Hydrogel-microcarrier composite systems for cell delivery in tissue engineering

Lau, Ting Ting

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NANYANG
TECHNOLOGICAL
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**HYDROGEL-MICROCARRIER COMPOSITE SYSTEMS
FOR CELL DELIVERY IN TISSUE ENGINEERING**

LAU TING TING

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

2014

**HYDROGEL-MICROCARRIER COMPOSITE SYSTEMS
FOR CELL DELIVERY IN TISSUE ENGINEERING**

LAU TING TING

School of Chemical and Biomedical Engineering

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TABLE OF CONTENTS

Chapter 1 : Introduction	1
1.1 Role of hydrogel in tissue engineering	1
1.2 The emergence of bioresponsive hydrogels	2
1.2.1 <i>Bioactive hydrogels for cell survival and proliferation</i>	4
1.2.2 <i>Biodegradable hydrogels for neo-tissue development</i>	8
1.3 Hydrogel/microspheres (GS) composite as bioresponsive hydrogel ..	13
1.3.1 Gelatin microspheres as cellular anchors in GS system	13
1.3.2 Genipin as a crosslinker for gelatin microspheres	14
1.3.3 Gelatin microspheres as porogens in GS system	15
1.4 Objective and organisation of this dissertation	16
Chapter 2 : Cell delivery with Genipin crosslinked gelatin microspheres in hydrogel/microcarrier composite	21
2.1. Introduction	21
2.2 Materials and Methods	22
2.2.1 <i>Gelatin spheres fabrication</i>	22
2.2.2 <i>Preparation of Genipin crosslinked microspheres</i>	23
2.2.3 <i>Cell seeding on microspheres</i>	24
2.2.4 <i>Cell loading efficiency</i>	25
2.2.5 <i>Cell viability testing</i>	25
2.2.6 <i>Encapsulation of cell-laden microspheres in hydrogel</i>	26
2.3 Results and Discussion	26
2.4 Summary	35
Chapter 3 : Genipin crosslinked microcarriers mediating hepatocellular aggregates formation and functionalities.....	36

3.1	Introduction	36
3.2	Materials and Methods	37
3.2.1	<i>Gelatin spheres fabrication</i>	37
3.2.2	<i>Preparation of Genipin crosslinked microspheres</i>	38
3.2.3	<i>Cell seeding on microspheres</i>	38
3.2.4	<i>Cell loading efficiency</i>	39
3.2.5	<i>Morphology of HepG2 on microcarriers</i>	41
3.2.6	<i>Liver-Specific Functions</i>	41
3.2.7	<i>Cytotoxicity of genipin microspheres degradation product</i>	41
3.2.8	<i>Statistical analysis</i>	41
3.3	Results	42
3.3.1	<i>Cell loading efficiency</i>	42
3.3.2	<i>Cell viability and proliferation</i>	42
3.3.3	<i>Morphology of HepG2 on microcarriers</i>	43
3.3.4	<i>Liver-Specific Functions</i>	45
3.3.5	<i>Cytotoxicity of genipin microspheres degradation product</i>	46
3.4	Discussions.....	47
3.5	Summary	52
Chapter 4 : Formation of model hepatocellular aggregates in hydrogel scaffold using degradable Genipin crosslinked gelatin microspheres as cell carriers		53
4.1	Introduction	53
4.2	Materials and Methods	55
4.2.1	<i>Preparation of Genipin crosslinked gelatin microspheres</i>	55
4.2.2	<i>Characterization of the crosslinked microspheres</i>	56

4.2.3	<i>Preparation of cell-laden microspheres</i>	56
4.2.4	<i>Fabrication of cell-laden microspheres hydrogel composite with or without MMP-9</i>	57
4.2.5	<i>In vivo subcutaneous implantation into nude mice</i>	58
4.2.6	<i>Cell viability and HepG2 spheroid morphology</i>	59
4.2.7	<i>Albumin and cytochrome P450 gene expressions</i>	59
4.2.8	<i>Albumin quantification.....</i>	60
4.2.9	<i>Histology staining</i>	61
4.2.10	<i>Statistical analysis</i>	61
4.3	Results	61
4.3.1	<i>Characterization of Genipin crosslinked gelatin microspheres ..</i>	61
4.3.2	<i>Cell viability and HepG2 spheroid morphology in constructs</i>	63
4.3.3	<i>Liver-Specific Functions of cell-laden microspheres hydrogel composite</i>	67
4.3.4	<i>In vivo nude mice subcutaneous implantation</i>	69
4.4	Discussions.....	70
4.5	Summary	74
Chapter 5 : Cells in hydrogel: Endochondral ossification in scaffold-free LhCG.....		75
5.1.	Introduction	75
5.2.	Materials and methods	79
5.2.1	<i>Preparation of LhCG prototype</i>	79
5.2.2	<i>Cell seeding to LhCG.....</i>	81
5.2.3	<i>Animal Study</i>	83
5.2.4	<i>Analytical Methods</i>	83
5.2.5	<i>Statistical analysis</i>	89

