30, 31]. The morphological change into a rounded cell further causes the accumulation and activation of the mentioned receptors [32, 33]. FasL binds to its receptor present on the cellular membrane, causing death-inducing signalling complex (DISC) to be formed. Subsequently, DISC causes the activation of caspase-8 that activates a caspase cascade involving caspases-3 and -7, causing the proteolysis and ultimately cell death (type I extrinsic apoptosis); caspase-8 also can cleave pro-apoptotic Bid into a form (t-Bid) that encourages apoptosome assembly and cytochrome c release, facilitating cell death in the second type of apoptosis [20].

Therefore, in order to maintain cell viability and function ex vivo, appropriate adhesion cues that suit the cell type must be provided.

2.1.1. Pluripotent stem cells

Naturally, the cell type for the engineered tissue is the functional cell type of that to be replaced, i.e. chondrocytes are sourced for cartilage repair. However, these terminally differentiated, functional cells usually would have exited the cell cycle and cannot be induced to proliferate to high cell numbers required for tissue

engineering. Stem cells therefore provide a good alternative due to their self-renewing property.

Pluripotent stem cells ESCs and iPSCs have become a promising alternative cell source for tissue engineering applications by virtue of two characteristics: self-renewal and pluripotency. In other words, they can proliferate immortally and differentiate into any type of cells in the body. They may therefore present a solution to the lack of donor cell sources and limited proliferative ability of isolated adult somatic cells, which also leads to the low cell numbers in the regenerated tissue.

However, the ethical issues involved in retrieving ESCs from human embryos as well as the problem of immune rejection in patients after allogeneic ESC transplantation are limitations ESCs have yet to overcome. A breakthrough to circumvent these problems was when Takahashi and Yamanaka reported the successful generation of murine embryonic-like stem cells from adult fibroblasts through the forced expression of four transcription factors (Oct4, Sox2, Klf4, c-Myc) found to maintain ESC pluripotency and phenotype, due to the transfection with retroviral vectors carrying the reprogramming genes [34]. These cells were thereby named iPSCs. A year later, chimeric mice were generated from iPSCs injected into blastocysts, which gave confirmation that the cells were indeed pluripotent [35]. Different groups also reprogrammed human adult fibroblasts [36] and newborn foreskin fibroblasts [37] through the transfection of 4

Figure 2-1 **I**deal model of patient-specific iPSC technology in regenerative medicine. A small amount of somatic cells readily available (e.g. keratinocytes and fibroblasts) are firstly collected from the patient and reprogrammed into iPSCs using different methods such as viral vectors, plasmids, mRNA and proteins. If the patient has a genetic defect, gene correction through homologous recombination or other methods can be used. The iPSCs undergo proliferation to reach the desired numbers and then differentiated to the required type of cells or tissue, such as erythrocytes and other functional cells. The cells can be seeded on a scaffold and implanted into the damaged site in the patient. An injection of functional blood cells can also be done. The patient then resumes normal function.

reprogramming genes, the former with Oct4, Sox2, Lin28 and Nanog and the latter with Oct4, Sox2, Klf4 and c-Myc.

Aside from fibroblasts, other types of somatic cells such as keratinocytes, blood cells and adipose stem cells were found to be reprogrammable into iPSCs [38-42], but most reports manipulated fibroblasts as the somatic cell source due to its abundance and convenience for extraction. However, there is evidence that

reprogramming capabilities differ between cell types: for example, neural progenitor cells only required the transfection of one reprogramming transcription factor Oct4 instead of the four factors required for fibroblasts [36, 43]. While a portion of the research community focuses on development of reprogramming techniques, another portion aims to differentiate iPSCs for cell replacement therapy.

In an ideal model of iPSC technology in regenerative medicine, a small amount of somatic cells are firstly collected from the patient and reprogrammed into iPSCs. If the patient requires replacement therapies, iPSCs can be grown quickly to the desired numbers and then differentiated to the required type of cells or tissue *in vitro*; if the patient has a genetic defect, the iPSCs can undergo gene correction by homologous recombination or similar methods, before proliferation and differentiation. Owing to its self-renewal capability, a large number of cells can be generated within a short period of time. Finally, the differentiated and functional cells can be transplanted into the patient through injection or a scaffold, and the patient can resume normal function (Figure 2-1). An ideal iPSC technology thus does away with donor organ transplantation, which is severely supply-strapped and requires long-term immunosuppressant medication for the organ acceptor.

Despite its immense potential as an unlimited cell source, two issues impede its progress towards becoming clinically available: risk of tumour formation and epigenetic memory. Firstly, both iPSCs and ESCs can form teratomas, or tumours

consisting of differentiated tissues from all three germ layers. Albeit its usefulness in the laboratory as the strictest confirmation of pluripotency [34], this tumourigenic potential of pluripotent stem cells becomes a safety concern in clinical applications as differentiated human iPSCs or ESCs were still capable of inducing teratomas *in vivo*, leading to a proposed explanation that residual undifferentiated iPSCs or ESCs were still present within the differentiated cell population [44]. Researchers must therefore develop differentiation protocols that can completely differentiate all or remove undifferentiated pluripotent stem cells prior to transplantation. Secondly, although iPSCs possess pluripotency and self-renewal capability like ESCs and share similar aspects e.g. cell doubling time and gene expression patterns, they are not one and the same. Kim et al. determined the presence of epigenetic memory in iPSCs of early passages through examination of methylation loci; iPSCs were found to retain certain methylation sites correlating to their somatic origin i.e. fibroblast-derived iPSCs had methylated regions similar to those in fibroblasts [45]. This phenomenon thus explains the biased differentiation towards lineages similar to that of the donor somatic cell and against those of a different type, and was confirmed by another study that B-lymphocyte-derived iPSCs yielded about ten times more hematopoietic colonies than those derived from fibroblasts [45]. While tissue engineering applications can exploit this by utilizing iPSCs derived from the same lineage, it is a problem when the desired cell type is depleted in the patient or requires invasive surgery to obtain. As fibroblasts and blood cells are currently the most convenient cell source for reprogramming into iPSCs [38, 41], this

translates to a lowered differentiation efficacy and efficiency into other cell types when compared to ESCs.

2.1.2. Stepwise differentiation of pluripotent stem cells into insulin-producing β -cells

Embryonic development into a fully formed fetus is a stepwise process. After fertilization of an ovum, the zygote undergoes cell division and form a blastocyst. The inner cell mass of the blastocyst then undergoes gastrulation to form three germ layers, namely the ectoderm, endoderm and mesoderm, which then differentiate stepwise into specialized, functional cells through biochemical signaling. As this proceeds, cells gradually lose plasticity or potency [46].

In vitro differentiation techniques draw guidance from the natural developmental process, seeking to imitate the step-wise commitment towards a functional cell type through providing timely and appropriate signals such as growth factors and small molecules known to activate or inhibit specific signaling pathways. The efficacy of the differentiation protocol is evaluated through stage-specific gene and protein expression profiles.

In the case of pancreas development, the inner cell mass firstly gives rise to definitive endoderm, from which a subset of cells will commit towards the pancreatic endoderm. The multipotent pancreatic progenitor cells are then specified towards endocrine or exocrine lineages, initially as precursors and finally as functional, terminally differentiated cells: endocrine islets are comprised of 80% insulin-producing β -cells surrounded by glucagon-producing

α -cells and a small percentage of somatostatin-producing δ -cells and pancreatic polypeptide-producing PP-cells; acinar cells of the exocrine function secrete digestive enzymes such as amylase, trypsin and lipases [47, 48]. This developmental pathway with the associated genetic expression is summarized in Figure 2-2.

In various stepwise differentiation protocols, scientists have sought to emulate this pathway for pluripotent stem cells in monolayer culture using various biological chemicals or factors [53-56]. Activin A is firstly supplemented to guide differentiation towards the definitive endoderm and its success determined by expression of specific markers Sox17 and Foxa2 as well as reduction in pluripotent markers Oct4 and Nanog expression [57-59].

Figure 2-2 Gene expression pattern of stepwise differentiation of pluripotent stem cells towards mature pancreatic β -cells, pathway in purple. Adapted from [48-52].

Subsequently, all-trans retinoic acid is secreted by the mesoderm to guide definitive endoderm cells towards pancreatic and duodenum homeobox 1 (Pdx1) expression, a transcription factor crucial for pancreatic commitment [60, 61]; its absence resulted in no Pdx1 expression and failure to develop the pancreas in murine embryos [62-65]. Differentiation studies that supplemented all-trans retinoic acid reported similar trends, increasing the efficacy of Pdx1-positive cells and decreasing the commitment towards the hepatic lineage [60]. During this stage (multipotent pancreatic progenitors), homeodomain markers Nkx2.2 and Nkx6.1 are also expressed [66]. These three genes are later downregulated but resurface to be expressed strictly in β -cells only. Several reports suggest that Gata4 expression is required for pancreatic development [67] but more recently, a study concluded that Gata4 is not essential as long as another transcription factor Gata6 is present [68]. As cells further commit to endocrine and exocrine fates, Gata4 then becomes an early marker exclusively expressed in exocrine differentiation [69].

The appearance of Neurogenin 3 (Ngn3) expression marks the commitment towards endocrine fate and is governed by Notch signaling [61, 70, 71]. Several genes are regulated downstream: Paired box-containing genes 4 and 6 (Pax4 and Pax6), Nkx2.2 and Nkx6.1. Pax4 deficiency has been reported in several studies to arrest differentiation towards β -cell lineage and increase the frequency of α -cells. Its expression is later restricted to β -cells only. On the other hand, Pax6

expression is concomitant with the endocrine fate: Pax6^{-/-} knockout mice studies reported a compromise in all pancreatic endocrine cell types, suggesting its importance in the precursor stage; Pax6 is expressed in all pancreatic endocrine cells but later expressed during α -cell differentiation [72, 73]. Nkx2.2 is suggested to act upstream of Nkx6.1 in β -cell commitment due to evidence that Nkx2.2^{-/-} knockout mice inhibited Nkx6.1 expression and subsequent endocrine cell development [74]. Basic fibroblast growth factor (b-FGF or FGF2) and epidermal growth factor (EGF) are usually supplemented at this stage to respectively increase β -cells' insulin secretion [75, 76] and proliferation of β -cells [77-79]. EGF is also reported to promote the differentiation towards β -cells, and reduce that towards α -cells [80]. As differentiation towards unipotent progenitors proceed, Ngn3 expression is progressively downregulated. Pdx1 and Nkx6.1 expression are reduced at this stage as well [61, 81].

Nicotinamide is then supplemented at the last stage to induce differentiation and increase β -cell mass [82, 83]. Mature β -cell markers include Insulin 1 and 2 (Ins1, Ins2), Nkx2.2, Nkx6.1, Pax4, MafA, and Pdx1. This is also marked by the reoccurrence of Nkx6.1 and Pax4 for β -cell specification [84-86] and Pdx1 for β -cell maturation and survival [81, 87].

2.1.3. Concluding remarks

The two categories of cell types, ADCs and non-ADCs have different morphologies and therefore require different scaffolding systems. The following cells were utilized or developed in this thesis: ADCs endothelial cells committed

towards vascularization, and non-ADCs HepG2, chondrocytes, pluripotent stem cells and pancreatic endocrine islets. In particular, pluripotent stem cells were introduced and utilized as an in-depth example for biochemical signaling and the resulting gene signaling pathways in its successful differentiation towards desired functional cell type.

In the next section, scaffold systems suitable for each cell category will be discussed and reviewed.

2.2. Scaffold system: hydrogels

Scaffolds can be broadly categorized into pre-formed or injectable systems. Pre-formed systems are fabricated *ex vivo*, and cells are introduced either during or after the fabrication process. The cell-laden macroscopic scaffolds are then cultured *in vitro* for a period of time prior to surgical implantation. On the other hand, injectable systems are either of cell-encapsulated micro-sized scaffold (microspheres) form or in a cell-suspension form that can be easily injected into the target site, thereby providing a minimally invasive means of implantation alongside the capability of filling up irregular defects. These would require material solutions that can be gelled *in situ* under mild conditions.

There are three fundamental scaffold structures: hydrogels, fibrous meshes and sponges. Depending on the target engineered tissue and therefore the cell type to be encapsulated, the scaffold structure and the corresponding polymer is then engineered. As this thesis is based on engineering soft tissues containing non-

ADCs, hydrogels and the associated polymers are chosen and discussed in more detail.

Hydrogels are a class of insoluble water-swollen polymeric networks formed from cross-linked water-soluble macromers. Usually, cells are mixed into the hydrogel precursor solution prior to gelation, hence achieving a homogeneous cell-laden hydrogel. Advantages of hydrogels include the highly hydrated environment and good diffusion of nutrients and waste as well as the mimicking of most tissues. Many hydrogels also offer the advantage of being injectable; they can be quickly gelled *in situ* under mild physiological conditions, thereby allowing moulding into irregularly-sized defects via a minimally invasive means of implantation. These properties have thus made hydrogels popular for cell and drug delivery. Through altering the porosity and crosslinking density, mechanical properties of hydrogels can be adjusted to suit the targeted cell type and site of implantation. Mechanical strength, however, is usually low compared to the meshes and sponges and hence hydrogels are only suitable for soft tissues such as cartilage. Furthermore, as hydrogels are highly hydrophilic, cells typically exhibit a rounded morphology when encapsulated within [88] therefore making hydrogels highly suitable for tissue engineering of non-ADCs.

For ADCs, proper cell adhesion is critical in determining the success of their delivery vehicles; otherwise they may undergo *anoikis* and decrease the efficacy of tissue engineering and cell delivery. Therefore, many studies have explored the post-fabrication incorporation of cell adhesive moieties in hydrogels with no

cell-adhesive properties. They can be functionalized through coatings or grafting of ECM proteins and ligands known to promote cell adhesion, e.g. fibronectin and laminin [89]. One of the most popularly incorporated bioactive ligands is the RGD sequence derived from fibronectin. It is known that RGD is the minimal recognition and binding sequence of cells' integrins to fibronectin, and can support adhesion and spreading of cells [90, 91]. Differentiated smooth muscle cells were demonstrated to express the contractile phenotype in PEGDA hydrogels coated with fibronectin or laminin, or functionalized with linear RGD sequences [92]. Cyclic RGD have been shown to have higher affinity and selectivity towards cell-binding, as well as greater stability against enzymatic degradation [93]. Patel et al. functionalized the synthetic polynorbornene hydrogel by grafting linear and cyclic RGD motifs and comparing between the two forms using HUVECs [94]. The group found that cyclic RGD was significantly more efficient in promoting cell adhesion – not only was the minimal concentration of the cyclic form (0.05%) required for cell adhesion 50 times lower than that required for linear RGD, the cyclic-RGD conjugated gel supported cell spreading within 15 minutes while the other required at least 90 minutes to achieve a similar state.

Larger ECM molecules such as gelatin and elastin can also be coated onto the biomaterials following the formation of the vehicle structure. Human fibroblasts and osteoblasts (both of which are ADCs) were viable and functional throughout the culture period on a novel gelatin-coated gellan gum microspheres [95]. A water-in-oil emulsion involving pre-heated FDA-approved gellan in peanut oil

yielded microspheres upon cooling, onto which gelatin was then grafted. PCL sponges were permeated with elastin using carbon dioxide gas foaming and crosslinked with glutaldehyde to create a mechanically suitable and cell-supportive scaffold for cartilage tissue engineering [96]. *In vitro* studies concluded that the integration of elastin not only increased the water content in the sponges, it also was able to support chondrocytes better than pure PCL sponges. For neural cells, laminin was coated onto electrospun fibres to present cell-adhesive moieties to mimic the native laminin-rich environment [97]. The basic bioactive sequence isoleucine-lysine-valine-alanine-valine (IKVAV) that interacts with neural cells and increases their adhesion and proliferation can also be isolated from laminin and conjugated onto biomaterials [98, 99].

2.2.1. Crosslinking mechanisms of hydrogels

Hydrogels can be crosslinked via physical or chemical crosslinking mechanisms, and possess unique advantages and disadvantages over each other in terms of physical and biochemical properties. Under suitable conditions, a solution consisting of macromers or unreacted monomers with crosslinking agents is converted into an insoluble 3D network upon gelation. In the following subsections, the polymers and their gelling mechanisms used in this thesis are discussed below. Other commonly used polymers are summarized in Table 2-1.

2.2.1.1. Physically crosslinked hydrogels and their role as sacrificial materials

Physical crosslinking involves hydrogen bonding, ionic bonding, electrostatic interactions or hydrophobic interactions, forming a hydrogel upon a change in environmental conditions e.g. temperature, pH and ionic concentration. These are usually reversible crosslinking reactions – upon reversion of conditions, the gel reverts to a solution form and can be removed. Physically crosslinked hydrogels are thus candidates for interim scaffolds, or sacrificial materials, and this property has been utilized heavily in our work.

One of the most popular hydrogels is alginate hydrogel derived from brown algae [100]. It is a naturally found polysaccharide comprised of α -L-guluronate (G) and β -D-mannuronate (M) block monomers (solution, sol) which, in the presence of divalent ions e.g. calcium or barium ions), form an ionic-crosslinked network (gel). This sol-gel process is mild, simple and allows direct encapsulation of cells. Together with the porosity, commercial availability, low cost, low toxicity, biocompatibility (when purified), its popularity as a cell encapsulation material is therefore fathomable. Its disadvantages are: weak mechanical properties as a hydrogel, absence of cell-adhesive moieties and perhaps its non-degradability *in vivo* as humans do not have the appropriate enzymes for alginate breakdown. Instead, ionic-crosslinked alginate naturally dissolute over time as divalent ions are leached from the gel and replaced with monovalent ions in the surroundings (e.g. sodium and potassium ions). This uncontrollable and slow process can be significantly quickened by oxidizing alginate to promote hydrolysis of its backbone instead [101-103]. As Bouhadir and colleagues reported, oxidation of alginate's uronate chains to a hydrolysable configuration resulted in complete

degradation within 10 days, whereas there was no significant degradation in the non-oxidized material (control) even after 15 days; also, implantation into mice did not elicit inflammatory response in the 7-week study [104]. This trend was also dependent on the extent of oxidation – degradation rate increases with higher extent of oxidation [105].

It is important to note the necessity of stringent purification in order to prevent inflammation and increase biocompatibility. Whilst there has been conflicting studies on G:M block monomer ratios on inflammation [106-108], other more recent studies have suggested the presence of contaminants was the cause of inflammation instead [109-111]. Indeed, the inflammatory reaction – judged by inflammatory cell density and fibrotic capsule thickness – towards implanted purified alginate was markedly reduced compared to crude alginate in rats [111, 112]. In another study, viability and phenotype of encapsulated pancreatic islets presented fewer and smaller necrotic cores in purified alginate than in crude alginate for at least 27 days [113]. From the consistent results of recent studies, the purification process of naturally-derived polymers is critically important.

Table 2-1 Properties of popular biomaterials

Polymers	Type, source	Common crosslinking mechanism	Reversible?	Advantages	Disadvantages
Natural polymers		Typically physical crosslinked		Biodegradable Low toxicity Low cost, widely available Typically mild crosslinking, allowing direct cell encapsulation	Batch-to-batch variation Presence of impurities, requires stringent purification Usually weak mechanical properties, suitable for mimicking soft tissue only Uncontrollable degradation, unstable
Alginate	Polysaccharide, brown algae	Ionic: Introduce divalent ions for sol-gel transition	Yes. Immersion in chelating agents (sodium citrate)	Suitable for direct cell encapsulation Mild crosslinking High water content	Non-cell adhesive; Susceptible to uncontrollable dissolution in presence of monovalent ions
Agarose	Polysaccharide, seaweed	Temperature: Decrease temperature for sol-gel transition	No.	Suitable for direct cell encapsulation High water content	Non-cell adhesive Brittle
Collagen	Protein, connective tissue of animal sources	Self-assembly	Yes. Enzymatic degradation by MMPs	Cell-adhesive ECM Good mechanical properties	Allogenic or xenogeneic source, may induce host immune response when transplanted

					Suitable for direct cell encapsulation	
Chitosan	Polysaccharide, deacylation of chitin	pH: increase pH for sol-gel transition	Yes. Enzymatic degradation by lysozyme	Cell-adhesive Good mechanical properties Possess amino groups for functionalization	Not able to encapsulate cells directly; cells can be seeded after fabrication Degree of deacylation is not precise Low hydrophilicity Slow degradation	
Fibrin	Protein, blood plasma	Self-assembly: Fibrinogen cleavage by thrombin to form fibrin monomers, self-assembly to form fibrous network	Yes. Enzymatic degradation by plasmin	Cell-adhesive Highly compliant Suitable for direct cell encapsulation	Allogenic or xenogeneic source, may induce host immune response when transplanted Chance of viral transmission (prepared from blood)	
Gellan	Polysaccharide, fermentation of <i>Spinghomonas elodea</i>	Ionic: Introduce multivalent cations for sol-gel transition; or Temperature	Yes. Increase in temperature	FDA-approved Good mechanical properties High acyl content gives compliant and elastic gels Suitable for direct cell encapsulation	Lack of cell-adhesive moieties; requires functionalization	

Gelatin	Protein, denatured collagen	Temperature: Decrease for sol-gel transition	Yes. Increase in temperature or enzymatic degradation by MMPs	Cell-adhesive ECM-like Suitable for direct cell encapsulation	Quick dissolution under physiological temperature; require covalent crosslinking
Synthetic polymers		Typically chemically crosslinked	No. Require modification e.g. photodegradable crosslinkers	Easily tunable mechanical and chemical properties Stable Consistent quality (no/ little batch-to-batch variation)	Lack of cell-adhesive moieties; requires functionalization Usually harsh fabrication conditions Expensive Non-degradable/ toxic degradation products
Poly(ethylene glycol) (PEG)	Polyester	Covalent	No	FDA approved; biocompatible Suitable for direct cell encapsulation	Lack of cell-adhesive moieties
Poly(lactic-co-glycolic acid) (PLGA)	Polyester	Solvent extraction/ evaporation	No	FDA approved; biocompatible Non-toxic Bioresorbable	Hydrophobic Lack of cell-adhesive moieties
Poly(caprolactone) (PCL)	Polyester	Solvent extraction/ evaporation	No	FDA approved; biocompatible Bioresorbable Slower degradation than PLGA	Lack of cell-adhesive moieties

Ease of blending with other
polymers

Instead, it may be simpler and more convenient to remove alginate completely prior to implantation, thereby bypassing the risk of immunogenicity. Decrosslinking, or a gel-sol transition, can be initiated by removing divalent ions. Masuda et al. developed the Alginate Recovered Chondrocyte (ARC) cell culture methodology to completely remove alginate: chondrocytes were encapsulated within alginate beads for a period of time before alginate was completely removed via immersion in a buffered sodium citrate solution [114]. The final product was a piece of cartilaginous tissue free of alginate and held together by the secreted ECM network. This ARC method was therefore adopted in this work.

Gelatin, agarose and poly(N-isopropylacrylamide) (poly(NIPAAm)) are examples of temperature-responsive and biocompatible polymers, i.e. they undergo a sol-gel transition when the temperature is altered. Gelatin, derived from denatured collagen, undergo gelation upon lowering the temperature below its USCT between 25 to 35°C, depending on the molecular weight [115]. Physically crosslinked gelatin may be used as a sacrificial material but not as a scaffolding system since it undergoes dissolution at physiological temperature (37°C) [115-118]. However, as its biodegradability and similarity to natural ECM, and therefore its possession of cell-adhesive moieties, also makes it suitable as a scaffold system for ADCs, researchers have functionalised gelatin with methacrylate groups to produce a chemically crosslinked gelatin scaffold suitable for cell encapsulation [13, 119], the mechanism of which is discussed in the next section.

2.2.1.2. Chemically crosslinked hydrogels

Chemically crosslinked hydrogels are linked via covalent bonds – radical initiators activate crosslinking agents that link monomers to a certain critical density that converts the precursor solution form into a gel form. Unlike physical crosslinking, chemical crosslinking is permanent and thus provides much stronger mechanical properties. By conjugating reactive functional groups and then initiating crosslinking using a chemical or enzyme crosslinker, many of the physically crosslinkable polymers can undergo covalent crosslinking, resulting in stronger and less degradable hydrogels. For example, naturally occurring genipin [120, 121] and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) [122] have been used to crosslink gelatin microspheres with significantly delayed degradation characteristics [120, 123].

Photocrosslinking utilizes a photo-initiator and ultraviolet light (UV) irradiation to form covalent bonds between monomers, and this convenient and simple process has become widely used. These monomers are typically conjugated with acrylate groups (e.g. methacrylate, diacrylate). Gelatin-methacrylate [13, 119], alginate-methacrylate [105], chitosan-methacrylate [124], Poly(ethylene glycol)-diacrylate (PEGDA) [125]. PEGDA and Irgacure (photo-initiator) have been utilized in clinical trials as a scaffold for knee cartilage repair, and no adverse reaction was reported in all 15 human subjects [126].

In this process, a photo-initiator in low concentrations is excited upon UV irradiation and forms free radicals, which then initiates a radical chain

polymerization reaction that crosslinks the reactive functional groups. The concerns associated with photocrosslinking are the biocompatibility of photo-initiator and duration of UV irradiation. A study concluded VA-086 and Irgacure 2959 to be the least toxic photo-initiators across several mammalian cell types including chondrocytes, osteoblasts and mesenchymal stem cells (MSCs), but the cytotoxic effect varies among different cell types [127, 128]. Additionally, Rouillard's study on the effect of 6 different photo-initiators and their concentrations on bovine chondrocytes in photocrosslinked alginate-methacrylate gave the following observations: 1) the choice of photo-initiator can affect the cell viability in both native form or its generated radicals; 2) increasing the duration of exposure to UV escalates cytotoxicity effect, and optimal duration depends on the photo-initiator; 3) the concentration of photo-initiator affects the mechanical properties; and 4) increasing the photo-initiator concentration can lead to a decrease in cell viability [128]. With these conclusions, crosslinking procedures must be carefully optimized for the target encapsulated cell.

2.2.2. Material-free tissue constructs

Ultimately, materials comprising the scaffold system have to be removed prior to implantation in order to completely eliminate the possible risks of biomaterial-related problems, including the mismatch of degradation rate to that of tissue development, immune responses and toxic degradation products. Yet, direct injection of therapeutic cells may not be an efficient and effective cell delivery method since cells are not retained within the target site (leakage) and survive

poorly without proper homing [129, 130]. This can be potentially solved by transplanting biomaterial-free microtissues which have been cultured *in vitro* using biomaterials as a temporary scaffolding system. In other words, cells can be firstly cultured on biomaterial scaffolds in the lab for a predetermined duration until they form micro-sized pieces of tissue comprising cells held together by their secreted ECM; subsequently they can be separated from the scaffolding system for implantation in the target site.

A confluent monolayer of cells, known as cell sheets, can be easily engineered in tissue culture plates by making use of responsive biomaterial surfaces to force the detachment of cells from the biomaterial surface without disrupting the cell-cell interactions and ECM holding the cells together. This strategy was pioneered by Kushida's group: temperature-responsive poly(NIPAAm) was coated on tissue culture treated polystyrene surfaces prior to cell seeding [131]. At the physiological temperature of cell culture, i.e. 37°C, the coating is mildly hydrophobic which is suitable for cell adhesion and culture; however, lowering the temperature to below 32°C (its lower critical solution temperature, LCST) increase the hydrophilicity of the coating and the cells are upheaved from the surface. This produced a material-free cell sheet held together by the cells' secreted ECM, which is removable from the attached surface without any enzymatic treatment that would have also broken down the ECM [132, 133]. The poly(NIPAAm) coating is currently commercially available as UpCell® and has been utilized in fabricating cell sheets of various cell types, including mesenchymal stem cells [134] and fibroblasts [135]. A monolayer of

mesenchymal stem cells implanted into infarcted myocardia of rats was able to reverse the thinning of the scarred myocardial wall, while promoting vascularization and differentiating into vascular cells, with the overall effect of improving function of the infarcted heart [134]. Other cell sheet fabrication strategies include the use of magnetic force and polyelectrolytes [136]. Ito et al. developed a magnetic nanoparticle – RGD-conjugated magnetite cationic liposomes (MCL) – that coated surfaces by applying magnetic force beneath the surface; fibroblasts as model cells were able to adhere to the RGD-conjugated surface coating and upon removal of the magnetic force, the cell monolayer was detached [137]. Kito et al. successfully utilized a similar MCL strategy to retrieve an iPSC monolayer for implantation to induce angiogenesis in ischemic tissues, except that MCLs were labelled in the cells instead of on a surface coating [138]. Like-wise, cells can be detached from RGD-conjugated polyelectrolyte coatings via altering the polarization [139, 140].

The difficulty of handling an extremely fragile monolayer of cells is a severe limitation of cell sheets in the clinical setting. Furthermore, being monolayer, it is difficult to scale up to form and replace a 3D macroscopic tissue defect since stacking multiple layers of monolayers is a laborious process. Biomaterial-free 3D systems are favourable for cell delivery as they can deliver a large amount of cells without unwanted leakage to non-target sites and circumventing any biomaterial-related problems. As an example, our previous work developed a material-free hyaline cartilaginous construct through using alginate as an interim scaffolding system. Upon reaching a cell and ECM dense state (35 days), the

ECM secreted by chondrocytes was able to support the construct such that alginate was removed without a collapse in structure [141]. Alginate, which crosslinks in the presence of calcium divalent ions, can be completely and quickly removed by immersing the scaffold in a sodium citrate-containing solution, whereby citrate ions act as calcium chelation. Implantation into rabbit osteochondral defects in the knee joint showed good integration with host tissue and expression of hyaline cartilage phenotype with no visible fibrosis.

2.2.3. Concluding remarks

Hydrogels are versatile and robust scaffold systems for engineering soft tissues. Although its highly hydrated environment is more suited for non-ADCs, they can be easily modified to suit ADCs such as chemical crosslinking of cell-adhesive gelatin, or inclusion of cell-adhesive moieties. In particular, alginate and gelatin are promising hydrogels as they are easily removable and can be utilized as sacrificial materials; alginate dissolves via immersion in a chelating solution, while gelatin naturally dissolves at physiological temperature. Therefore, gelatin is desirable as a sacrificial material since it does not require any additional removal step. Yet, its cell-adhesive properties are also desirable as a convenient and inexpensive scaffold system for ADCs. In this case, chemical crosslinking provides a simple and quick way of creating gelatin hydrogels for tissue engineering of ADCs, through incorporation of photo-crosslinkable moieties such as acrylate groups.

2.3. Overcoming diffusion constraints in conventional hydrogel scaffold systems

Conventionally, a cell-laden hydrogel construct is fabricated by mixing the cells into the hydrogel precursor solution and injecting into a macroscopic mould where crosslinking is then initiated. A macroscopic cell-laden hydrogel is subsequently suspended in cell culture medium and cultured for tissue development. However, this platform is not feasible due to oxygen diffusion constraint of maximum 200 μm [1]. There are several proposed strategies to overcome this bottleneck: increasing diffusion through increasing porosity within the construct, using a bottom-up approach of creating micro-sized cell-laden constructs which may then be used as-is or put together to form macroscopic constructs, or inducing vascularization throughout the construct. Below, we discuss the strategies in more detail.

2.3.1. Macroporous hydrogels

Macroscopic 3D platforms are not feasible for prolonged cell culture due to diffusion constraints; particularly, oxygen diffusion constraint has been reported to be of maximum 200 μm [1]. To increase cell viability and ingrowth, attempts have been made to improve the diffusion through increasing the effective porosity through different strategies, including phase separation [142, 143], cryogelling [144], freeze drying [145, 146], foaming [147, 148] and particle leaching [116, 149, 150]. Most techniques are either laborious or utilize harsh chemicals and processes, and therefore utilize a two-step procedure: firstly by

manufacturing the porous scaffold and then seeding cells on it. This poses two problems: incomplete penetration of cells into the core of the macroscopic construct, as well as its non-suitability for non-ADCs.

In particular, we will utilize gelatin microspheres to create interconnected spherical pores within a hydrogel as a simple one-step particle leaching strategy for non-ADCs; cells and gelatin microspheres are coencapsulated in a macroscopic hydrogel, with the latter being sacrificed naturally upon culture at physiological temperature. Exploiting the temperature-responsive behaviour of gelatin, which undergoes a gel-sol transition upon increasing the temperature above its UCST, several research groups have also manufactured gelatin microspheres and incorporated them into hydrogels as porogens where gelatin is naturally leached after 2-3 days [117, 118, 150, 151]. Porosity can be easily tuned based on the diameter and concentration of gelatin microspheres selected.

In 2010, Gong and colleagues developed a novel scaffolding strategy for cartilage tissue engineering, named Phase Transfer Cell Culture (PTCC) [150]. Through the co-encapsulation of empty gelatin microspheres and chondrocytes in an agarose hydrogel scaffold followed by incubation at 37°C, gelatin in the constructs completely dissolved within 2 days and left behind same-sized cavities within the hydrogel bulk. The strategy capitalizes on the natural edge flourish (EF) phenomenon of non-ADCs such as chondrocytes encapsulated in a non-cell-adhesive hydrogel bulk (alginate, agarose). The cells at gel edges outgrow the gel-medium interface and secrete ECM to form neo-tissue much faster than in

the hydrogel bulk. A study on this EF phenomenon concluded that the cell outgrowth was driven by an outward stress from hydrogel to medium [152]. Cavities were completely filled up with cells and ECM to form neo-tissue islets which, given time, further developed into the agarose gel bulk as well and merged with neighbouring islets to form a macroscopic cartilaginous construct. Chondrocytes were observed to maintain its hyaline phenotype, as collagen type I (observed in fibrosis) was minimal and unobservable and collagen type II (typically comprising 90% of total collagen in hyaline cartilage) was abundant. Compared to conventional chondrocyte-encapsulated agarose hydrogel constructs, the EF phenomenon allowed for superior ECM production and cell proliferation. Albeit its superior hyaline cartilage-like phenotype, the complete degradation of agarose in the construct is unascertainable. Therefore, in a follow-up study, agarose was replaced with alginate (mentioned in Section 2.2.2). After 35 days of culture, alginate was removed via the ARC method as mentioned previously, yielding a material-free construct which was not reproducible with a conventional cell-laden hydrogel without gelatin microsphere-induced pores [141]. These results imply the advantage of macroporosity in scaffolds in promoting cell proliferation and ECM secretion.