5.3.	Results and discussion.....	89
5.4.	Summary	109
Chapter 6 : Hepatogenesis of murine induced pluripotent stem cells (iPSC) in 3D microcavitary hydrogel system for liver regeneration		110
6.1	Introduction	110
6.2	Materials and Methods	112
6.2.1	<i>Cell Culture</i>	112
6.2.2	<i>Setting up of cell-laden micro-cavitary hydrogel (MCG)</i>	113
6.2.3	<i>Hepatic differentiation of murine iPSC and ESCs in the hydrogel construct</i>	116
6.2.4	<i>Cell viability</i>	116
6.2.5	<i>Gene expressions</i>	117
6.2.6	<i>Immuno- Histochemisty</i>	119
6.2.7	<i>Quantification of urea and albumin</i>	120
6.2.8	<i>Statistical analysis</i>	121
6.3	Results	121
6.4	Discussion	137
6.5	Summary	141
Chapter 7 : Conclusion and Future work.....		142
7.1	Conclusion.....	142
7.2	Future work	144

LIST OF TABLES

Table 4.1. List of primer sequence used for real-time PCR. A.T denotes annealing temperature while P.S denotes product size.....	60
Table 5.1. Human osteogenic primers used for qPCR. A.T, P.S and Ref stand for annealing temperature, product size and reference respectively.....	86
Table 5.2: Primers used in polymerase chain reaction (PCR). A.T, P.S and Ref stand for annealing temperature, product size and reference respectively.....	87
Table 6.1. Primers used for PCR. P.S stands for product size.....	118
Table 6.2. Primers used for qPCR. P.S stands for product size.....	119

LIST OF FIGURES

Figure 1.1 Schematic illustration of two main types of bioresponsive hydrogel.

(A) Bioactive hydrogel with cell adhesive moieties to facilitate cell adhesion

(B) Biodegradable hydrogel containing either weak interaction and are

responsive to physical changes or degradable moieties that are cleavable upon

exposure to MMP.....4

Figure 2.1: Gelatin microspheres after 48 hours in 4°C (A) 100% EtOH, (B)

90% EtOH (C) 80%27

Figure 2.2: (A) Gelatin microspheres after 24 h cross-linking with genipin at 37

°C in various concentration of ethanol (Left to right: 100%, 90%, 80% and 50%)

(B-D) Photomicrographs of gelatin spheres in 100%, 90% and 80% ethanol

concentration respectively. No picture is available at 50% ethanol concentration

due to heavy aggregation. Scale bar applies to B, C and D.29

Figure 2.3: Small gelatin spheres after (A) 14 h (B) 24 h (C) 36 h and (D) large

spheres after 36 h (E) Small spheres at 48 hours. Images showed that no further

change in color intensity after 36 hours.31

Figure 2.4: Cell loading efficiency onto microspheres after 2 days31

Figure 2.5: Live/dead staining of osteoblast attached on (A) small (B) large

microspheres after 1 day32

Figure 2.6: Cell proliferation of osteoblast seeded on Genipin crosslinked microspheres using WST-1 colorimetric assay.....	32
Figure 2.7: Live/dead staining of hMSC on small microspheres (A) 40X (B) 100X and Large micropsheres (C) 40X (D) 100X 2 days after encapsulated in agarose hydrogel	34
Figure 3.1: Schematic illustration of cell-laden genipin crosslinked gelatin microspheres. In Step 1, genipin is dissolved in 90% aqueous ethanol solution and gelatin microspheres are added. After 24h in 4°C fridge and subsequently 36h at 37°C in the incubation, genipin crosslinked microspheres were obtained via oxygen radical-induced polymerization and dimerization forming intramolecularly and intermolecularly crosslinked products with a heterocyclic structure [108]. In Step 2, HepG2 cell suspension were added to genipin crosslinked microspheres in a agarose pre-treated well and incubate overnight for maximum cell attachment. It is then transferred to a strainer of mash size 40µm where non-attached cells would fall to the bottom of the well.....	40
Figure 3.2: Graph of HepG2 cell loading efficiency on microcarriers	42
Figure 3.3: Cell proliferation profile of cells on microcarriers using WST-1 assay. *p<0.05 between BS and SS	43
Figure 3.4: Live/dead staining of the cells on small (A,C,E) and big (B,D, F) microspheres on Day 3 (A-B), Day 7 (C-D) and Day 17 (E-F).....	44