2.3.2. Microspheres as scaffolds

A second strategy to bypass the diffusion constraint is via a bottom-up approach – creating micro-sized cellular constructs which may then be used as-is or put together to form macroscopic constructs. Notably, microspheres are being

developed as cell-laden scaffolds for two main classes: *in vitro* cell culture for cell expansion and biomolecule manufacturing, and *in vivo* cell delivery for cell replacement or therapy. They are relatively easy to fabricate and handle, and provide a large surface area to volume ratio for cell culture. It also promises a means of minimally or non-invasive, localized delivery and immune-isolation into patients. These delivered cells may secrete (either naturally or forcefully through genetic modification) therapeutic factors in a sustained manner at the site, which therefore circumvents the need for multiple administration of drugs.

Microspheres designed for biological cells to attach on or be encapsulated within are generally spherical polymerized networks of 100-400 μm in diameter to counteract the oxygen diffusion limits. The former is usually fabricated to possess cell-adhesive moieties, after which ADCs are seeded on. On the other hand, microcapsules are typically fabricated via crosslinking a polymer-cell suspension to entrap non-ADCs within the core of the microsphere. The seemingly simple product, however, requires much thought in its design and optimization in order to provide the appropriate microenvironment for biological cells to survive, reside and maintain their desired function in.

Microsphere synthesis can be broadly categorized into firstly choosing the fabrication technique, and then by modifying different parameters to achieve desired size, physical and chemical properties that are suitable for the desired outcomes, cell survival and proliferation.

2.3.2.1. Emulsions as a fabrication technique

Although the different applications have specific requirements, it is not so much the choice of fabrication technique than the polymers and modifications that can be customized to fit the applications. Instead, fabrication technique is generally chosen based on the desired simplicity, scalability, size and size distribution. In Table 2-2, 4 fabrication techniques are summarized, amongst which emulsion is explained in further detail due to its usage in this work.

Emulsions are a simple and low-cost fabrication technique utilizing a benchtop magnetic stirrer plate, but it can be more tedious and time-consuming as washing steps to remove the continuous phase are required. A simple emulsion comprises a mixture of two immiscible phases (oil and aqueous). The dispersed phase will contain the polymer solution which may contain biological cells, and the continuous phase will be a contrasting phase of a greater volume than the former. Once an equilibrium is reached, crosslinking is initiated through mechanisms appropriate for the polymer. This technique is scalable towards industrial fabrication of microspheres, but lacks uniformity in microsphere sizes. If uniformity is important, either selection through sieving or an extrusion method will be necessary.

Microspheres are fabricated using a water-in-oil (W/O) emulsion if the polymer is of aqueous phase such as alginate and PEG hydrogels. Washing steps are necessary to remove the oil from microcapsules [95, 153]. An oil-in-water (O/W) emulsion technique followed by solvent extraction and evaporation is conversely used for organic polymers such as PLGA. PLGA is dissolved in an organic

solvent prior to emulsification, which is then extracted by the continuous phase and finally evaporated to form solid microspheres [154-156].

Many factors play a part in determining the size and distribution of microspheres: size of vessel, the volumetric ratio of both phases, viscosity of each phase (related to polymer concentration as well), stirring speed, presence and concentration of surfactant [157-159]. Increasing the stirring speed (usually defined in terms of revolutions per minute, rpm) increases the shear force, which translates to a decrease in microsphere size [155, 158]. In Heiskanen's study, the mean diameter of microspheres increased from 19.1 μm to 40.5 μm as the stirring speed decreased from 600 rpm to 450 rpm [157]. Another study observed an increase in mean diameter of collagen microspheres from 75 μm to 182 μm when stirring speed was decreased from 1000 rpm to 600 rpm [122].

To reduce interfacial tension and stabilize the dispersed phase, a surfactant (also known as emulsifier) may be added e.g. Span 20 (Sigma) [122], sodium dodecyl sulfate (SDS) and poly(vinyl alcohol) (PVA). The principle is: surfactant molecules are amphiphiles which insert themselves between the phases. Under a certain limit, increasing the surfactant concentration can result in smaller microspheres, after which the droplet surface is fully saturated and excess surfactant molecules will form micelles [158].

Table 2-2 Common microsphere fabrication techniques and properties

Technique	Principles	Advantages	Disadvantages	Factors affecting microsphere size	Example ref
Emulsion	<ul style="list-style-type: none"> - Two immiscible phases. Dispersed phase contains polymer solution. - Mechanical agitation/stirring to break up polymer solution into droplets 	<ul style="list-style-type: none"> - No specialized equipment required - Scalable - Multiple emulsions achievable - High throughput 	<ul style="list-style-type: none"> - Large size distribution - Washing steps required - Batch-wise process 	<ul style="list-style-type: none"> - Size of vessel - Viscosities of phases - Stirring rate - Presence and concentration of surfactant 	Collagen [122], Gelatin [160], Chitosan [161], PLGA [162], Fibrin [163]
Extrusion	<ul style="list-style-type: none"> - Polymer solution is extruded through a nozzle and fall into collector 	<ul style="list-style-type: none"> - Uniformly sized microspheres - Continuous process achievable 	<ul style="list-style-type: none"> - Specialized equipment required - Low throughput (1 bead at a time) 	<ul style="list-style-type: none"> - Flow rate - Viscosity of polymer solution - Diameter of nozzle 	Chitosan [164, 165], Alginate [166, 167]
Atomization	<ul style="list-style-type: none"> - Atomized droplets fall into a collector 	<ul style="list-style-type: none"> - Small microspheres with narrow size distribution - High throughput - Continuous process achievable - Scalable 	<ul style="list-style-type: none"> - Specialized equipment required 	<ul style="list-style-type: none"> - Flow rate - Viscosity of polymer solution - Frequency - Height of collector and air flow direction (for spray drying) - Temperature 	PVA [168], alginate [169]

Microfluidics	-	Two immiscible phases flowing continuously, with continuous phase pinching off beads of dispersed phase (polymer solution)	-	Uniformly sized microspheres	-	Easy to customize	-	Small platform	-	Continuous process achievable	-	Multiple emulsions achievable	-	Washing steps required	-	Not scalable	-	Low throughput (1 bead at a time)	-	Flow rates	-	Viscosities of phases	-	Diameter of nozzle	-	Dimension of setup	-	Presence and concentration of surfactant	PEG [170], Agarose [171], Poly(NIPAAm) [172]
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2.3.2.2. Using microspheres as a bottom up approach to create macroscopic tissues

Microspheres containing functional cells implanted into human subjects was initially reported in 1994 – allogenic pancreatic islets in alginate-PLL microcapsules into a Type 1 diabetic patient. The subject's blood glucose level was stable throughout the 9-month study [173]. Synthetic photocrosslinked hydrogel PEGDA was also explored and proven to maintain viability and functionality of porcine pancreatic islets for at least 110 days in diabetic mice [174]. Since then, microspheres were widely studied as cell delivery vehicles.

Additionally, cell laden microspheres can also be utilized as building blocks of a macroscopic construct which can further localize microspheres to the desired site. Forming a macroscopic construct through a bottom-up approach is simple: cell-laden microspheres can undergo a secondary encapsulation in a polymer [160, 175], fusion via sintering [12] or aggregation [10, 176]. In the first instance, a secondary polymer encapsulation creates a macroscopic construct containing cell-laden microspheres, which can simulate a tissue model *in vitro* or be an injectable platform that further localizes and immune-isolate the microspheres *in vivo*. Our lab utilized this strategy to doubly immune-isolate macrophages beside a tumour mass in the aim of preventing leakage and proliferation whilst providing anti-tumour effects; unlike doubly encapsulated macrophages, cells were observed to leak from microspheres after a few days, which would have incurred an immune response *in vivo* [177]. A second strategy utilizes perfusion

bioreactors to compact microspheres over a period of time to allow development of ECM to hold the macroscopic construct together [9, 10]. Lastly, sintering utilizes solvent or heat treatment to fuse microspheres into an interconnected macroporous structure, onto which cells are seeded. This is however mainly confined to PLGA-based microspheres for bone tissue engineering due to its similar mechanical properties to bone [12, 178].

2.3.3. Inducing vascularization in scaffolds

Finally, another scientific community aims to develop blood vessels within the macroscopic scaffolds to recapitulate the natural architecture of most biological tissues with the exception of naturally avascular ones [8]. An imbedded microvascular network will allow efficient nutrient supply to and waste removal from cells in the interior, and proper integration into the host when implanted.

As mentioned, tissue engineering consists of three aspects: cells, scaffold and biochemical signals. All blood vessels have a layer of anchorage-dependent endothelial cells as the innermost lining; capillaries are simple structures with mainly endothelial cells while larger vessels have an outer layer of smooth muscle cells. Being ADCs, endothelial cells therefore require a cell-adhesive scaffold such as collagen and gelatin for survival and proper function. However, being terminally differentiated, endothelial cells are plagued with low proliferation rates and availability [179]. Endothelial progenitor cells (EPCs) that possess greater proliferation rates [180] and can differentiate towards the endothelial lineage [181, 182] have therefore become a popular alternative cell

source. They are found in peripheral blood, umbilical cord blood and bone marrow and observed to contribute to new blood vessel formation [182-184]. EPCs have been shown to successfully differentiate into endothelial cells and vascularize engineered tissue, with the presentation of mature blood vessel markers [185-187].

In order to increase efficiency of vascularization in constructs, studies have developed complex growth factor supplementation and surface modification of scaffolds as described below:

It is well known that supplementation of angiogenic factors such as the potent vascular endothelial growth factor (VEGF) and b-FGF are important in vascular structure assembly [7, 188-190]. However, manual addition of such factors to macroscopic scaffolds not only results in inconsistent concentrations in the cell culture medium over time, but also throughout the construct due to hampered diffusion towards the core of the construct. To overcome this limitation, growth factor immobilization [191-193] and gene delivery [167, 189, 194] ensures sustained release. Gene delivery, although very promising in providing an inexpensive and sustained release of growth factors within the construct, still face concerns for clinical use such as low efficiencies in non-viral gene delivery and immunogenicity of viral vectors [195-197].

Conversely, other groups have utilized micropatterning to restrict and confine endothelial cell growth to encourage self-assembly of vascular structures. In the native vascular environment, endothelial cells are stretched and aligned along the

longitudinal axis of blood vessels, i.e. parallel to blood flow. When organized in this orientation on manufactured biomimetic scaffolds, endothelial cells were able to retain their morphology, cell-specific gene expressions and possess athero-resistant qualities with less adhesion of platelets and monocytes [198]. Nikkhah et al. recently demonstrated the importance of topography in directing endothelial cell adhesion and attachment with the maintenance of its functionality in vascularization [199]. Longitudinal tubes of photo-initiated crosslinkable gelatin methacrylate hydrogel were fabricated with micropatterns by exposing selective areas to light using a mask (photomasking) [199]. HUVECs were of an elongated and spreading morphology and reorganized themselves to align along the length of the tubes by day 5, forming a 3D stable cord-like structure in 15 days. On the other hand, HUVECs on the unpatterned gelatin methacrylate gel block were randomly aligned, therefore affirming the effect of topography on cell behaviour and functionality.

All the mentioned strategies require complex manipulation of scaffolds, which directly translates to time and cost, and may not be scalable. Therefore, there exists a need for a simple system that can induce vasculogenesis throughout the macroscopic scaffold.

2.3.4. Concluding remarks

Here, the need for a simple system to overcome diffusion constraints is highlighted. Sacrificial gelatin microspheres may hold great promise as a potential integrated strategy of all three approaches: firstly, it can be used to

create macroporous hydrogel; secondly, it can be utilized as a cell-laden microsphere for delivery into a macroscopic hydrogel thereby also creating macroporosity; and finally, its high permeability does away with the need to immobilize growth factors on the macroscopic scaffold. Further, the macroporosity may also physically constrain vascular-committed cells and encourage their self-assembly into vascular networks in a 3D macroscopic construct.

Chapter 3 Directed differentiation of pluripotent stem cells into insulin-producing islets in a sacrificial microsphere/ hydrogel system

Summary

Sacrificial gelatin microspheres were utilized to create a MCG system for pluripotent stem cell proliferation and EB development prior to stepwise differentiation towards pancreatic β -cell islets. The high permeability of MCG system allows rapid formation of dense EBs throughout the construct. This study evaluates the feasibility of developing a 3D pluripotent stem cell-derived β -cell macroscopic construct as a replacement strategy for patients inflicted with Type 1 diabetes. Through gene expression and immunohistochemistry analyses, ESCs were shown to commit to β -cells but was unresponsive to varied glucose concentrations; iPSCs were found to lag behind in terms of differentiation capability, but may be attributed to epigenetic memory. Further optimization of differentiation protocol to suit the 3D macroscopic nature is required, instead of adaptation from previously established protocols from monolayer studies. However, this study still shows potential of the MCG system as a simple platform for proliferation and development of a cell-dense tissue construct.

3.1. Introduction

Type 1 diabetes, also known as insulin-dependent diabetes, is a condition in which insulin-producing pancreatic β -cells are destroyed [200]. Although not as common as Type 2 diabetes, the incidence of Type 1 diabetes is rapidly increasing across the globe, with the number of new cases projected to increase by 70% in adolescents from 2005 to 2020 [201]. Patients inflicted with type 1 diabetes require exogenous insulin treatment as well as careful management throughout their lifetime, which respectively lead to complications and loss in quality of life.

Cadaveric and xenogeneic β -cell islet transplantations have been performed, but are plagued by not only greatly limited donor sources but also complications arising from lifelong reliance on immunosuppression drugs [202]. Furthermore, results were not favorable long-term, with approximately 90% of recipients requiring exogenous insulin treatment 5 years post-transplant [203]. A tissue engineering approach for cell replacement therapy can be realized by using pluripotent stem cells ESCs and iPSCs, attractive as cell sources due to their unlimited self-renewal and differentiation potential. Several groups have reported successful step-wise differentiation of pluripotent stem cells imitating that of *in vivo* embryonic pancreas development: firstly towards the definitive endoderm, then commitment to pancreatic lineage, and subsequently towards the endocrine lineage and further maturation into insulin-producing β -like cells [53, 204-206]; however, they are limited to monolayer or suspension culture conditions, which restricts its potential for cell delivery.

A 3D platform provides not only a biomimetic environment, but also a means of easy handling and implantation. The MCG platform further provides a one-step encapsulation protocol in which pluripotent stem cells can proliferate and rapidly form EBs within prior to differentiation, unlike conventional two-step procedures of firstly forming EBs and subsequently encapsulating them [59].

3.2. Materials and Methods

All cell culture related reagents were purchased from Invitrogen, and all chemical reagents from Sigma-Aldrich unless otherwise stated.

3.2.1. Cell culture

mESC (E14tg2A) was purchased from American Type Culture Collection (ATCC) and miPSC (APS0003) [207] was purchased from RIKEN Bio Resource centre (Ibaraki, Japan). Both miPSCs and mESCs were cultured under feeder-free conditions on 0.1% w/v gelatin type B-coated tissue culture dishes. Pluripotent cell culture medium composition was as follows: Dulbecco's Modified Eagle's Medium (DMEM) containing 15% Foetal Bovine Serum (FBS, 'Gold' Standard, PAA Laboratories), 0.1 mM NEAA, 0.1 mM 2-Mercaptoethanol and 1000 U/ml leukaemia inhibitory factor (LIF, Millipore). Only cells from passages 9-12 were utilized in this study.

3.2.2. Fabrication of MCG construct

Gelatin microspheres were fabricated as previously described [141] and 150 – 180 μm diameter microspheres were selected for use as porogens. 1×10^7 cells

were separately suspended per 1 mL of 4 °C 1.5% w/v alginate solution (dissolved in 0.15 M NaCl and autoclaved. The cell suspension was subsequently mixed with 0.3 g gelatin microspheres per 1 mL of alginate solution. 80 µL of the mixture was transferred to each circular mould of 5 mm diameter, laid on a carpet of 15% w/v gelatin substrate containing 102 mM CaCl₂ and placed in 4°C for 4 min. 10 µL of 102 mM CaCl₂ was carefully pipetted onto the surface of the construct and replaced in 4°C for another 4 min for gelling to take place. Finally, constructs were transferred to 24-well agarose-coated plates containing cell culture medium and maintained at 37 °C, 5% CO₂. Gelatin microspheres dissolve at 37 °C to leave behind pores of corresponding diameters within two days, forming a MCG system [141]. Constructs were maintained in pluripotent cell culture medium for 10 days to allow proliferation and EB formation [59]. Cell culture medium was changed daily.

3.2.3. Step-wise differentiation towards pancreatic β-cell islets

After 10 days, both mESC and miPSC constructs were induced towards pancreatic differentiation with a step-wise differentiation protocol modified from previous protocols [53, 56]. This time point is defined Day 0. Cells were firstly induced towards definitive endoderm by culture in serum-free RPMI 1640 medium (PAA Laboratories) supplemented with 1× B27 supplement and 100 ng/mL Activin A (R&D Systems) for 5 days [54, 59, 208]. Subsequently, for pancreatic commitment, medium was replaced with serum-free RPMI 1640 medium supplemented with 2 µM retinoic acid (RA), 0.5% bovine serum

Figure 3-1 Timeline of cell culture conditions. Day 0 is defined as the start of differentiation procedure; the establishment of MCG construct is therefore depicted as Day -10.

albumin (BSA) and 1× Insulin-Transferrin-Selenium (ITS) (Gibco) for 2 days. In the third step for endocrine commitment, medium was changed to DMEM/F12 supplemented with 10 ng/mL murine basic fibroblast growth factor (b-FGF, Millipore), 20 ng/mL murine epidermal growth factor (EGF, Gibco), 0.2% BSA and 1× ITS for 3 days. Finally, for pancreatic islet maturation, medium was changed to DMEM/F12 supplemented with 10 ng/mL murine b-FGF, 10 mM nicotinamide (Sigma-Aldrich), 0.2% BSA and 1× ITS for 5 days. The differentiation process is depicted in Figure 3-1.

3.2.4. Cell viability assays

Live/Dead cell viability assay was performed as per manufacturer's instruction (Invitrogen). Briefly, each construct was incubated in respective cell culture/differentiation medium with the addition of calcein AM and ethidium homodimer-1 for 30 min, prior to observation under fluorescence microscopy.

3.2.5. Gene expression analysis using quantitative real-time polymerase chain reaction (qPCR)

At each predetermined time point, 3 constructs per sample group were homogenized in 1 mL TRIzol® (Invitrogen) prior to RNA extraction as per manufacturer's instructions. 500 ng RNA were subsequently reverse transcribed into cDNA as per manufacturer's instruction (Promega). qPCR was performed with CFX Connect qPCR system (Bio-Rad). Primer sequences (AIT Biotech Singapore) used are listed in Table 3-1. Relative gene expression values to reference gene β -actin were calculated by comparative ΔC_T method, and subsequently normalized against the respective genes of control, Day 0 for both mESC and miPSC to obtain gene expression fold-values.

Table 3-1 qPCR primer sequences for murine gene markers used in Chapter 3. Forward (F) and Reverse (R). PS stands for product size.

Gene	Accession number/ Reference	Primer Sequences (both 5'-3')	PS/ bp
Oct4	[59]	F: AAAGCAACTCAGAGGGAACCTCCT R: TAGCTCCTTCTGCAGGGCTTTCAT	122
Nanog	[59]	F: TGGTGTCTTGCTCTTTCTGTGGGA R: ACACTCATGTCAGTGTGATGGCGA	180
Sox17	BC060612.1	F: TGTAAGGTGAAAGGCGAGGTGGT R: GCATCTTGCTTAGCTCTGCGTTGT	182
Gata4	[59]	F: TCAAATTCCTGCTCGGACTTGGA R: GTTTGAACAACCCGGAACACCCAT	163
PDX1	NM_008814.3	F: TTGGGTATAGCCGGAGAGAT R: TCCCAGGAAAGAGTCCTAGAG	124
Ngn3	[209]	F: ATCCAGTGTTGCGTCTTACC R: GGACTAAGGCAGAATGGAGAAG	122
Nkx6.1	[209]	F: GGATGACGGAGAGTCAGGTC	104

		R: CGAGTCCTGCTTCTTCTTGG	
Nkx2.2	[210]	F: CCGGGCGGAGAAAGGTATG R: CTGTAGGCGGAAAAGGGGA	156
Pax4	[211]	F: TCCCAGGCCTATCTCCAAC R: TATGAGGAGGAAGCCACAGG	65
Pax6	AF44322 3.1	F: GAGAGAACACCAACTCCATCAG R: CCTCAATCTGCTCTTGGGTAAA	120
Ins1	[211]	F: GAAGCGTGGCATTGTGGAT R: TGGGCCTTAGTTGCAGTAGTTCT	76
Ins2	[211]	F: AGCCCTAAGTGATCCGCTACAA R: CATGTTGAAACAATAACCTGGAAG A	178
Glut2	AK00506 8.1	F: CTTACAGTCACACCAGCATACA R: AGACAGAGACCAGAGCATAGT	96
Gcg	AK00791 1.1	F: CCTTCAAGACACAGAGGAGAAC R: TGTAGTCGCTGGTGAATGTG	122
Sst	AK00301 4.1	F: CAAAGCTGGCTGCAAGAAC R: GCCAGGAGTTAAGGAAGAGATATG	123
B-actin	[59]	F: TCATGAAGTGTGACGTTGACATCC GT R: CCTAGAAGCATTGCGGTGCACGA TG	285

3.2.6. Immunohistochemistry

At predetermined time points, samples were fixed in 4% w/v paraformaldehyde in PBS overnight prior to dehydration and embedding in paraffin. 8 µm-thick sections were mounted on glass slides and stained separately for Nanog (Santa Cruz Biotechnology), Sox17 (Santa Cruz Biotechnology), PDX1 and Insulin (Ins). Paraffin was removed from sectioned samples by immersion in xylene. After fixing in 2.5% glutaraldehyde solution for 30 min, samples were blocked in 10% horse serum at 37°C for 20 min.

Samples were separately incubated overnight at 4°C with one of the following primary antibodies: 2 µg/mL rabbit polyclonal IgG Nanog (Santa Cruz Biotechnology), 2 µg/mL goat polyclonal IgG Sox17 (Santa Cruz), 1 µg/mL goat polyclonal IgG PDX1 (Abcam) and 1 µg/mL rabbit polyclonal IgG Ins (Abcam). After washing 3 times in PBS, samples were incubated with their respective secondary antibodies: Nanog samples with goat anti-rabbit IgG AlexaFluor 488 (5 µg/ml in PBS, Invitrogen); Sox17 and PDX1 samples with rabbit anti-goat IgG AlexaFluor 488 (5 µg/ml in PBS, Invitrogen); and Ins samples with goat anti-rabbit IgG-FITC (2 µg/ml in PBS, Santa Cruz). Washing step was repeated followed by counterstaining of nuclei using 4', 6-diamidino-2-phenylindole (DAPI) for 5 min.

3.2.7. Quantification of insulin release

At Day 15, each mESC MCG construct were firstly washed with Krebs Ringer Bicarbonate (KRB) buffer and incubated with 1 mL KRB buffer at 37°C for 90 minutes. The KRB buffer was then replaced with 500 uL KRB buffer containing varying concentrations of glucose (2.5 mM, 12.5 mM and 27.5 mM) and 30 mM KCl as control. Samples were then incubated at 37°C for 60 min and the resulting medium was collected [212]. Quantification of insulin was performed with rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Merck Millipore) as per manufacturer's protocol.

Figure 3-2 Live/Dead (L/D) assay of mESC and miPSC 3D constructs at 10× magnification at each time point prior to cell culture medium change. Live cells were stained green and dead cells red. Scale bar represents 200 μm .

3.2.8. Statistical analysis

Results are presented as mean $\pm$ s.d. All samples were analysed as triplicates ($n=3$). One-way ANOVA was performed to measure statistically significant differences ($p < 0.05$).

3.3. Results and Discussion

3.3.1. Cell viability and morphology

Live/Dead assay was performed to observe the cell viability (Figure 3-2) at the end of each differentiation step. After 10 days in pluripotent stem cell medium, viable EBs were clearly formed and about 100 to 150 μm in diameters, as similar to a previous study [59]. The EBs were generally viable and rounded throughout

Figure 3-3 qPCR analysis of pluripotency markers for mESC and miPSC constructs at the end of each differentiation stage. Fold values for each gene was calculated based on the expression value of the particular gene at Day 0, represented as 1 unit for both cell types. * indicates $p < 0.05$ as compared against Day 0 ($n=3$); nd stands for non-detectable.

the differentiation period and increased in size to about 200 μm in diameter. However, at the end of the differentiation protocol (Day 15), some dead cells were observed. This may be due to the prolonged serum-free culture conditions, as well as the inevitable cell death during the differentiation process.

3.3.2. Gene expression

Quantitative gene expression analysis by qPCR was performed to determine the gene profiling patterns throughout the stepwise differentiation protocol. Pluripotency markers Oct4 and Nanog showed a general decreasing profile after differentiation was induced (Figure 3-3). Particularly, from Day 7 onwards, Nanog and Oct4 were at least 50 times lower or non-detectable for mESC samples. miPSC samples followed the same trend, albeit with a delay where similar fold-values were only observed from Day 10 onwards.

Intermediate gene markers specific for definitive endoderm, pancreatic multipotent progenitor and endocrine progenitor cells are shown in Figure 3-4. Day 5 marks the end of definitive endoderm induction through Activin A pathways [59]. Definitive endoderm marker Sox17 was highly expressed from Day 5 onwards and peaked at Day 7. Although not significantly different on Day 5 for mESC samples, Sox17 was about 20 folds higher on Day 7. As cells committed to a pancreatic lineage, its expression decreased.

In the second stage of stepwise differentiation by Day 7, successful commitment to the pancreatic lineage (pancreatic multipotent progenitor cells) is determined by Pdx1, Nkx2.2, Nkx6.1 and Gata4 expressions. However, whilst the former 3 are continually expressed in endocrine lineage [206], Gata4 is later involved in development towards the exocrine lineage [69]. mESC and miPSC samples registered high expression levels for Pdx1 and Nkx6.1. miPSC samples highly expressed Nkx2.2 and Gata4 as well, but these genes were downregulated in mESC samples. Nkx2.2 would later become upregulated to 3.2 ± 0.17 folds by Day 15, an indication of endocrine lineage commitment. On the other hand, Gata4 has been suggested as a non-essential marker for pancreas organogenesis [67, 68] and therefore should not affect mESCs' differentiation potential into β -like cells. Furthermore, it also should not be expressed in mature endocrine islets [69], which was observed in mESC samples.

Figure 3-4 qPCR analysis of intermediate stepwise markers for mESC and miPSC constructs at the end of each differentiation stage. Fold values for each gene was calculated based on the expression value of the particular gene at Day 0, represented as 1 unit for both cell types. * indicates $p < 0.05$ as compared against Day 0 (n=3).

In the third differentiation step for pancreas endocrine induction by Day 10, specific markers Ngn3, Pax4 and Pax6 are expected to be expressed, while Pdx1, Nkx2.2 and Nkx6.1 are downregulated. Generally, both mESC and miPSC samples adhered to the trend. Particularly, a sharp increase of Pax4 levels to 24.5 ± 1.3 folds was observed in mESCs unlike miPSCs, which registered little increase in Pax4 but an approximate doubling in Pax6 levels.

Gene expression profile of mESCs and miPSC samples continued to indicate differentiation and maturation towards β -cells by Day 15, with the high Pax4 expression levels and recurrence of high Pdx1, Nkx6.1 and Nkx2.2 levels [51]. However, miPSCs were only slightly upregulated for Pax4 as compared to that in mESCs (6 to 8 times lower than the latter), whereas Pax6 expression levels were consistently more than 3 times that of mESCs; these trends suggest an inclination towards α -cells [72].

Finally, hormonal markers of terminally differentiated cells were analyzed (Figure 3-5). Both mESC and miPSC samples highly expressed all markers at Day 15, but gene expression levels of miPSCs were visibly of a lower extent. At D15, Insulin (Ins1 and Ins2) mRNA levels were respectively 27.9 ± 2.6 and 62.9 ± 10.7 folds of control Day 0, while Glucose transporter 2 (Glut2) was also significantly higher than control in mESC samples, indicating an inclination towards mature β -cell phenotype [213, 214]. This was further supported by the maintenance of high Pax4, Nkx6.1 and Pdx1 expression levels representative of mature β -cells [48, 51, 215]. On the other hand, miPSC samples registered

Figure 3-5 qPCR analysis of mature markers for mESC and miPSC constructs at the end of each differentiation stage. Fold values for each gene was calculated based on the expression value of the particular gene at Day 0, represented as 1 unit for both cell types. * indicates $p < 0.05$ as compared against Day 0 (n=3).

8.3±2.3 and 7.1±0.9 folds for Ins1 and Ins2 respectively, a substantial difference from mESC samples. Furthermore, glucagon Gcg (α -cell) and somatostatin Sst (δ -cell) expression levels were increased to a much larger extent for mESC (25.2±4.3 and 1564±60 respectively) compared to 10.6±0.7 and 341±22.7 for miPSC samples.

In conclusion, mESC samples had higher responsiveness to the stepwise culture conditions, as reflected in gene expression trends at each step of the differentiation. Furthermore, by the end of the differentiation protocol, all mature markers were highly expressed, indicating the presence of terminally differentiated cells. miPSC samples, however, was generally lagging behind that

of mESCs, and may require a longer duration for maturation. It is known that exocrine acinar cells (Gata4-overexpressing) can transdifferentiate into β -cells [216], and polyhormonal-expressing precursor cells or α cells can be further matured or transdifferentiated into β -cells [217-219].

3.3.3. Immunohistochemistry

Qualitative examination of various proteins was performed by immunohistochemistry stains (Figure 3-6). In both mESC and miPSC samples, pluripotency marker Nanog was clearly only present at Day 0, while definitive endoderm marker Sox17 was expressed in Day 5. Sox17 was also detected at Day 7 in mESC samples, but was unobservable after that, similar to miPSCs. The presence of PDX1 throughout Days 5 to 10 as well as that of Ins at Day 15 confirmed the successful differentiation of mESCs towards insulin-producing pancreatic cells (Figure 3-6A). On the other hand, both PDX1 and Ins were unobservable in miPSC samples (Figure 3-6B), consistent with gene expression analyses which were lagging behind that of mESCs. Therefore, insulin secretion assays were performed for mESC samples only.

Figure 3-6 Immunohistochemistry stains of (A) mESC and (B) miPSC samples at 10× magnification, for the following: pluripotency marker Nanog, definitive endoderm marker Sox17, pancreatic progenitor PDX1 and β -cell secreting Insulin (Ins). Markers were stained green and nuclei stained blue by DAPI. Scale bar represents 200 μ m.

3.3.4. Glucose-stimulated Insulin production assay

The final functional test of successful differentiation into pancreatic β -like cells is its ability to secrete insulin in a responsive manner when exposed to glucose as part of the homeostatic mechanism. However, as shown in Figure 3-7, mESC samples was not responsive to varying glucose concentrations. High glucose

Figure 3-7 Insulin quantification of mESC constructs after stimulation by varying glucose concentrations. * indicates $p < 0.05$ between samples ($n=3$).

conditions at 27.5 mM glucose-containing buffer resulted in a significant increase in insulin concentration compared to both low (2.5 mM) and medium (12.5 mM), but was similar to that without glucose (30 mM KCl). Moreover, albeit the significant differences, high glucose concentrations only resulted in a marginal 12% increase from low glucose conditions. Therefore, although insulin-secreting pancreatic cells were successfully obtained from mESCs via the stepwise differentiation protocol, they did not function well as glucose-responsive true β -cells, which would require further optimization for a protocol suitable for 3D culture, or a more detailed 7-step differentiation protocol similar to that by Rezania et al. [204].

On the other hand, epigenetic memory of fibroblast-derived miPSCs used in this experiment may have contributed to the depressed stage-specific genetic and

protein patterns unlike mESCs [45]. In future, exploitation of this same property by using pancreatic β -cell derived iPSCs is postulated to provide greater differentiation efficacy and efficiency towards insulin-secreting β -cells [220, 221].

3.4. Conclusion and future work

In this work, a 3D MCG system was used to encapsulate murine pluripotent stem cells and differentiate them into insulin-producing islets after EB formation in a 25-day process. mESCs were successfully differentiated into insulin-producing cells as evaluated from its genetic and protein secretion patterns, but were not responsive towards glucose challenge. On the other hand, miPSCs were not able to secrete insulin at the end of the differentiation procedure, and typically had a lower genetic expression pattern compared to mESCs at each stage of differentiation, which may be due to the epigenetic memory hindering its efficacy in differentiating to endoderm and pancreatic β -cell lineage. More refinement to the differentiation protocol is required to impart responsiveness to glucose challenge, as well as removing the issue of epigenetic memory in iPSCs. With these two issues resolved, unlimited and patient-specific cell sources for a transplantable construct of functional β -cell islets is envisioned.

Chapter 4 Vasculogenesis in a sacrificial microsphere/ hydrogel system

Summary

Successful vasculogenesis, or the differentiation or self-assembly of endothelial progenitors to form a capillary network, may overcome the oxygen diffusion constraints hindering the advent of macroscopic engineered tissue. In this chapter, the one-step MCG strategy was applied but for ADCs: endothelial progenitor outgrowth cells (EPOCs) in a photocrosslinked cell-adhesive MCG reported increased proliferation and subsequent successful angiogenic induction into an interconnected microvascular network surrounding the cavities, as compared against a conventional gel construct without micro-cavities. High gene and protein expressions of vascular and mature vascular markers von Willibrand Factor (vWF) as well as intercellular contacts and tubular structures around the cavities were consistently observed in MCG constructs. This simple setup may be potentially used to vascularize engineered tissues or therapeutic cells delivered in microspheres.

4.1. Introduction

The bottleneck of tissue engineering is the oxygen diffusion constraints of maximum 200 μm [222], which can potentially be resolved by mimicking native tissue: developing a network of microvascular structures throughout the engineered tissue construct to increase diffusion of oxygen and other nutrients to cells embedded within the construct [8]. Vasculogenesis, defined as the differentiation and self-assembly of endothelial progenitors into a network of capillaries [223], has been investigated using angiogenic growth factor immobilization [191, 224] and micropatterning [225, 226]. However, such techniques require skill and laborious optimization.