Figure 3.5: SEM micrographs of small microspheres (A-B) and big microspheres (C-D) on day 7 (A, C) and Day 20 (B, D)	45
Figure 3.6: Relative percentage increase in albumin secretion of HepG2 on microcarriers at various time points. * indicates $p < 0.05$ between BS and SS...	46
Figure 3.7: Cell proliferation profile of HepG2 cultured in supernatant of microspheres/MM-9 using WST-1 assay. * $p < 0.05$ compared with the control group	47
Figure 3.8 Graph of Free energy in relation to microsphere diameter. Indicated in the graph, the range of free energy gives the size range of the BS and SS. ..	50
Figure 4.1:(a) Schematic illustration of fabrication steps for cell-laden microsphere hydrogel composite construct (control, MM and MG); insert picture is of 10× magnification (b) Phase contrast picture of control, MM and MG constructs with or without MMP-9 treatment	58
Figure 4.2: Overview of gelatin microspheres after crosslinking in different concentrations of genipin (0.1wt%, 0.25wt% and 0.5wt %). First column: Genipin crosslinked microspheres after swelling in PBS. Approximation of crosslink degree is indicated by the intensity of blue pigment formed. Second column: genipin crosslinked microspheres in PBS incubated at 37°C for 30min. Third column: GM incubated in 100µg/ml MMP-9 containing media at 37°C for 4 hours	63

Figure 4.3: Cell proliferation profile of cells in control, MM and MG constructs using WST-1 assay. * represents $p < 0.05$ when compared to control sample of that day.....	64
Figure 4.4: Live/dead staining and phase contrast images of the cells in control, MM and MG constructs on Day 4, 7 and Day 14.	66
Figure 4.5: Gene expression of albumin and cytochrome P4501A1 (CYP1A1) of control, MM and MG constructs at various time points. * represents $p < 0.05$ when compared to control sample of that day	68
Figure 4.6: Albumin secretion from control, MM and MG constructs at various time points. * represents $p < 0.05$ when compared to control sample of that day	69
Figure 4.7: Histochemical staining of control, MM and MG constructs 14 days after subcutaneous implantation in nude mice. Red dotted lines outline the cavities while arrows indicate HepG2 cell aggregates within the constructs. ...	70
Figure 5.1: (a) Schematic illustration of the establishing in vitro endochondral osteogenesis niche using LhCG prototype (b) Timeline showing various conditions applied to different co-culture constructs on different days.....	85
Figure 5.2: Assessment of LhCG as a possible open platform for cell seeding. (a) Green fluorescent protein (GFP) labeled osteoblasts were used to illustrate cell seeding was feasible in LhCG+FOB (Day 14). The image on the left is at 40× magnification while the one on the right is at 100× magnification. (b) Live/dead	

staining of cells in LhCG+FOB showed both chondrocytes and osteoblasts were viable in this co-culture system on Day 14. Furthermore, osteoblasts, being typical anchorage dependent cell, were able to maintain the desired stretched morphology. (c) Gross overview of LhCG-FOB and LhCG+FOB constructs at various time points92

Figure 5.3: qPCR of osteogenic markers of LhCG+FOB before and after treatment with differentiation conditions compared to monolayer osteoblasts. Differentiation conditions were introduced from Day 14 to Day 17 and proliferation conditions resumed on Day 17 till Day 30. The results indicated up regulation of osteogenic markers in osteoblasts within LhCG+FOB construct after osteoblast differentiation conditions. The obtained values represent the means $\pm$ standard deviations (N = 3). *Relative gene expression of sample LhCG+FOB on that day compared to Day 14 is significant ($p < 0.05$)93

Figure 5.4: Calcium assay of LhCG-FOB and LhCG+FOB construct at various time points. The obtained values represent the means $\pm$ standard deviations (N = 3). *Differences between different constructs are significant ($p < 0.05$)94

Figure 5.5: Histochemical and immune-fluorescent staining of LhCG-FOB and LhCG+FOB at various time points. Clockwise beginning from top left corner: Alizarin Red S staining; immunostaining of OCN; Col II and Col I. Nuclei were stained blue by DAPI.96

Figure 5.6: (a) Assessment of LhCG for cell seeding of hMSCs. LhCG+MSC refers to LhCG with seeded hMSC while LhCG-MSC refers to a plain LhCG construct undergoing the same OG condition with LhCG+MSC. Live/dead staining of cells in LhCG-MSC and LhCG+MSC revealed that cells were viable in the both constructs. Chondrocytes were rounded in morphology while hMSCs had typical stretched morphology. (b) Gross overview of LhCG-OG and LhCG+MSC constructs at various time points indicating the gradual whitening of the constructs. 200µm scale bar is applied to all 100× images while 100µm scale bar is applied to all 200× images.98

Figure 5.7: Gel electrophoresis osteogenic and chondrocytic marker expressions in LhCG-OG and LhCG+MSC using of species specific primers. ‘-’ represents LhCG-MSC while ‘+’ represents LhCG+MSC. There were co-expression of both chondrocytic and osteogenic markers in both constructs.99

Figure 5.8: qPCR of human osteogenic markers in hMSCs of LhCG+MSC. It showed a general up-regulation of the osteogenic markers with OG condition treatment. The obtained values represent the means $\pm$ standard deviations (N = 3). *Relative gene expression of sample L LhCG+MSC on that day compared to Day 14 is significant ($p < 0.05$)..... 101

Figure 5.9: (a) Calcium assay of LhCG-MSC and LhCG+MSC constructs at various time points. *Differences between different constructs are significant ($p < 0.05$) (b) ELISA assay of osteocalcin (OCN) secretion in LhCG and