We postulate that encapsulating vascular-committed cells in a cell-adhesive hydrogel with an interconnected macroporous structure will not only increase the metabolic exchange between cells and the environment, but also form tubular structures surrounding the cavities upon angiogenic induction (Figure 4-1). Although some studies have utilized porous scaffolds, they are usually two-step: fabricating porous scaffold and subsequently seeding of endothelial cells [224, 227, 228]. However, this is time-consuming and successful and complete penetration of cells into the scaffold is not guaranteed.

In this work, we report a simple one-step technique that induced successful vasculogenesis: a micro-cavity gelatin-methacrylate (Gelatin-MA) gel encapsulating endothelial progenitor outgrowth cells (EPOCs), a subtype of endothelial progenitors that is committed to the endothelial lineage [229, 230].

Figure 4-1 Schematic diagram and timeline of experiment. Endothelial progenitor outgrowth cells (EPOCs) and gelatin microspheres were mixed evenly in gelatin-methacrylate (Gelatin-MA) and injected into moulds before gelatin via UV irradiation. The constructs were cultured in mEPOC medium for 7 days to allow proliferation. During this process, temperature-responsive gelatin microspheres dissolve to form cavities in the construct by Day 2 hence a micro-cavity gel (MCG) is established. Constructs were then cultured in angiogenic cell culture medium for 14 days to develop vascular structures.

We used EPOCs to firstly increase the cell numbers within the construct [231, 232], and subsequently induced them towards vascularizing throughout an interconnected macroporous/ MCG construct as established in our previous work [141, 233]; an EPOC-laden gelatin-methacrylate template containing sacrificial gelatin microspheres allowed the development of a microvascular network around the cavities left behind by microspheres within 21 days.

4.2. Methods and Materials

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich, and all cell-culture related reagents from Gibco.

4.2.1. Fabrication and characterization of gelatin-methacrylate (Gelatin-MA)

6 g gelatin type A (porcine skin) was dissolved in 150 mL phosphate-buffered saline (PBS) under stirring at 60°C, followed by the addition of 40 mL N, N-dimethylformamide (DMF). 10 g triethylamine (TEA) and 20 g glycidyl methacrylate (GMA) was added prior to stirring for 5 days at 37°C. Subsequently, the solution was concentrated for 3 days using a rotary evaporator, dialyzed (molecular weight cut-off 14000) against deionized water, and lyophilized to obtain gelatin-MA.

The gelatin-MA was characterized with ^1H NMR spectra (Bruker Avance-300 spectrometer) at room temperature, using deuterium oxide (D_2O) as the solvent. ^1H NMR spectroscopy was employed to determine the degree of methacrylation of gelatin-MA.

4.2.1.1. Fabrication of acellular micro-cavity gel (MCG) and control (Gel) constructs

12% w/v Gelatin-MA and 0.05% w/v Irgacure 2959 dissolved in $1\times$ PBS was sterilized via filtration (0.2 μm) and referred to as precursor solution. 0.3 g sterile gelatin microspheres of 150-180 μm diameters were suspended per 1 mL of precursor solution. Gelatin microspheres were prepared using a double oil/water/oil emulsion and sterilized as previously described [141, 233]. 50 μL of the suspension was carefully pipetted into each cylindrical mould (diameter 5.2 mm) and exposed to ultraviolet (UV) light (365 nm, 30 mW/ cm^2) for 4 min for

gelation. The covalently crosslinked constructs were then transferred to wells containing PBS at 37°C, allowing dissolution of entrapped gelatin microspheres and forming MCG constructs. Fabrication of Gel constructs (control) was performed via the same procedure, except without gelatin microspheres.

4.2.1.2. Characterization of MCG and Gel constructs

Fabricated constructs were immersed in PBS at 37°C. At predetermined time points, samples were gently dabbed to remove extra liquid prior to determining their wet weight (W_w) by gravimetric analysis. They were then freeze-dried to obtain the corresponding dry weight (W_d). The mass-based equilibrium swelling ratio (Q_m) was calculated as follows [234]:

$$Q_m = \frac{W_w}{W_d}$$

The volume-based equilibrium swelling ratio (Q_v) was then determined by the following equation [235]:

$$Q_v = 1 + \frac{\rho_p}{\rho_s}(Q_m - 1)$$

Wherein, ρ_p and ρ_s respectively represents the density of polymer (1.44 g/mL for gelatin) and solvent (1.0 g/mL) [236]. M_c , number-average molecular weight between cross-links, was estimated with a simplified Flory-Rehner equation [237, 238]:

$$M_c = Q_v^{5/3}(0.5 - \chi)^{-1} \frac{V_1}{v}$$

Wherein, v and V_1 respectively denote specific volume of the polymer and molar volume of the solvent (18 mL/mol). χ , the polymer-solvent interaction parameter, is 0.49 for gelatin [236].

The effective crosslink density (v_e) of hydrogel was determined by the following equation [239]:

$$v_e = \frac{\rho_p}{M_c}$$

4.2.2. Fabrication of cell-laden MCG and Gel constructs

4.2.2.1. Cell culture

Murine endothelial progenitor outgrowth cells (EPOCs) were purchased from Biochain Institute Inc. (Hayward, CA, USA) and cultured as per manufacturer's instruction in EPOC growth medium at 37°C, 5% CO₂. Cells of passages 14-17 were used in this work.

4.2.2.2. Fabrication of cell-laden constructs

Fabrication was similar to that of acellular construct, except with the addition of 2×10^6 cells/ mL precursor solution. Briefly, 2×10^6 cells (and 0.3 g sterile gelatin microspheres) were suspended per 1 mL of precursor solution, transferred to moulds and exposed to UV light to obtain MCG (and Gel) constructs.

Constructs were then cultured in agarose-coated wells containing EPOC growth medium for 7 days and then in angiogenic cell culture medium for a further 14 days at 37°C, 5% CO₂. Angiogenic medium comprises: 2 mM MCDB131 with

1.176 g/L sodium bicarbonate and 2% FBS (“Gold”-defined, PAA Laboratories), supplemented with 2 mL/L Bovine Brain Extract (Hammond Cell Tech, Windsor, CA, USA), 50 µg/mL L-ascorbic acid, 1 µg/mL hydrocortisone, and the following murine growth factors: 20 ng/mL basic fibroblast growth factor (b-FGF, ImmunoTools), 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL vascular endothelial growth factor (VEGF, Millipore). The process is depicted in Figure 4-1.

4.2.3. Cell viability assays

Cell viability assays Live/Dead and Prestobblue were performed as per manufacturer’s instruction (Invitrogen). Qualitative Live/Dead fluorescence staining was observed via fluorescence microscopy (Olympus) following incubation of constructs in culture medium containing calcein AM and ethidium homodimer-1 for 30 min. Quantitative Prestobblue cell viability assay was performed by incubating each construct in 1 mL Prestobblue working solution for 1 h and then read under fluorescence at $\lambda_{\text{ex}} = 560 \text{ nm}$ and $\lambda_{\text{em}} = 590 \text{ nm}$ (Tecan Infinite).

4.2.4. Gene expression analysis using quantitative real-time polymerase chain reaction (qPCR)

At each predetermined time point, 3 constructs per sample group were homogenized in 1 mL TRIzol® (Invitrogen) prior to RNA extraction as per manufacturer’s instructions. 500 ng RNA were subsequently reverse transcribed into cDNA as per manufacturer’s instruction (Promega). *qPCR was performed*

with *iQ™ qPCR system* (Bio-Rad). Primer sequences (AIT Biotech Singapore) used are listed in Table 4-1. Relative gene expression values to reference gene β -actin were calculated by comparative ΔC_T method, and subsequently normalized against the respective genes of control, mEPOC cultured in monolayer in mEPOC medium, to obtain gene expression fold-values.

Table 4-1 qPCR primer sequences for murine gene markers used in Chapter 4. Forward (F) and Reverse (R). PS stands for product size.

Gene	Accession number/ Reference	Primer Sequences (both 5'-3')	PS/ bp
VE-cadherin	AK167620 .1	F: CTTCAACGCTACCAAGCAAAC R: GATGAGTTACAGCCTCCCTTTAG	109
CD31	AK037551 .1	F: CACCCATCACTTACCACCTTATG R: TGTCTCTGGTGGGCTTATCT	102
vWF	AK044921 .1	F: GAGAATGAGCCTGTCTGTGTATC R: TAGGGCATGGAGATGCTTTG	99
B-actin	[59]	F: TCATGAAGTGTGACGTTGACATCCG T R: CCTAGAAGCATTTGCGGTGCACGAT G	285

4.2.5. Histology and immunohistochemistry

At each time point, samples were fixed in 4% w/v paraformaldehyde in PBS overnight before embedding in Tissue Freezing Medium (Leica) for cryosectioning. 8 μ m-thick slices were mounted on glass slides. Hematoxylin and eosin (H&E) stain was performed as per manufacturer's instruction (Sigma-Aldrich).

For immunohistochemistry, sections were stained for vascular endothelial (VE)-cadherin and von Willibrand Factor (vWF) respectively using rabbit polyclonal IgGs VE-cadherin and vWF antibodies (Santa Cruz Biotechnology) at a concentration of 2 $\mu\text{g}/\text{mL}$ in PBS overnight at 4°C. After washing 3 times in PBS, specimens were incubated with goat anti-rabbit IgG AlexaFluor 488 (5 $\mu\text{g}/\text{mL}$ in PBS, Invitrogen) for 1 h in the dark at room temperature. Washing step was repeated followed by counterstaining of nuclei using 4', 6-diamidino-2-phenylindole (DAPI) for 5 min.

4.2.6. Statistical analysis

Results are presented as mean $\pm$ SD. All samples were analysed as triplicates (n=3). One-way ANOVA was performed to measure statistically significant differences ($p < 0.05$).

4.3. Results

4.3.1. Gelatin-MA synthesis and characterization

The methacrylation of gelatin was confirmed by ^1H NMR spectroscopy (Figure 4-2B): the peaks at 5.65 and 6.06 ppm (b) belong to the methylene protons, which were not observed in original gelatin. This result demonstrated the successful grafting of photocrosslinkable methacrylate groups onto gelatin to achieve gelatin-MA. Degree of methacrylation was estimated to be 45.1% based on comparing the integral intensity of methylene protons (δ 5.65 and 6.06 ppm, b) to that of the aromatic groups (δ 7.23, a) [119].

Figure 4-2 Fabrication of gelatin-MA. (A) Reaction pathways for methacrylation of gelatin with GMA to obtain photocrosslinkable gelatin-MA; and (B) ¹H NMR spectroscopy of gelatin and gelatin-MA.

Figure 4-3 presents the physical properties of acellular constructs. M_c of MCG $(5.93 \pm 0.42) \times 10^5$ g/ mol was about one-fold higher than Gel $(3.07 \pm 0.06) \times 10^5$ g/ mol; the average crosslink density of MCG was half that of Gel at 2.43 ± 0.17 vs 4.68 ± 0.1 mol/ m³. Q_v of MCG was significantly higher than Gel, at 26.2 ± 1.1 vs 17.6 ± 0.2 .

Figure 4-3 Characterization of physical properties, number-average molecular weight between cross-links (Mc) and average crosslink density, of acellular Gel and MCG samples. *represents $p < 0.05$ between groups ($n = 3$).

4.3.2. Cell morphology and viability

Cells were viable and proliferative as observed through both Prestoblue and Live/Dead assays (Figure 4-4). In the quantitative Prestoblue assay (Figure 4-4A), no statistically significant differences between samples (Gel and MCG) at similar time points were observed, but there was a statistically significant increasing trend i.e. increased number of viable cells as the experiment progressed (Days 2 to 14) for both groups, with a doubling in values from Day 7 to 14. The values stagnated after Day 14, but was not statistically significant.

Figure 4-4 Assessment of cell viability in Gel and MCG samples. (A) Prestoblu, *represents $p < 0.05$ between different time points for both Gel and MCG samples ($n=3$); and (B) Live/Dead assay at $10\times$ magnification. Live cells were stained green and dead cells red. Scale bar represents $200\text{ }\mu\text{m}$.

Live/Dead assays consistently showed a highly viable cell population (Figure 4-4B); few dead cells were observed in both Gel and MCG constructs. Especially from Day 14 onwards, cell densities were much higher in MCG as compared to Gel constructs; cells were also observed to be elongated and interconnected in the former, whilst those in Gel remained sparse. Lastly, macroporous structures of MCG constructs were evidently definable by the lack of cell growth in specific areas.

4.3.3. Gene expression

Expression of vascular genetic markers CD31, VE-cadherin and vWF were evaluated quantitatively via qPCR and groups were compared against each other at similar time points. Generally, higher expressions of all three markers were detected in MCG constructs throughout the experiment (Figure 4-5).

Figure 4-5 Gene expression analysis of vascular markers through qPCR. Fold values were normalized against mEPOC cultured in monolayer with mEPOC medium. *represents $p < 0.05$ between groups ($n = 3$) at the same time point.

VE-cadherin fold-expression values for MCG were consistently about 2 times higher than Gel constructs, peaking at Day 14 (62.7 ± 14.8 vs 28.3 ± 3.8 folds) and remaining at similar levels at Day 21. CD31 fold-expression values were consistently higher for MCG although not statistically significant at Days 14 and 21. Lastly, vWF was highly expressed in MCG constructs after angiogenic induction; fold-expressions of MCG constructs were approximately 2 and 4 times higher than Gel constructs at Days 14 and 21 respectively.

Figure 4-6 Various histology (H & E) and immunohistochemistry stains of vascular markers VE-cadherin and vWF for Gel and MCG samples. In immunohistochemistry stains, markers were stained green and nuclei counterstained blue (DAPI). Scale bar represents 200 μm .

4.3.4. Histology & Immunohistochemistry

Figure 4-6 shows general histology (H&E) and immunofluorescence stains for VE-cadherin and vWF. H&E stains clearly displayed the macroporous structure of MCG left behind by sacrificial gelatin microspheres, which were absent from Gel constructs. Cell nuclei were stained purple (haematoxylin) and proteins stained pink (eosin). From Day 14 onwards, MCG specimens were observed to have much higher cell numbers.

VE-cadherin and vWF were highly stained (green) in MCG specimens from Day 14 onwards, indicating the development of interconnected endothelial cells and vascular structures. On the other hand, both proteins were negligible or sparse in Gel specimens, except on the edges. DAPI (blue) staining the cell nuclei also indicated much greater cell densities in MCG specimens.

4.4. Discussion

Gelatin was chosen as the gel setup as it provides cell-adhesive moieties for ADCs to attach, spread, proliferate and differentiate [119, 240]. However, the dense polymer network of gelatin gel constrains cell proliferation and metabolic exchange. In this study, the MCG was designed and fabricated with photocrosslinkable gelatin-MA precursor and sacrificial gelatin microspheres; the latter provides cavities within the gelatin-MA construct to overcome the mentioned limitations. As gelatin microspheres are temperature-sensitive and lose their structural integrity upon exposure to heat, a quick and simple gelling mechanism was required and photocrosslinking was chosen.

Firstly, methacrylate groups were grafted to confer photocrosslinkable properties to gelatin. The synthesis of gelatin-MA mainly relied on the reaction between GMC and amino groups of gelatin (Figure 4-2A). TEA served as the catalyst while the presence of DMF increased the solubility of GMA as compared to PBS [241]. Similar to other reports, both ring-opening and nucleophilic acyl substitution reactions of GMA through amino groups should exist simultaneously [241, 242]. NMR spectroscopy confirmed the successful grafting of methacrylate groups to gelatin.

Acellular MCG setup was then characterized for crosslink density, M_c and swelling density, which indicated better permeability for nutrient transport compared to Gel (control). During the establishment of MCG, dissolution and diffusion of sacrificial gelatin microspheres into medium at 37°C left behind

Supplementary Figure 4-1 Microscopy images of MCG constructs at Day 14, 20× magnification (A) Bright field microscopy; and (B) H&E. Scale bar represents 100 μm .

micro-cavities of corresponding sizes within the construct [233]. Due to the presence of micro-cavities, the MCG exhibited different physical properties (swelling ratio Q_v , M_c , crosslink density) compared to Gel [243].

After validating the physical properties, EPOCs were then encapsulated in gelatin-MA to study the vascularization in MCG system for 21 days (Figure 4-1). The viability, gene expression and histology analyses showed that the MCG structure provided a favourable environment for vasculogenesis. H&E stains validated the dissolution and removal of gelatin microspheres in MCG constructs from Day 2 onwards. As the experiment progressed, a much greater number of cells (nuclei stained purple) were found surrounding the cavities. At a higher magnification, the intercellular contact and tubular structures were clearly visible at the cavities' boundary (see Supplementary Figure 4-1). Without the macroporous structure, cells in Gel construct were sparsely distributed, therefore there are fewer or unobservable intercellular contacts.

Furthermore, the greater abundance of intercellular contacts observed were supported by results from qPCR and immunohistochemistry stains. VE-cadherin is crucial for intercellular contact between endothelial cells and formation of vascular structures [244] and vWF is a marker for blood vessel identification [245]; both gene markers and proteins were highly expressed in MCG constructs, especially after angiogenic induction. Through the immunohistochemistry results, it can be concluded that formation of microvascular structures, or vasculogenesis, was successful in a MCG system.