LhCG+MSC. The obtained values represent the mean $\pm$ standard deviations (N = 3). *Differences between different constructs are significant ($p < 0.05$)..... 102

Figure 5.10: hMSCs was seeded onto LhCG (LhCG+MSC) on Day 7. Both LhCG and LhCG+MSC were cultured in chondrocyte culture (CC) medium was switched to osteogenic (OG) medium on Day 14. Samples were evaluated for evidence of osteogenic differentiation after 3 weeks (Day 35) and 6 weeks (Day 56) in OG medium respectively using histochemical and immunostaining of LhCG and LhCG+MSC. (a) Alizarin Red S for calcification; immunostaining of (b) OCN ; (c) Col I and (d) Col II. Nuclei were stained blue by DAPI. 104

Figure 5.11: After implantation of constructs in nude mice, the samples were retrieved on Day 45 and Day 56 respectively for evaluations. (a) Gross overview of LhCG-MSC and LhCG+MSC constructs at various time points (b) Calcium assay of LhCG-MSC and LhCG+MSC. (N=3) *Differences between different constructs are significant ($p < 0.05$) 105

Figure 5.12: Histochemical and immune-fluorescent staining of LhCG-OG and LhCG+MSC. (a) Alizarin Red S staining; immunostaining of (b) OCN; (c) Col I and (d) Col II. Nuclei were stained blue by DAPI. 106

Figure 6.1: Overview of the MCG establishment and cell culture conditions (a) Schematic illustration of the protocol for cell encapsulation and various stages of cell growth in MCG construct. (b) Timeline of cell culture conditions at

various stages. Day 0 marks the establishment of cell-laden MCG construct. The samples were cultured in LIF DMEM, which contains DMEM media, 10% FBS, 10^3 unit/ml of LIF, 0.1mM 2-ME and 0.1M NEAA, for 10 days. Subsequently, media was changed to RPMI+ActA. It is made up of RPMI 1640 medium supplemented with B27 and 100ng/ml of activin A for 4 days to induce endoderm formation. For hepatic lineage specification, RPMI+HGF is used. It is made up of RPMI 1640 medium supplemented with B27 and 20ng/ml of HGF. Hepatic lineage specification was carried out for 6 days until Day 20. For final hepatic maturation, cells were cultured in HCM media supplemented with 10ng/ml of oncostatin (HCM+OCM) for 5 days and subsequently maintained in HCM media till Day 30. (c) Phase contrast pictures of cell-laden MCG construct at various stages of growth and differentiation showing the formation and maintenance of EBs within the construct. 115

Figure 6.2: Comparison between MCG and non-MCG constructs. Phase contrast and live/dead images revealing the ability of MCG in facilitating viable EB formation compared to non-MCG constructs. Insert images are of 10× magnification. 123

Figure 6.3: Assessment of cell viability in MCG constructs. (a) WST-1 assay indicating general increase in absorbance. (b) Live/dead staining of ESCs and iPSCs in the microcavitary construct. Cells remained highly viable throughout the culture period and EBs formation was observed on Day 10. * indicates $p < 0.05$ 125

Figure 6.4: Gel electrophoresis of pluripotent markers, endoderm markers and hepatic progenitor markers from Day 0- 30 in both mESCs and miPSCs samples.....	128
Figure 6.5: Gene expression of mESCs and miPSCs samples at different stages of differentiation by qPCR. All data are normalized to Day 0 of their respective group (N = 3) where Day 0 is represented as 1 unit. * indicates $p < 0.05$ when compared to its Day 0 counterpart.	130
Figure 6.6: (a) Histochemical (H&E) and immuno-histochemical staining of pluripotent marker (Nanog), endoderm markers (Sox17 and Foxa2) and (b) hepatic lineage marker (HNF4 α) and mature hepatic markers (ALB and CYP1A1) in murine ESC samples at from Day 5 to 30.	132
Figure 6.7: (a) Histochemical (H&E) and immuno-histochemical staining of pluripotent marker (Nanog), endoderm markers (Sox17 and Foxa2) and (b) hepatic lineage marker (HNF4 α) and mature hepatic markers (ALB and CYP1A1) in miPSCs samples from Day 5 to 30.	136
Figure 6.8: Assessment of liver-specific functionalities. Quantification of (a) urea and (b) albumin produced in mESCs and miPSCs after hepatic lineage induction. * indicates $p < 0.05$	137

LIST OF ABBREVIATIONS

ADC	anchorage-dependent cells
REDV	Arg-Glu-Asp-Val
RGD	Arg-Gly-Asp
BMPs	bone morphogenetic proteins
BDNF	brain-derived neurotrophic factor
CB	carboxybetaine
DMEM	Dulbecco's modified Eagle's medium
ESCs	embryonic stem cells
ECM	extra cellular matrix
FBS	fetal bovine serum
FGF	fibroblast growth factors
FN	fibronectin
Fmoc-FF	fluorenylmethoxycarbon–diphenylalanine
GA	glutaraldehyde
hMSCs	Human mesenchymal stem cells
HUVECs	human umbilical vein endothelial cells
HA	hyaluronic acid
GS	hydrogel / microspheres
IKVAV	Ile-Lys-Val-Ala-Val
iPSCs	induced pluripotent stem cells
LN	laminin