Generally, it was consistently observed that cells proliferate at gel boundaries at a much higher rate compared to within the gel. Particularly in Figure 4-6, VE-cadherin and vWF stains of Gel constructs' edges indicated great numbers of interconnected endothelial cells (numerous nuclei stained by DAPI and the presence of vascular markers stained fluorescent green), in contrast to the sparse cells in the interior. This may be due to the differential outward pressure exerted from the gel to its outer environment (cell culture medium), a phenomenon reported in several studies [141, 233]. Also, it may be the ease of nutrient and waste exchange for cells at the gel boundaries, as oxygen diffusion constraints are a bottleneck in macroscopic engineered tissues [8, 246]. The MCG system increases the surface area of such boundaries through sacrificial gelatin microspheres, providing higher diffusion and therefore supporting the high cell numbers around the cavities and constructs' edges. It is postulated that quantitative Prestoblue cell viability assays were unable to detect significant differences between Gel and MCG constructs due to this phenomenon – Gel

Supplementary Figure 4-2 Live/Dead images of interior and surface of MCG and Gel samples at Day 14, 10× magnification. Scale bar represents 200 μm .

constructs also possess gel boundaries on the outer surface where proliferation of EPOCs also take place (Supplementary Figure 4-2). MCG constructs additionally have inner surfaces (cavities), therefore although not statistically significant, their absorbance values were still generally higher than Gel constructs'. However, as blood vessels form and mature from Day 14 to 21 and thereby increasing the intercellular contacts and specifically its associated molecule VE-cadherin are known to inhibit cell proliferation [247], a slight decrease in Prestobluo absorbance values was therefore observed.

4.5. Conclusion

In this work, successful vasculogenesis in a macroscopic cell-adhesive gelatin hydrogel was achieved through incorporating sacrificial microspheres throughout a cell-adhesive hydrogel bulk. As compared to conventional gel without micro-cavities, endothelial progenitors proliferated greatly along the gel boundaries (cavities) within the gel bulk and upon angiogenic induction,

developed microvascular structures. Such a setup is promising for engineering vascularized tissues: therapeutic cells such as pancreatic islets may be encapsulated in the sacrificial microspheres and further encapsulated in the EPOC-laden gelatin-MA hydrogel; a microvascular network enveloping and providing nutrients to the therapeutic cells may thus be created in a simple, one-step process.

Chapter 5 Development of hepatocarcinoma spheroids as a drug testing platform using a sacrificial gelatin microsphere system

Summary

Sacrificial gelatin microspheres can be developed as a cell delivery vehicle for non-ADCs – its incorporation into a macroscopic scaffold system not only allow the cells to be cultured in suspension within cavities left behind by the sacrificial material, it also allows tissue development to be confined within the cavities. The previous two chapters utilized acellular gelatin microspheres, while in this chapter, the feasibility of developing sacrificial cell-laden gelatin microspheres in a simple and quick manner is studied. Hepatocarcinoma cells HepG2 were used to optimize conditions and the fabricated cell carriers, named temperature-cured dissolvable gelatin microsphere based cell carriers (tDGMC), were then evaluated and characterized for tumour spheroid formation. This strategy was successful in developing dense and viable material-free spheroids of controlled sizes, by means of a suspension culture within the cavities in the hydrogel bulk. Therefore, by initial selection of microsphere (tDGMC) size and distribution, spheroid size can be controlled for various applications, e.g. uniform tumour spheroids as a reproducible drug screening and testing platform.

5.1. Introduction

Cancer has become the leading cause of death globally, and its incidence is set to rise as the population grows and ages [248]; 14.1 million new cases were diagnosed in 2012 according to GLOBOCAN [249]. Anticancer drug therapy is a widely studied field which relies heavily on monolayer cell culture and mouse models with human tumour xenografts as preclinical drug screening and testing platforms [250, 251]. However, monolayer conditions are not representative of the nature of tumours *in vivo*, which are 3D in structure [252] and mouse models have not been accurate models of human cancer cell responses [253]. Furthermore, control and regular inspection of tumour development cannot be performed in mouse models. A 3D *in vitro* tumour model that mimics the *in vivo* nature of tumours is therefore imperative to provide a more accurate prediction, thereby reducing animal testing as well as the risk of failure at clinical trial phases [254, 255].

Self-assembly of 3D multicellular tumour spheroids (MCTS) can be achieved by culturing cells in a non-cell adhesive environment e.g. suspension cultures, round bottomed or coated plates [255, 256] and moulds [257]. However, they may either require specialized and expensive equipment, expertise or intensive labour. 3D polymeric matrices may be used as a convenient scaffolding system, on which cells are seeded onto after fabrication [258, 259] or encapsulated within [260], but the presence of material may affect the recapitulation of *in vivo* phenomena. Furthermore, to our knowledge, regulation of MCTS size has not been reported,

although size control may provide a reproducible and convenient method of drug screening and testing.

To overcome these limitations, we propose the use of sacrificial gelatin microsphere as a cell delivery vehicle for formation of size-controlled tumour spheroids in a hydrogel bulk. Temperature-responsive gelatin dissolute quickly at physiological temperature and can therefore be used as a sacrificial material [115, 116] when encapsulated into another hydrogel bulk. In this study, a temperature-cured dissolvable gelatin microsphere based cell carrier (tDGMC) was developed as a cell delivery vehicle for hepatocarcinoma cells through a simple and scalable emulsion technique. As gelatin dissolute, similarly-sized cavities are formed and the delivered cells are developed into material-free spheroids akin to suspension culture, but with an additional advantage of size regulation – spheroid size is limited to the cavity size by the surrounding hydrogel. tDGMC can thus serve as a versatile platform for size-customized spheroid formation of non- ADCs for various applications such as tissue engineering and drug testing.

5.2. Materials and Methods

All cell culture related reagents were purchased from Invitrogen, and all chemical reagents from Sigma-Aldrich unless otherwise stated.

5.2.1. Cell culture

Human hepatocarcinoma cell line HepG2 were purchased from American Type Culture Collection (ATCC) and cultured in monolayer with Dulbecco's Modified

Eagle's Medium (DMEM) containing 10% Foetal Bovine Serum (FBS, 'Gold' Standard, PAA Laboratories) at 37°C, 5% CO₂. Cells were harvested by trypsin for encapsulation when 80% confluence is reached.

5.2.2. Fabrication of tDGMC

Five percent w/v gelatin type A was prepared by dissolving 0.50 g gelatin in 5 mL 1× PBS and 5 mL cell culture medium through boiling. The gelatin solution and filtered soya oil were maintained in a 37°C water bath prior to use.

1×10^7 HepG2 cells were suspended in 1 mL of 37°C gelatin solution and added into a 50 mL beaker containing 15 mL 37°C soya oil. A water-in-oil emulsion was formed by stirring for 2 min at 350 revolutions per min (rpm) at room

Figure 5-1 Schematic diagram of experiment. (A) tDGMC fabrication using a water-in-oil emulsion technique. A HepG2-gelatin suspension is added to a beaker of soya oil at 37°C and stirred to form an emulsion. Gelatin undergoes a sol-gel transition upon lowering the temperature using an iced water bath. The tDGMC-oil emulsion is then centrifuged and washed with 1× PBS twice. (B) Fabrication and developmental process of HepG2-tDGMC constructs. 0.3 g of tDGMC is added to 1 mL alginate solution and injected into moulds prior to crosslinking of alginate using Ca²⁺ ions. Upon culture at 37°C, gelatin in tDGMC dissolve and diffuse out of the construct to form cavities with HepG2 cells suspended within. HepG2 grows as per suspension culture into spheroids which can be retrieved via alginate removal (sodium citrate treatment).

temperature. The beaker was then transferred to an iced water bath and stirred for 10 min at 300 rpm for cooling and gelation of gelatin to take place. The emulsion was then transferred to a 50 mL centrifuge tube and centrifuged at 700 rpm for 3 min. After removing the supernatant, the pellet of gelatin microspheres with encapsulated cells, named HepG2-tDGMC, was resuspended in 15 mL 4°C 1× PBS for washing. The suspension was centrifuged at 700 rpm for 3 min and washed again. A brief schematic of the procedures, which were done under sterile conditions, is shown in Figure 5-1A.

The procedure was repeated for two other stirring speeds for optimization. The settings are: 500 rpm for 2 min at room temperature, and 350 rpm for 10 min in an iced water bath for the first setting; and 650 rpm for 2 min at room temperature and 350 rpm for 10 min in an iced water bath for the second setting.

For quantification of size distribution, microspheres were suspended in a suitable amount of 1× PBS in a 10 mm petri dish. 40 random images were taken under light microscopy (Carl-Zeiss) for subsequent diameter measurements and size distribution analysis using ImageJ software. At least 300 microspheres were analysed for each setting.

500 rpm was selected as the initial stirring speed for subsequent experiments.

5.2.3. Fabrication of 3D constructs

HepG2-tDGMC were firstly sieved using a 40 µm cell strainer (BD Falcon) and mixed in 1.5% alginate solution (dissolved in 0.15 M NaCl, autoclaved and kept

at 4°C) at 0.3 g tDGMC per 1 mL alginate. For gelation, 80 µL suspension was pipetted into each cylindrical silicon mould laid on a gelatin substrate comprising 15% gelatin and 102 mM CaCl₂ dissolved in distilled water. The setup was then placed in 4°C refrigerator for 4 min. Subsequently, 5 µL 102 mM CaCl₂ was carefully added onto the surface of the suspension, and the setup was returned to 4°C for 4 min for gelation. HepG2-tDGMC constructs were retrieved and cultured in agarose-coated wells containing cell culture medium at 37°C, 5% CO₂. Upon elevation of temperature to 37°C, dissolution of gelatin occurred to leave behind suspended cells in the cavities for further development. The process is depicted in Figure 5-1B.

For comparison, a similar number of HepG2 cells was encapsulated in a conventional hydrogel bulk setup and named HepG2-alginate, assuming 30% w/v tDGMC in 1 mL alginate translated to 3×10^6 cells in 1 mL alginate. Therefore, 3×10^6 cells were mixed with 1 mL alginate solution and gelled as per above.

5.2.3.1. Removal of alginate

Sodium citrate (SC) solution comprising 55 mM sodium citrate in 0.15 M NaCl was used to remove alginate [114]. Briefly, each construct was immersed in 3 mL 55 mM SC solution in a centrifuge tube for 10 min prior to centrifugation at 700 rpm for 3 min. The pellet was gently resuspended in PBS.

5.2.4. Cell viability assays

Live/Dead cell viability assay was performed as per manufacturer's instruction (Invitrogen). Briefly, each construct was incubated in respective cell culture/differentiation medium with the addition of calcein AM and ethidium homodimer-1 for 30 min, prior to observation under fluorescence microscopy.

WST-1 assay (Roche, Switzerland) was used to quantify cell viability. Each sample was incubated in 10% v/v WST-1 reagent in cell culture medium for 1.5 h in the dark at 37°C, 5% CO₂. The conditioned medium was then transferred to 96-well plate (Iwaki) and absorbance was measured at 450 nm against 690 nm reference absorbance by a microplate spectrum reader (Multiskan[®] Spectrum, Thermo).

5.2.5. Drug testing

At Day 10, constructs were treated with Doxorubicin (Dox)-supplemented cell culture medium at the following concentrations: 0, 5, 10, 25, 50 and 100 µM. After 24 h, cytotoxic effect of Dox was assessed using WST-1 assay. Briefly, each construct was immersed in 375 µL 10% v/v WST-1 reagent in cell culture medium for 1 h in the dark at 37°C, 5% CO₂ prior to measurement as before (Section 5.2.4).

5.2.6. Quantification of DNA

Samples were collected and frozen at -20°C and lyophilized for 12 h. The dry weight was measured prior to digestion procedure: alginate was removed by

firstly immersing samples in SC solution for 10 min and then centrifuged at 14000 rpm for 10 min. Each cell pellet was then digested overnight in 500 μ L papain solution (0.3 mg/ mL in 0.1 mM disodium ethylene diamine tetra acetic acid (EDTA) and 0.2 mM dithiothreitol (DTT)).

DNA content was quantified using Hoechst 33258 fluorescent assay. Briefly, 200 μ L 2 μ g/ mL Hoechst 33258 dye was mixed with 5 μ L sample (with appropriate dilutions in distilled water) in each well of a 96-well IWAKI microplate and fluorescence was measured at $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 465$ nm (Tecan Infinite).

5.2.7. Statistical analysis

Results are presented as mean $\pm$ s.d. All samples were analysed as triplicates (n=3). One-way ANOVA was performed to measure statistically significant differences ($p < 0.05$).

5.3. Results and Discussion

5.3.1. Size distribution

The size distribution of the different stirring speeds are presented as percentage values of total microsphere count in Figure 5-2. As initial speed increased, the range in size narrowed: microspheres from 25 to 300 μ m diameters were obtained from 350 rpm; 0 to 200 μ m from 500 rpm; and 0 to 150 μ m from 650 rpm. Furthermore, the mean diameters of cell-laden microspheres (tDGMC) decreased with higher stirring speed as shown in Figure 5-2D. These observations were similar to other reports in emulsion-derived microparticles [157-159, 261].

Figure 5-2 Size distribution of microspheres obtained from various initial stirring speeds, as a percentage of total number of microspheres. (A) 350 rpm; (B) 500 rpm; (C) 650 rpm; and (D) comparison of size distribution of cell-laden microspheres (tDGMc) obtained with the various stirring speeds. Diameters of at least 300 microspheres per setting were analysed by ImageJ software.

It was also observed from Figure 5-2A-C that higher initial speed resulted in more empty microspheres (light grey, without cells): approximately 12%, 32% and 41% of total microspheres did not contain cells at respective stirring speeds of 350, 500 and 650 rpm. This may be attributed to probability; assuming an evenly distributed cell-gelatin suspension, as diameter and hence volume of gelatin droplet increases, the probability of cells being present in the droplet increases.

Supplementary Figure 5-1 Bright field microscopy images of microspheres obtained from different initial stirring speeds, at 10 $\times$ magnification. Clumping and dissolution are indicated with yellow and red arrows respectively. Scale bar represents 200 μ m.

For subsequent experiments, 500 rpm was chosen as the initial stirring speed for the following reasons: it produced a narrower size distribution than 350 rpm and higher cell loading efficiency than 650 rpm conditions; also, microspheres derived from 650 rpm condition tended to clump and quickly dissolve, making it difficult to handle in subsequent encapsulation processes (Supplementary Figure 5-1).

5.3.2. Cell viability and morphology

Cell viability of HepG2-tDGMC encapsulated in alginate was assessed by qualitative Live/Dead staining and quantitative WST-1 assay over the course of 14 days (Figure 5-3). HepG2 cells were observed to be highly viable after encapsulation in gelatin microspheres and proliferated into dense and viable spheroids with tight boundaries in 10 days, as shown by the strong green fluorescence signals (live cells) and absence of red fluorescence which would have indicated the presence of dead cells (Figure 5-3A). Bright-field images

Figure 5-3 Cell viability assays of HepG2-tDGMC constructs over 14 days. (A) Live/Dead and corresponding bright-field microscopy images at 4 $\times$ magnification, scale bar represents 200 μ m; and (B) WST-1 quantitative assay. *indicates $p < 0.05$; differences from the previous time point are significant.

showed progressive opacity of the spheroids from Days 3 to 10, and most spheroids were approximately 85 to 140 μ m in diameters (data not shown). However, mass cell death was observed at Day 14, which may be related to density dependent apoptosis [262] as surrounding alginate matrix imposes a physical limitation on further spheroid growth [263, 264]. Alginate, a polysaccharide hydrogel with no cell-adhesion moieties unlike natural ECM, does not allow tumour migration into its hydrogel bulk [265, 266], thereby confining HepG2 growth to the cavities left behind by gelatin. WST-1 results in Figure 5-3B supported these results – there was rapid proliferation from Day 3

Figure 5-4 Investigation of spheroid-forming ability of conventional HepG2-alginate constructs over 10 days. (A) Live/Dead and corresponding bright-field microscopy images at 4 $\times$ magnification, scale bar represents 200 μ m; and (B) DNA quantification by Hoechst 33258 assay, normalized against dry weight of construct. *indicates $p < 0.05$; differences between groups at the same time point are significant.

onwards and plateauing was observed from Day 10 to 14. These results indicate the encapsulation technique did not affect the cell viability and proliferation into dense spheroids, but culture duration should be optimized in order to avoid cell death as observed in Day 14. Therefore, in subsequent experimental procedures, HepG2-tDGMC constructs at Day 10 were used.

A comparison with conventional cell-laden hydrogel, i.e. HepG2 cells encapsulated in alginate bulk, was performed. Similar cell numbers as that of HepG2-tDGMC constructs were used. As shown in Figure 5-4A, cells in HepG2-alginate constructs failed to form spheroids, in contrast to cells in tDGMC (Figure 5-3A). Small and irregularly shaped HepG2 aggregates of approximately 40 μ m diameters were formed by Day 7, but further culture led to mass cell death on Day 10. This similar trend may also be attributed to alginate hydrogel matrix

Figure 5-5 Characterization of material-free HepG2 spheroids obtained after alginate removal at Day 10. (A) Representative bright-field microscopy image of material-free HepG2 spheroids at 10× magnification, scale bar represents 200 μm; and (B) size distribution of spheroids (grey bars) and HepG2-tDGMC (blue line), presented as percentage values of total spheroid and cell-laden HepG2-tDGMC microspheres.

imposing a physical constraint on its proliferation. A comparison of DNA content per dry weight of construct in Figure 5-4B indicated a general increase in cell numbers but at a rate lower than HepG2-tDGMC constructs, especially evident in Day 10 where HepG2-tDGMC constructs had significantly higher cell densities than HepG2-alginate constructs.

5.3.3. Establishment of material-free constructs

At Day 10, HepG2-tDGMC constructs have reached a cell-dense yet viable state as noted in Figure 5-3. Alginate was removed via immersion in SC solution – citrate ions act as a chelating agent for divalent Ca^{2+} ions and therefore dissolves the alginate hydrogel. In Figure 5-5A, HepG2 spheroids mostly maintained their structural integrity except for a small amount of dissociated cells. Analysis of the material-free spheroids' size distribution (grey bar graph) showed a correlation with that of original HepG2-tDGMC (immediately after fabrication

and prior to encapsulation, blue line), as shown in Figure 5-5B. This suggests that the initial diameter of HepG2-tDGMC and the eventual cavity size imposes a size constraint on the resulting HepG2 spheroid. Therefore, spheroid size can be customized by selecting the initial size of tDGMC to be encapsulated in alginate hydrogel.

tDGMC is a cell delivery vehicle that ultimately creates a cavity in which delivered cells are suspended within. This platform provided HepG2 cells to proliferate similar to suspension culture, but provided a size constraint requirement using the surrounding non-cell adhesive alginate hydrogel bulk. The tDGMC platform can be therefore used to customize and control HepG2 spheroid size in a reproducible and convenient manner.

5.3.4. HepG2-tDGMC construct as drug testing model

Drug testing and screening is conventionally performed on monolayer cultures which are not representative of the *in vivo* 3D nature of tumours. To better mimic the *in vivo* environment and therefore provide more reliable projections on drug efficacy, a 3D drug testing model should be used.

Doxorubicin (Dox) is a chemotherapy drug used against a wide range of cancers [267-269], including hepatocarcinomas [270, 271]. It acts as a DNA intercalating agent – by insertion of Dox molecules between base pairs, the DNA secondary structure is disrupted and DNA replication is hindered, resulting in inhibition of cell proliferation or cell death [272, 273]. Therefore, Dox was used to test the responsiveness of hepatocarcinoma cell line HepG2 in a 3D HepG2-tDGMC

Figure 5-6 Cytotoxic effect of chemotherapy drug Doxorubicin (Dox) on HepG2-tDGMC constructs at Day 10, as measured by WST-1 assay and normalized against quantity of DNA in each construct. *indicates $p < 0.05$; differences between sample and untreated control (0 μM) are significant.

platform at Day 10, where dense spheroids have been developed. Figure 5-6 showed a significant cytotoxic effect after 24 h treatment with 50 μM Dox, and a further decrease in viability or proliferation when treated with 100 μM Dox.

5.4. Conclusion

In this work, sacrificial gelatin microspheres were utilized as a cell delivery vehicle into a non-cell adhesive hydrogel bulk, and shown to be successful in developing similarly-sized dense and viable material-free spheroids, by means of a suspension culture within the cavities in the hydrogel bulk. Therefore, by initial selection of microsphere (tDGMC) size and distribution, spheroid size can be controlled for various applications, e.g. uniform tumour spheroids as a reproducible drug screening and testing platform. This is a simple and convenient platform that does away with laborious or expensive conventional spheroid

culture in round bottom microwells and moulds. Furthermore, tDGMC platform can be further used as complex study models of tumour migration and coculture systems by using an ECM hydrogel (for example, collagen) and incorporating other cell types in them.

Chapter 6 Development of a sacrificial gelatin microsphere-based chondrocyte cell carrier for cartilage tissue engineering

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Summary

In this study, tDGM was developed and investigated in therapeutic tissue engineering. As a cell for cartilage tissue engineering, chondrocytes were successfully encapsulated in gelatin-based microspheres (~75-100 micron diameter) with maintenance of cell viability. These cell-laden microspheres were then encapsulated in alginate based hydrogel constructs. By elevating the temperature to 37°C, the chondrocytes were released from the microspheres and suspended inside the cavities to be cultivated for further development. In this cell delivery system, the microspheres played a dual role as both removable cell vehicles and porogens for creation of the intra-hydrogel cavities in which the delivered cells were provided with both free living spaces and better permeable environment. This temperature-cured dissolvable gelatin microsphere based cell carrier (tDGM) associating with cell-laden hydrogel scaffold was attempted and evaluated through WST-1, quantitative PCR, biochemical assays and various immunohistochemistry and histology stains. The results indicate that tDGM technology can facilitate the delivery of chondrocytes, as a therapeutic non-ADC, with significantly higher efficiency.

6.1. Introduction

Microspheres have been used widely as delivery vehicles for drugs and cells as they provide a minimally invasive means of transplantation [274-279]. In particular, many materials and fabrication methods have been explored in delivery of cells for regenerative medicine purposes due to their advantages: the simplicity of large-scale culture of cells in microspheres of controlled sizes, the provision of a tuneable three-dimensional (3D) environment for cells, the ability to incorporate biochemical signals and biomechanical moieties as well as the simplicity of direct injection of cell-loaded microspheres into defect site without trypsinization [278, 280, 281]. Studies were usually done through a two-step method of firstly fabricating microspheres, for example through single or double emulsion method [282-284], electrospraying [285] and thermally induced phase separation [286] and subsequently seeding cells onto them. Although the abovementioned methods were able to support cells, the microsphere fabrication techniques usually require specialized equipment, for example freeze dryer; or a significant amount of time as thorough washing steps were necessary after chemical-based treatment. Furthermore, these techniques were largely catered for ADCs such as fibroblasts [287]. Several other groups reported techniques of direct cell encapsulation into microspheres using either synthetic polyethylene glycol diacrylate (PEGDA) [285, 288] – which require surface modification and addition of enzyme-degradation sites – or naturally occurring alginate-gelatin [289], which possess batch-to-batch variation as well as uncontrollable degradation rates [290].

In this study, we aimed to overcome the abovementioned pitfalls of current tissue engineering technologies through using tDGM platform as previously mentioned: a simple and non-toxic water-in-emulsion technique involving cell-gelatin suspension and soya oil was developed for manufacturing gelatin type A microspheres with therapeutic non-ADCs – in this case, chondrocytes. Gelatin type A microspheres would have then dissolved given a time window of several hours (less than one day) at 37°C incubation.

tDGM is a versatile platform for the delivery of therapeutic non-ADCs such as hepatocytes, pancreatic β -cells, chondrocytes and pluripotent stem cells, all of which are naturally cell aggregate-forming [291-296]. As chondrocytes were used in this experiment, tDGM were utilized by combining with our previously established PTCC technology [150] to ultimately construct a 3D biomaterial-free LhCG [141]. Through implementation of tDGM technology upon the PTCC system, we aimed to accelerate the LhCG formation process, in which tDGM plays dual roles: creating cavities for better nutrient and waste diffusion and space for cell growth as in the PTCC system, whilst also delivering additional cells into the gel so as to accelerate development.

6.2. Materials and Methods

6.2.1. Fabrication and size characterization of tDGM

Five percent w/v gelatin was prepared by dissolving 0.50 g gelatin (gelatin type A from porcine skin, Sigma) in 5 mL 1× PBS and 5 mL chondrocyte medium (CC medium). The composition of CC medium was as follows: DMEM with 20%

v/v FBS, 0.4 mM proline, 0.1 mM nonessential amino acids, 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 0.05 mg/ mL Vitamin C, 100 mg/ mL streptomycin and 100 units/ mL penicillin. 1×10^7 of passage 1 porcine chondrocytes were suspended in 1 mL of 37°C 5% w/v gelatin solution. The suspension was added into a 50 mL beaker containing 15 mL soya oil (filtered and pre-warmed to 37°C) and stirred for 2 min at 500 rpm at room temperature. The beaker was then transferred to an iced water bath and stirred for 10 min at 500 rpm. The emulsion was centrifuged at 700 rpm for 3 min. After removing the supernatant, the pellet of gelatin microspheres with chondrocytes encapsulated within, named tDGMC, was resuspended in 15 mL 4°C 1× PBS for washing. The suspension was centrifuged at 700 rpm for 3 min and washed again. A brief schematic of the procedures, which were done under sterile conditions, is shown in Figure 6-1A.

For quantification of size distribution, tDGMC were suspended in a suitable amount of 1× PBS in a 10 mm petri dish. 20 random images were taken under light microscopy (Carl-Zeiss) for determination of size distribution.

6.2.2. Fabrication of 3D constructs

6.2.2.1. Fabrication of PTCC-tDGMC constructs

PTCC-tDGMC constructs were fabricated by mixing both 1×10^7 of passage 1 porcine chondrocytes and 0.30 g tDGMC in 1 mL alginate solution (1.5% w/v alginic acid in 0.15 M NaCl) as shown in Figure 6-1B. 80 μ L of suspension was

injected into each cylindrical silicon mold cavity, which molds were placed on a 100 mm petri dish pre-coated with CaCl_2 -containing gelatin substrate (15% w/v gelatin and 102 mM CaCl_2 in distilled water). Each mold cavity was of 7 mm

Figure 6-1 Schematic diagram of fabrication processes of tDGMC and constructs. (A) Fabrication process of tDGMC similar to Figure 5-1A; (B) PTCC-tDGMC and LhCG-tDGMC fabrication process. A suspension of chondrocytes in alginate is added to tDGMC (0.30 g/ mL alginate). The well-mixed suspension is then transferred to a silicon mould and gelation of alginate is completed through the addition of calcium chloride solution to form PTCC-tDGMC [150]. Upon incubation at 37°C, gelatin dissolves and cavities are formed. Cells suspended within the cavities proliferate into cell islets while cells from alginate gel bulk infiltrate cavities. Neotissue consisting of chondrocytes and their secreted extracellular matrix (ECM) fills up the pores and merge together. LhCG-tDGMC is obtained by removal of alginate via sodium citrate (SC) treatment of PTCC-tDGMC construct after 21 days in culture [141]. (C) PTCC-blkMC and LhCG-blkMC fabrication process (previously established) [141, 150]. A suspension of chondrocytes and blank gelatin microspheres in alginate is transferred into silicon moulds; gelation is as above. Cells from alginate bulk infiltrate cavities left behind by gelatin microspheres and neotissue develops. After 35 days of culture, alginate is removed via SC treatment to yield a biomaterial-free 3D LhCG-blkMC. [160]

diameter $\times$ 2 mm depth. For gelation of alginate to occur, the dish was placed in 4°C for 4 min, after which 15 μ L 102 mM CaCl_2 was gently added to the surfaces of each construct and placed in 4°C for 4 min again. Upon incubation at 37°C, gelatin dissolved, leaving behind chondrocytes suspended within the cavities whilst chondrocytes were encapsulated within the alginate gel bulk.

6.2.2.2. Fabrication of PTCC-blank MicroCarrier (PTCC-blkMC) constructs

Blank gelatin microspheres were prepared by an oil-in-water-in-oil emulsion technique as previously described [141]. Briefly, 30 mL 10% w/v gelatin solution (preheated to 70°C) was added into a 100 mL beaker containing 10 mL ethyl acetate and stirred at 700 rpm for 1 min. The gelatin/ ethyl acetate emulsion was transferred to another 100 mL beaker containing 60 was used to wash the suspension thrice to remove the soya oil. Finally, the gelatin microspheres were dried in a 70°C oven and sieved for size quantification.

PTCC-blkMC constructs were then fabricated using blank gelatin microspheres as previously described [141]. Briefly, blank microspheres of diameter 100 - 125 μ m were cosuspended with 1×10^7 of passage 1 porcine chondrocytes in 1 mL alginate solution at a concentration of 0.3 g/mL, and gelled as above (Figure 6-1C). Upon culture at 37°C, the gelatin dissolved and left behind pores within the constructs. Chondrocytes within the alginate gel bulk naturally proliferated towards the cavities and proceeded to fill up the pores with chondrocytes and their secreted ECM.

6.2.2.3. Fabrication of blank GEL-tDGMC (blkGEL-tDGMC) constructs

0.30 g tDGMC was suspended in 1 mL alginate solution, injected into moulds and gelled as above. blkGEL-tDGMC constructs were solely used to observe proliferation and viability of cells that underwent tDGMC technique.

6.2.2.4. Acquisition of biomaterial-free constructs

Each construct was placed in a 15 mL tube containing 5 mL sodium citrate (SC) solution (55 mM in 0.15 M NaCl) for 10 min at room temperature. These were done at weekly time points: Days 14, 21, 28 and 35. After SC treatment, PTCC-blkMC and PTCC-tDGMC constructs were renamed LhCG-blkMC and LhCG-tDGMC respectively.

All constructs were cultured in CC medium with gentle shaking (every alternate 12 h) on an orbital shaker at 50 rpm, 5% CO₂ and humidity at 37°C.

6.2.3. Cell viability assays

Cell viability of the PTCC-blkMC and PTCC-tDGMC constructs were quantified by means of WST-1 assay (Roche, Switzerland). Briefly, each sample was incubated in 10% v/v WST-1 reagent in CC medium for 1.5 h in the dark at 37°C, 5% CO₂. Medium was transferred to 96-well plate (Iwaki) and absorbance was measured at 450 nm against 690 nm reference absorbance by a microplate spectrum reader (Multiskan[®] spectrum, Thermo). Live/Dead staining (Invitrogen) was also used.

6.2.4. Biochemical analysis

Samples collected were frozen at -20°C and lyophilized for 24 h prior to overnight digestion using 1 mL papain solution (0.3 mg/ mL in 0.1 mM disodium ethylene diamine tetra acetic acid (EDTA) and 0.2 mM dithiothreitol (DTT)). Hoechst 33258 assay was used to measure DNA content, which related to number of chondrocytes (7.7 pg DNA per cell) [297]. Glycosaminoglycan (GAG) content was determined with dimethylmethylene blue assay while total collagen content was measured using proline/hydroxyproline assay [298, 299].

6.2.5. Gene expression analysis using quantitative real-time polymerase chain reaction (qPCR)

All constructs were treated with sodium citrate solution to remove alginate before homogenization in 1 mL TRIzol® (Invitrogen), where applicable. RNA was extracted and converted to cDNA via reverse transcription. qPCR was executed with iQ™ SYBR® Green Supermix (Bio-Rad) and iQ™ qPCR system (Bio-Rad). Table 6-1 lists the qPCR primer sequences (AIT Biotech, Singapore) used in this experiment. For analysis, gene expression values relative to housekeeping gene TATA-Binding Protein (TBP) were calculated using ΔC_T method, and subsequently normalized against the respective genes of PTCC-blkMC Day 0 to obtain gene expression fold-values. All reverse transcription and PCR reagents were purchased from Promega (Madison, MI), unless otherwise stated.

Table 6-1 qPCR primer sequences for porcine gene markers used in Chapter 6. Forward (F) and Reverse (R). PS stands for product size.

Gene	Accession number/ Reference	Primer Sequences (both 5'-3')	PS/ bp
Collagen Type 1	[300]	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCCTT	84
Collagen Type 2	[301]	F: GCTATGGAGATGACAACCTGGCTC R: CACTTACCGGTGTGTTTCGTGCAG	256
Aggrecan	[301]	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC	177
RhoA	NM_001664.2	F: AGCTGGGCAGGAAGATTATG R: TGTGCTCATCATTCGAAGA	200
Integrin β1	NM_002211.3	F: TGCCAAATCATGTGGAGAATGTAT R: GTCTGTGGCTCCCCTGATCTTA	297
Sox9	NM_000346.3	F: GCTGGCGGATCAGTACCC R: CGCGGCTGGTACTTGTA	165
Cartilage oligomeric matrix protein (COMP)	NM_007112.3	F: GGCACATTCCACGTGAACA R: GGTTTGCCTGCCAGTATGTC	127
TBP1 (Reference gene)	NM_001172085.1	F: ACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG	152

6.2.6. Histology and immunohistochemistry staining

Paraffin-embedded PTCC-blkMC and LhCG-tDGMC samples from each time point were cut into 10 μ m-thick sections using a microtome and subsequently mounted on glass slides. Sections were stained with haematoxylin and eosin (H & E), Masson Trichrome or Safranin O. Anti-IgG immunohistochemistry staining was performed for collagen types 1 and 2 (Col1 and Col2 respectively).

Sections were incubated with primary antibody for Col2 (2 $\mu\text{g}/\text{ml}$ in $1\times$ PBS, MAB8887, Chemicon) at 4°C overnight followed by anti-IgG (5 $\mu\text{g}/\text{ml}$ in $1\times$ PBS, AlexaFluor 488, Invitrogen) at room temperature for 1 h in the dark. For Col1 immunohistochemistry staining, primary antibody for Col1 (2 $\mu\text{g}/\text{mL}$, goat polyclonal IgG; Santa Cruz Biotechnology) and anti-IgG (5 $\mu\text{g}/\text{mL}$; Alexa Fluor 543; Invitrogen) were used. 4', 6-diamidino-2-phenylindole (DAPI) was used to counterstain for nuclei.

6.2.7. Statistical analysis

All results are presented as mean $\pm$ S.D. Cell viability, biochemical and qPCR data were compared against PTCC-blkMC constructs at the particular time points. ANOVA was used to measure statistically significant differences ($p < 0.05$) between the groups (3 samples per group).