LhCG	living hyaline cartilaginous graft
MMPs	matrix metalloproteases
MSC	mesenchymal stem cell
MCG	microcavitary hydrogel
PEG	polyethylene glycol
PVA	polyvinyl alcohol
3D	three-dimensional
2D	two dimensional
YIGSR	Tyr-Ile-Gly-Ser-Arg
VEGF	vascular endothelial growth factor
VN	vitronectin
PTCC	Phase transfer cell culture

SUMMARY

Amongst the diversity of scaffolding systems, hydrogel remains a popular choice for tissue engineering applications. However, good structural support and adequate biocompatibility are no longer the only emphasis in scaffold design; new generation of scaffolds are also expected to play a role in developmental guidance of the regenerated tissues. Thus, a simple hydrogel system could not satisfy the current demand.

Instead of carrying out chemical modifications to hydrogel, we proposed the incorporation of microspheres into hydrogel to develop a hydrogel/microspheres (GS) composite system. By employing the microspheres as cell-affinitive interfaces for cell attachment or degradable moieties within hydrogels, the microspheres act as the bioresponsive entity within the composite model. Accordingly, this dissertation presents three distinct GS composite systems to deliver different cell types for cell-based therapy purposes.

Based on the type of cell to be delivered, modification to GS system could be made respectively to suit the target cell development. Thermally stable microspheres were employed as permanent cellular attachment site within the GS system for delivery of typical anchorage-dependent cells (ADCs) like osteoblasts and mesenchymal stem cells (MSCs).

For delivery of another type of model cell, HepG2 cell, partially crosslinked microspheres were used in the second GS system. Upon

introduction of collagenase, microspheres in the GS system degrade, creating micro-cavities in the hydrogel bulk for further cell expansion and development. This design serves to use microsphere as a transient cell delivery vehicle and a porogen to establish a micro-cavitary hydrogel (MCG) construct. The dual functionality of the microspheres in the GS system enables direct cell delivery to the cavities of the hydrogel and also permits formation of controlled size cellular aggregates.

In all previous set ups, cells were delivered on the microspheres while in this last GS system proposed, cells were delivered in the hydrogel phase of the composite system. As the cells would be encapsulated and maintained their rounded morphology, primary chondrocytes, a typical non-ADC was selected. This third GS composite is designed to be a thermally responsive system where microspheres dissolved upon exposure to physiological temperature without the need for collagenase. The beneficial effects of MCG are further demonstrated in this study where a macroscopic scaffold-free living hyaline cartilaginous graft (LhCG) consisting of only chondrocytes and ECM proteins could be derived. Given the high purity of LhCG construct, it served as an excellent in vitro cartilaginous template for mimicking endochondral ossification process. It is shown that the LhCG construct, rich in ECM protein, favours osteoblast and MSC attachment. Osteogenesis of the seeded cells on LhCG was achieved based on the positive expression of bone markers and calcification.

Besides the ability to derive a scaffold-free LhCG construct, the thermally responsive GS system was also employed for delivery and differentiation of stem cells. Murine embryonic stem cells (mESCs) and induced pluripotent stem cells (iPSCs) were encapsulated respectively in this third GS system. Our results indicate that this system is able to facilitate embryoid bodies (EBs) formation and also allow differentiations to take place. Spontaneous formation of EBs and subsequent endodermal and hepatic differentiation could all occur in the continuous MCG system.

In summary, the studies in this dissertation proved that GS composite system is an easily customizable platform for delivery of various cell types – model cell lines, primary cells, and pluripotent stem cells.

Chapter 1 : Introduction

1.1 Role of hydrogel in tissue engineering

Biomaterial scaffolds play essential roles in tissue engineering, executing mechanical support and developmental guidance for cellular growth and neo-tissue deposition. Traditional scaffolds were designed to be biocompatible and stable in structure – both physically and bio-chemically [1, 2]. Among the myriad of scaffolding systems, hydrogel is one of the most extensively explored categories for tissue engineering applications [3-5]. Its intrinsic high water content, good capacity for nutrient exchange and tunable mechanical properties make it a close biophysical analog of natural extra cellular matrix (ECM). Furthermore, cells can be homogeneously suspended in the three-dimensional (3D), aqueous matrices that closely mimic certain native tissues. However, the major drawback of hydrogel is that the frameworks of most common hydrogels do not support optimal cell adhesion and outstretching but confine the encapsulated cells into a spheroidal shape. As a result, a large family of anchorage-dependent cells (ADCs) such as osteoblasts and mesenchymal stem cells (MSCs) – of which the survival requires full achievement of cellular focal adhesion – has been found to settle or commit unfavourably in the hydrogel bulk [6-8]. This has become a major challenge for hydrogel-mediated cell delivery and associated tissue engineering applications.

1.2 The emergence of bioresponsive hydrogels

In recent years, the paradigm for scaffold design has shifted where the emphasis is no longer placed solely upon structural support with adequate biocompatibility but more towards developmental guidance[9, 10]. Neo-tissue development consists of three stages: cell proliferation, differentiation and tissue morphogenesis. Ideally, a scaffold should be multifunctional, mimicking both the biochemical and biophysical properties in the natural ECM. Namely, it should be able to support initial cell growth along with desired phenotypic commitment and also facilitate neo-tissue remodeling eventually. Given the inherently favorable biophysical and biochemical properties of hydrogel, further improvements in bio-functional properties become the new focuses in recent studies. For this purpose, it is essential to integrate bioresponsive cues into hydrogel for better construction of microenvironment comparable to that in natural ECM. The process of incorporating bio-functional additives into hydrogels could be achieved by either physically blending relevant biomolecules with hydrogel precursors [10-12] or chemically grafting the biomolecules into the hydrogel network [13-16]. Based on the targeted tissue species and their specific developmental stages, a myriad of biomolecular additives are employed to decorate hydrogel framework as bio-functional signals. These modifying additives may include various extracellular ligands, enzymes, antibodies, cytokines, hormones, nucleic acids and so on. These modified hydrogels, carrying signaling biomolecules, are capable of responding

to cell behaviors, generating constructive mutual interactions with cells; they are hence defined as bioresponsive hydrogel.

Generally, bioresponsive hydrogels are classified into two main groups: namely, bioactive hydrogels and biodegradable hydrogels (**Figure 1.1**). Bioactive hydrogels contain biomolecules like ECM proteins[17, 18] or protein-derived short peptides[19] to provide cell attachment site or as instructive molecules[20, 21] for initial tissue development stages: cell survival and cell differentiation. Bioactive hydrogels are referred to as the hydrogels that may directly exert biological related responding behaviors rather than degradation at certain research stage of interest, for example, when mechanical support still remains essential. On the other hand, later developmental stages such as tissue morphogenesis may involve drastic changes in shape and structure that favors the use of biodegradable hydrogel to make room for such remodeling to take place[10, 22]. These degradable hydrogels contained bioresponsive linkages, substrates[23] or proteases that could react to changes in the biological microenvironment like pH, temperature, ions concentration or presence of enzyme.

Figure 1.1 Schematic illustration of two main types of bioresponsive hydrogel. (A) Bioactive hydrogel with cell adhesive moieties to facilitate cell adhesion (B) Biodegradable hydrogel containing either weak interaction and are responsive to physical changes or degradable moieties that are cleavable upon exposure to MMP.

1.2.1 Bioactive hydrogels for cell survival and proliferation

As hydrogels are generally bio-inert, the need to functionalized hydrogels with ECM-derived proteins or short peptides is essential to for encapsulation of ADCs. ADCs require binding of integrin receptors to their specific ligands for cell survival[9, 24]. Over the years, studies have revealed that the integrin-mediated signaling is a complex cascade of events which implicates multiple cellular events such as proliferation, differentiation, migration and apoptosis. The understanding of matrix biology has provided necessary knowledge in the

use of ECM proteins to modulate specific cellular responses. This section discusses various approaches explored to incorporate ECM proteins or their derived peptides within hydrogel scaffolds.

The class of bioactive molecules for cell survival and proliferation can be divided into two groups, cell adhesive proteins (eg. collagen, fibronectin (FN), vitronectin (VN) and laminin) and growth factors (eg. fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF)). Based on the tissue of interest, different bioactive molecules could be employed in hydrogel systems.

For instance in bone regeneration, collagen type I, being the predominant type of collagen present in bones, was simply blend with hydrogel precursors to form hybrid hydrogels for pre-osteoblast cell attachment and osteogenic differentiation [25, 26]. Similarly, Diab et al have shown that encapsulation of BMP2 in silk hydrogels results in bone repair and formation in large bone defect in rats[12]. On the other hand, for pro-angiogenic properties, implantation of VEGF encapsulated alginate in rats, the density of vascular network increase compared to the blank alginate control[27].

The most straightforward method for protein and growth factor incorporation is to add them into the hydrogel precursor solution during fabrication process. However, this type of direct protein incorporation typically results in rapid burst release profile which could not be sustained over a long time. To attain better control over the release profile of the proteins, they could

be first encapsulated in nano/microspheres before embedding them in hydrogel. When VEGF and brain-derived neurotrophic factor (BDNF) encapsulated microspheres were combined into hydrogel systems, it was shown that sustained release could be maintained for up to 30 days which supports survival of neural stem cells[28]. Alternatively, growth factors could be grafted onto the hydrogel network for more intimate cell interactions. Porter and colleagues have fabricated a biomimetic polyethylene glycol (PEG) hydrogel where VEGF is grafted onto PEG backbone, enhancing the survival of endothelial cells [29]. It was also reported that when acryloyl-modified FN is photocrosslinked into hyaluronic acid composite hydrogel (GMHA/FN-PEG-Ac), more FN is retained in the gel network compared to unmodified FN (GMHA/FN). When compared to GMHA hydrogel (without FN incorporation), GMHA/FN-PEG-Ac supported human umbilical vein endothelial cells (HUVECs) cell adhesion and survival[30].