6.3. Results and Discussion

6.3.1. Establishment of tDGMC

In this study, we have fabricated tDGMC, which are chondrocyte-laden gelatin-based microspheres, using a single water-in-oil emulsion method illustrated in Figure 6-1A. When encapsulated in a hydrogel scaffold, tDGMC serve two purposes: as a cell delivery vehicle and as a porogen since gelatin would melt and diffuse out of the scaffold within a few hours of incubation at 37°C . The size distribution of tDGMC, based on manual counting of tDGMC in 20 random bright-field microscopy images, is presented in Figure 6-2. tDGMC were mostly in the diameter range of 50 to 125 μm . Larger tDGMC tend to have cells

Figure 6-2 tDGM size distribution based on 20 random bright-field microscopy images. [160]

encapsulated within (a large proportion of black column) as compared to those of smaller sizes, which can be attributed to probability. As larger tDGM have larger volume of gelatin, more cells were expected to be encapsulated within them. blkGEL-tDGM constructs were fabricated for the sole purpose of easy observation of tDGM alone in a 3D environment. Through Live/Dead assays in Figure 6-3, fabrication process was observed to be non-toxic to the cells. At Day 0, majority of cells were viable, albeit with some dead cells scattered throughout the construct. However, the cells were observed to have proliferated quickly to become cell islets with no necrotic cores by Day 21. The dense cell islets had increased in size dramatically, notably in Day 35 as the islets were about 500 μm in diameter, or approximately 5-6 times larger than average tDGM at Day 0. The islets had outgrown the cavity boundaries and into the alginate hydrogel,

Figure 6-3 Live/ Dead staining and corresponding bright-field microscopy images of tDGMC constructs at various time points at 4 $\times$ magnification. Scale bar represents 500 μ m. [160]

finally merging with other similarly dense cell islets. The fabrication process, which was simple, quick and free of chemical treatment, was hence concluded to be non-toxic to chondrocytes.

6.3.2. Application in PTCC system

We further applied the tDGMC technology to a previously established PTCC approach for hyaline cartilage regeneration (Figure 6-1B), where chondrocytes and blank gelatin microspheres were co-encapsulated in an alginate hydrogel and named PTCC-blkMC; after 35 days of culture, the alginate was removed from the construct to produce LhCG-blkMC, a biomaterial-free construct composed of chondrocytes and their secreted ECM [141]. In the PTCC-blkMC constructs, blank gelatin microspheres played the role of porogens which then created gel-medium boundaries within the alginate scaffold, to capitalise on the natural

Figure 6-4 Assessment of cell viability through WST-1 assay. *indicates $p < 0.05$; differences between different constructs at the same time point are significant. [160]

phenomenon that chondrocytes – as a type of non- ADCs – proliferated and formed neo-tissue quickly at the edges of non-cell adhesive gels (Figure 6-1C) [150]. The replacement of blank gelatin microspheres with tDGMC not only created similar gel edges as in the PTCC approach, but also provided extra chondrocytes suspended within the cavities left behind by the dissolved gelatin to be further cultured. Cell growth was then expected in two ways: outgrowth from alginate gel bulk into the cavities (as in PTCC approach) and cell islet formation within the cavities (contributed by tDGMC).

Figure 6-5 Photograph of constructs after treatment with sodium citrate solution at Day 21. Left: LhCG-blkMC and right: LhCG-tDGMC. Integrity of LhCG-tDGMC construct was retained, but visibly less so for the LhCG-blkMC construct which partially collapsed. Red arrows indicate pieces of debris. [160]

6.3.2.1. Cell viability and morphology

As seen in WST-1 assay results in Figure 6-4, PTCC-tDGMC constructs were able to proliferate and remain viable, with values significantly higher than previously established PTCC-blkMC controls at the latter half of the experiment. This can be attributed to the increase in cell numbers in the PTCC-tDGMC constructs due to tDGMC. There was a general drop in absorbance values on Day 35 from those on Day 28; this could be explained by the lack of space within the constructs for further cell proliferation. It also correlated with why alginate was removed from PTCC-blkMC constructs on Day 35 in previous studies [141]. As absorbance values of PTCC-tDGMC constructs started decreasing from Day 21 onwards, it was hypothesised that PTCC-tDGMC constructs had reached a similar cell-dense and ECM-rich state to those of PTCC-blkMC at Day 35. Hence, alginate in the construct was removed via sodium citrate treatment at Day 21. As shown in Figure 6-5, whilst PTCC-blkMC construct partially collapsed into

pieces of debris (red arrows) during sodium citrate treatment, the PTCC-tDGMC construct proved to withstand the process and retained its structural integrity with no visible debris and was thereby renamed LhCG-tDGMC. WST-1 absorbance values of LhCG-tDGMC constructs were maintained with respect to PTCC-tDGMC constructs. From these results, the fabrication of a biomaterial-free construct based on chondrocytes and their secreted ECM was accelerated by 40%, or 14 days.

6.3.2.2. Gene expression analysis

Gene expression studies were carried out via qPCR to investigate chondrocyte markers (Figure 6-6). All samples were normalized to that of control PTCC-blkMC Day 0 for each gene, with PTCC-blkMC Day 0 designated a fold-value of 1. Generally, cells expressed increasingly higher Col2 with time for both PTCC-blkMC and PTCC-tDGMC constructs. On Days 21 and 28, PTCC-tDGMC constructs had significantly higher Col2 fold-expressions than PTCC-blkMC; the former was approximately 6 to 8-fold higher than control, whereas the latter had measured 4 to 5-fold. A suitable environment had been produced more quickly in PTCC-tDGMC system, hence cells were stimulated to produce hyaline cartilage-specific ECM i.e. Col2. The consecutive 2-fold drops in the PTCC-tDGMC system from Day 21 to 35 could be explained by the space constraint and sufficient ECM detected by cells. This was further proven by the 10 to 12-fold high values measured in LhCG-tDGMC constructs: space left

Figure 6-6 Analyses of various chondrocyte markers expression. Fold values for each gene was calculated based on the expression value of the particular gene in PTCC-blkMC construct at Day 0. *indicates $p < 0.05$; differences between different constructs at the same time point are significant. [160]

behind by alginate removal was detected by cells which therefore became stimulated to produce ECM.

On the other hand, Col1 gene expression was constantly much lower than the control and had depressed to less than 20% of the control on Day 35. This was a favourable result as Col1, a major component of fibrosis, is minimal in hyaline cartilage ECM; the PTCC-tDGMC system was hence inclined towards hyaline cartilage tissue development. Generally, ECM components were highly expressed with time, with higher values for PTCC-tDGMC than PTCC-blkMC constructs especially during the latter half of the study period. Taken together, the PTCC-tDGMC system had similar expression patterns as those of PTCC-blkMC, albeit with higher tendencies towards hyaline cartilaginous tissue formation.

6.3.2.3. Biochemical analysis

Two cartilaginous ECM components – GAG and collagen – produced in both systems were evaluated through biochemical assays (Figure 6-7). The PTCC-tDGMC constructs consistently measured significantly higher amount of GAG and collagen throughout the study period, when compared against those of PTCC-blkMC. ECM components produced by cells were critical in maintaining structural integrity of the construct after alginate was removed.

As seen in total GAG and collagen content (Figure 6-7, bottom), PTCC-tDGMC Day 21 reached a similar level of ECM components per unit construct's dry weight to Day 35 for PTCC-blkMC constructs; hence, alginate could be

Figure 6-7 Biochemical analysis for GAG and collagen content. Top: GAG and collagen per cell; bottom: GAG and collagen normalized to dry weight. *indicates $p < 0.05$; differences between different constructs at the same time point are significant. [160]

successfully removed from the PTCC-tDGMC constructs at that particular time point without collapse in structure. Whilst GAG content was evidently higher after alginate removal, i.e. for LhCG-tDGMC constructs, it was not as clear-cut for collagen content. Nonetheless, the overall results were promising as there was an overall increase in ECM content, which therefore allowed for a purely biomaterial-free cell-laden 3D construct within 21 days of culture.

Figure 6-8 Various histochemical and immunohistochemistry staining comparing PTCC-blkMC and LhCG-tDGMC constructs at 10× magnification. (A) Haematoxylin & Eosin staining; (B) Masson Trichrome staining; (C) Safranin O staining; (D) Immunofluorescence staining for collagen type 2; and (E) Immunofluorescence staining for collagen type 1. In all immunohistochemistry images, nuclei were counterstained blue (DAPI). Scale bar represents 200 μm. [160]

6.3.2.4. Histology and immunohistochemistry

Finally, a qualitative analysis of construct development towards a hyaline cartilage phenotype was done through various histological and immunohistochemistry stains (Figure 6-8). LhCG-tDGMC constructs were typically visibly denser in terms of cell numbers and ECM content when compared against those of PTCC-blkMC constructs at similar time points; ECM was more equally distributed in the former (Figure 6-8A). Furthermore, lacunae

were observed more frequently and distinctively in LhCG-tDGMC constructs, which are also typically observed in hyaline cartilage tissues. These observations were further substantiated by Masson Trichrome (Figure 6-8B) and Safranin O (Figure 6-8C) which respectively stained blue for collagen and orange-red for proteoglycans. The darker blue stain surrounding the lacunae in LhCG-tDGMC in Figure 6-8B and the uniform and darker orange-red Safranin O stained images in Figure 6-8C implied a denser ECM structure was secreted and modelled. Especially on Day 21 of Figure 6-8C, voids were observed in PTCC-blkMC construct, which could be attributed to the slow chondrocyte infiltration into the cavities left behind by gelatin microspheres. This phenomenon, however, was unseen in LhCG-tDGMC at the same time point and could be explained by tDGMC's delivery of viable cells into the cavities. Col2 was abundant in both PTCC-blkMC and LhCG-tDGMC constructs, but visibly more compact in the latter (Figure 6-8D), whilst Col1 was negligible in both (Figure 6-8E), supporting the gene expression studies and biochemical assays that tDGMC had accelerated the formation of a dense biomaterial-free hyaline cartilaginous tissue.

tDGMC, when combined with the previously established PTCC approach, was shown to greatly aid hyaline cartilage regeneration. Cartilage-specific gene markers such as Col2 were highly expressed and translated to higher ECM production such that a biomaterial-free construct (through removal of alginate) was realised as early as Day 21 of culture. This was attributed to the higher viability and proliferation rates of cells, which also produce markedly higher amounts of ECM per cell. Favourably, fibrosis due to presence of Col1 was

consistently insignificant as observed in both gene expression and immunohistochemistry. Therefore, tDGMC not only provided additional viable cells into the construct, the extra cells also retained their phenotype and contributed to hyaline cartilage regeneration.

tDGMC is hence proven to be a viable cell delivery option. Through encapsulation in a hydrogel, tDGMC's gelatin component dissolves and diffuses out, leaving behind cells suspended in pores. Cells were shown to be viable through Live/Dead staining assays, proliferating to fill up the pores as cell islets. This phenomenon can be expected for other non-ADC types, for example hepatocytes and pluripotent stem cells, since they have tendencies towards cell islet formation.

6.4. Conclusion

In this work, we have demonstrated the non-toxicity of a fabrication technique of cell-encapsulated gelatin microspheres, tDGMC, using porcine chondrocytes. Post fabrication, chondrocytes were still viable and formed cell islets. When the platform was combined with a cell-laden gel bulk, it was shown to increase the scaffold's stability with heightened cell proliferation and hyaline cartilage-specific ECM production, hence accelerating the previously established PTCC approach of forming a dense biomaterial-free construct LhCG, based solely on chondrocytes and its secreted ECM, by 40%, or from 35 days to 21 days. Hence, tDGMC technology was revealed to be able to facilitate delivery of chondrocytes into a gel bulk with significantly higher efficiencies for hyaline cartilage

regeneration. This promising platform is envisaged to be versatile for the delivery of similar non-ADC types which have the tendencies to form cell islets.

Chapter 7 Conclusion and Future work

7.1. Conclusion

In this thesis, sacrificial gelatin microspheres were proposed and evaluated for two main purposes: simply as a porogen and additionally as a cell delivery vehicle into a macroscopic sized scaffold system. The natural property of gelatin to dissolve at physiological temperatures was exploited – no additional treatment was required to remove the sacrificial component from the macroscopic construct; instead, gelatin dissolved and diffused out of the construct upon culture at 37°C.

In the first purpose, acellular gelatin microspheres were used to create a MCG system which has been established to overcome diffusion constraints in macroscopic tissue engineering constructs. This system was evaluated for its versatility in engineering different 3D tissues *in vitro*. In both studies, the MCG system have provided ample diffusion rates and space to increase cell proliferation throughout the construct, after which differentiation into functional cells was carried out. Firstly, non-ADCs pluripotent stem cells ESCs and iPSCs were encapsulated within non-cell adhesive MCG system for development into EBs and subsequently differentiation into pancreatic insulin-producing cells. ESCs successfully committed to the pancreatic β -cell lineage and secreted insulin, but was not capable of responding to different glucose levels, while iPSCs showed a reduced differentiation capacity towards β -cells. Secondly, vascular-committed endothelial progenitor outgrowth cells, a type of ADCs, were

encapsulated in a cell-adhesive MCG for the development of a microvascular network.

In the second purpose, gelatin microspheres were developed to contain non-ADCs through a one-step water-in-oil emulsion fabrication technique. Similarly, this platform was demonstrated to enable neotissue formation of 2 different cell types: hepatocarcinoma cell line HepG2 and primary chondrocytes. The cell-laden microspheres, tDGMC, were sacrificial components in a macroscopic alginate hydrogel scaffold; their dissolution allowed delivered cells to develop akin to suspension culture in the cavities. HepG2 cells were shown to form viable spheroids of controllable sizes based on tDGMC size selection, and can be used as a platform for chemotherapy drug testing and screening. Similarly, tDGMC technique was demonstrated to be non-cytotoxic to primary chondrocytes; dense cell islets were formed. tDGMC were applied to a previously established MCG system containing chondrocytes for the eventual formation of a material-free hyaline cartilage graft, LhCG. Here, tDGMC acted as both porogen to create a MCG system, as well as a cell delivery vehicle; LhCG establishment was accelerated 40% from a 35-day to a 21-day process.

Collectively, the above findings demonstrated the versatility and simplicity of sacrificial gelatin microspheres in a variety of tissue engineering applications. The temperature-responsiveness of gelatin was exploited to form highly porous macroscopic scaffold systems in a one-step procedure and thereby overcoming diffusion constraints typically faced by conventional systems through three

different approaches: increasing the overall porosity, developing a microvascular network through self-assembly, and micro-sized cell carriers as a bottom-up tissue engineering strategy.

7.2. Future work

The *in vitro* engineering of tissue constructs using single cell types do not recapitulate the natural architecture of biological organs, which comprise a main tissue type that performs the organ function, and stroma tissue such as nerves and blood vessels. In order for engineered tissue to be viable as an organ replacement, vascularization of the construct and connection to the host blood supply to provide nutrients and oxygen is necessary.

The findings in this thesis separately demonstrated the self-assembly of a microvascular network around cavities in an MCG system, as well as the successful delivery and development of cells in sacrificial gelatin microspheres, tDGMC. Collectively, they can create a complex vascularized functional tissue construct, whereby spheroids of the functional tissue type developed in tDGMC are embedded within a microvascular network. However, this system is limited to functional tissues comprised of non-ADCs, such as hepatocytes in liver and endocrine islets in the pancreas.

The same strategy can also be applicable for *in vitro* study models of tumour development, angiogenesis and metastasis. Engineering a tumour model that is mimetic of *in vivo* tumours will also provide more accurate predictions of drug efficacy and dosage than currently used monolayer cultures and mouse models,

reducing the risk of failures at clinical or pre-clinical stages which incur huge costs.

However, in such co-culture systems, cell culture conditions must be optimized to ensure that encapsulated cell types maintain their viability, genotype and phenotype. Addition of cell-specific small molecules and growth factors specific for one cell type may affect the other cell type; one strategy may be to immobilize them – in the case of EPOCs, VEGF for angiogenic induction can be conjugated and immobilized on gelatin-MA.

List of publications

Journal articles

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Manuscripts in progress

Leong, W. & Wang, D. -A. Cell-laden polymeric microspheres for biomedical applications. (*Submitted and in revision, Trends in Biotechnology, review article*)

Leong, W.*, Fan C.J.* (Equal contribution) & Wang, D. -A. Vasculogenesis in a simple micro-cavity gel system for tissue engineering. (*Submitted manuscript*)

Leong, W. & Wang, D. -A. Directed differentiation of pluripotent stem cells into insulin-producing islets in a microsphere/ hydrogel system. (*In preparation*)

Leong, W. & Wang, D. -A. Development of hepatocarcinoma spheroids as a drug testing platform using a sacrificial gelatin microsphere system. (*In preparation*)

List of patents

“Massive cell delivery and its carrier in cross-linked polymer matrices”, *U.S. Patent*, Provisional Application No. 61/680,854; *WO* Provisional Application #PAT/064/12/13/PCT. (by: Wang, D.-A., Lau, T. T., **Leong, W.**).

“Method of Manufacturing A Scaffold for Tissue Engineering or Repair”, *U.S. Patent*, Application No. 20130171111 (CIP of U.S. Patent, Application No. 20120134968, by: Wang, D.-A., Lau, T. T., Su, K., **Leong, W.**, Gong, Y. H.).

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