Due to the complex whole protein tertiary structure folding, presentation of specific bioactive motifs within the 3D hydrogel matrix could not be controlled. Hence, short peptides have also emerged as popular candidates in fabrication of bioactive hydrogels as exposure of cell binding domains could be ensured. Furthermore, unlike the whole proteins, short peptide sequences are comparatively more stable and resistant to denaturation[16]. Numerous peptide sequences have been studied and introduced into hydrogels such as Arg-Gly-Asp (RGD)[31-33] and Arg-Glu-Asp-Val (REDV)[34] from FN, Tyr-Ile-Gly-Ser-Arg (YIGSR)[35, 36] and Ile-Lys-Val-Ala-Val (IKVAV) from laminin

(LN)[20, 37]. The most extensively studied short peptide is RGD which binds to a variety of heterodimeric integrin receptors like $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [38, 39]. Owing to their small molecular sizes, short peptides are generally bioconjugated on to hydrogel backbone to prevent loss by rapid diffusion. There are a myriad choices of hydrogel material used for short peptide grafting includes hyaluronic acid (HA) [40, 41], alginate[42, 43], polyvinyl alcohol (PVA)[44] and carboxybetaine hydrogel[19] but the most common hydrogel used for this application is PEG[15, 45, 46]. Basic PEG structure is PEG diol consisting of two hydroxyl end groups that can be easily modified to other functional groups like carboxyl, amine, thiol etc.[16]. Therefore, most of the recent functionalized PEG gels are multi-functional, usually containing 2 or more bioactive groups [14, 47, 48]. Ehrbar and colleagues have designed an intricate PEG based hydrogel consisting of multiple bioresponsive moieties. In their work, multi-arm PEG macromers were functionalized with two different transglutaminase peptide substrates. Each peptide substrate is further conjugated with either a glutamine acceptor substrate or a matrix metalloproteases (MMPs) substrate conjugate lysine donor peptide (MMP-Lys). As the crosslinker transglutaminase enzyme factor XIIIa was coupled with RGD ligand, the cell adhesive peptide was incorporated in the gel crosslinking process [14]. This resultant PEG hydrogel not only supports cell attachment and survival of human dermal fibroblasts, it can also be degradable by proteases (MMPs) secreted by encapsulated fibroblasts.

In addition to modifying natural or synthetic hydrogel, peptide-based hydrogel has also emerged as an attractive alternative hydrogel since biocompatibility and biodegradability are ensured. Generally, recombinant peptides were specially designed to self-assemble at physiological conditions so as to establish a more favorable environment for cell encapsulation compared to typical chemical crosslinking/conjugation process[49]. Zhou et al. have employed fluorenylmethoxycarbon–diphenylalanine (Fmoc-FF) and Fmoc-RGD as building blocks for construction of self-assembled peptide-based bioactive hydrogel. By changing the pH and the temperature, self-assembled Fmoc-FF/RGD hydrogel could be formed within a minute. This peptide-based hydrogel supports cell attachment and proliferation in encapsulated human dermal fibroblasts[50].

1.2.2 Biodegradable hydrogels for neo-tissue development

ECM is a dynamic niche that takes part in all three tissue development processes; its biochemical composition and mechanical structure determines cell proliferation and differentiation, its degradation rate on the other hand influences cell morphogenesis[51-53]. The process of cellular remodeling during tissue morphogenesis includes cell migration, cell invasion and changes in the cellular structural architect so as to take shape of target functional organ or tissue. Considering that biomaterials can function as a biophysical barrier that hinders cell invasion and remodeling, progression in morphogenesis is dependent on rate of material degradation. Material degradation could occur via

surface erosion or bulk degradation [54-56]. Rate of surface erosion is influenced by surface area exposed and is limited to surface of the materials. Bulk degradation on the other hand is determined by various factors like diffusion of water, temperature, pH, ionic concentrations and presence of enzyme.

1.2.2.1 Bioresponsive physical degradation in hydrogel

Physical changes such as temperature, pH in the environment could be stimuli to initiate hydrogel degradation. However, as the cell practices rigorous hemostasis in its microenvironment, thermal and pH induced hydrogels degradation are less popular for cell delivery; rather, they are extensively used for drug delivery [57-59]. Compared to temperature and pH, ionic concentration in cells is a relatively more volatile parameter. Given a myriad of ions participate in various signaling pathways during cellular processes, intra- and extra-cellular ion concentrations are frequently subjected to fluctuations [60-62]. Hence, changes in ion concentration could be a bio-stimulus to initiate hydrogel degradation for morphogenesis.

Alginate and gellan gum are polysaccharide hydrogels that crosslink through ionic bonding or a combination of hydrogen bonding and ionic bonding respectively. Gel concentration and concentration of divalent cations such as Mg^{2+} and Ca^{2+} strongly influenced both structural and mechanical properties of these gels [63-65]. Based on their crosslinking nature, it has made alginate and gellan gum intrinsic bioresponsive during encapsulation chondrocytes.

Cartilage being a non-mineralizing tissue[66, 67], has the ability to avoid calcification in the ECM. This suggests that as the chondrocytes grow and expand within the hydrogel, the calcium-free regions within the hydrogel expand as well, breaking down bulk alginate and gellan gum gel.

Comparing alginate and gellan gum, alginate gelation depends solely upon presence of calcium ions while gellan gum gelation is determined by both hydrogen bonding (affected by temperature) and calcium ions. In alginate, integrity of the hydrogel bulk is lost once calcium ions were removed but in gellan gum, even with the loss of ionic linkages, the hydrogen bonding remaining could still attenuate gel bulk disintegration. This suggests that encapsulated cells could be easily recovered from alginate hydrogel by treatment with citrate to remove calcium ions from the matrix [68-70] but full degradation of gellan gum is not possible.

In general, gellan gum gellates at too high a temperature for cell encapsulation that restricts its applications. To circumvent this problem, Gong et al have lowered the gelling temperature by decreasing the molecular weight of the gellan gum through repeat oxidation with NaIO_4 [71]. It was shown that gelation temperature could be adjusted to as low as 27°C , suitable for cell encapsulation. In addition, hydrogel degradation was much more significant in gellan gum after 150 days compared to agarose. This facilitates higher GAG and collagen type II deposition into the ECM matrix in gellan gum constructs than agarose as well.

1.2.2.2 Enzymatic degradation of hydrogels

To facilitate tissue morphogenesis, cells secrete proteases like MMP, driving changes in ECM properties and structure[10]. Several strategies have been investigated to mimic the proteolytic sensitivity of ECM in hydrogel scaffolds. The approaches commonly involve incorporation of enzyme substrate as the bioresponsive component in the gel matrix for degradation. Ideally, degradation rate of the hydrogel should be closely related to tissue formation rate so that it remains mechanically stable until the newly developed tissue attains sufficient integrity. Furthermore, it is important to note that degradable hydrogel should be cyto-compatible both before and after degradation; that is to say that the hydrogel can support cell viability and its degradation product would not induce cytotoxicity. Chien and co-workers have fabricated a degradable carboxybetaine (CB) hydrogel, functionalized with RGD for encapsulation of fibroblasts. The disulfide-containing crosslinker within the CB hydrogel served as cleavage site for degradable when exposed to biological reducing agents like glutathione or cysteine. Fibroblasts proliferated within CB hydrogel and when the cells remained viable after recovering from hydrogel degradation, suggesting degradation products do not affect cell viability[19].

Brandl et al. have functionalized PEG based hydrogel with collagenase sensitive peptide (Ala-Pro-Gly-Leu) and laminin derived peptide, YIGSR, for preadipocytes culture[21]. During adipose tissue formation, MMPs is expressed by the adipocytes to facilitate remodeling. In non-degradable gels, isolated

adipocytes containing multiple oil droplets were observed after induction. In contrast, intracellular lipid droplets and many unilocular cells (a feature of mature adipocytes) were present in enzymatically degradable PEG hydrogel, suggesting a more favorable microenvironment for differentiation of adipocytes[21]. Besides applications in adipose tissue engineering, Kraehenbuehl and colleagues have also employed MMP responsive peptide, thymosin β 4, in PEG hydrogel for vascular development. The presence of thymosin β 4 significantly induced MMP-2 and MMP-9 secretion in encapsulated HUVEC which subsequently accelerated localized hydrogel degradation and matrix remodeling[23].

The success of cell-mediated scaffold degradation is also demonstrated in cartilage tissue engineering. Human MSCs were encapsulated in MMP7 peptide substrates containing PEG-based hydrogel. As chondrogenesis progresses, MMP7 secreted by differentiating MSCs gradually degrades the hydrogel matrix, making room for neocartilage formation[52]. In another study, Anderson et al have fabricated cell-adhesive PEG hydrogels using thiol-ene photopolymerization incorporated with various concentrations of MMP sensitive peptides to achieve different degrees of hydrogel degradation[72]. MSCs encapsulated in these hydrogels were directed toward chondrogenesis, osteogenesis and adipogenesis respectively. It was shown that cell-degradable hydrogel increases GAG and collagen deposition in chondrogenic MSCs; greater alkaline phosphatase activity in osteogenic MSCs and lipid droplets in adipogenic MSCs. Both studies strongly suggest that cell-responsive degradable

hydrogel which permits dynamic cellular remodeling of the ECM and scaffold favors differentiation and morphogenesis.

1.3 Hydrogel/microspheres (GS) composite as bioresponsive hydrogel

Besides direct manipulation of the hydrogel, a combination of two scaffold systems – hydrogel / microspheres (GS) composite was developed as a bioresponsive system. By employing the microspheres as cell-affinitive interfaces for cell attachment [7, 8, 73] or degradable moieties [69, 74] within hydrogels, the microspheres played act as the bioresponsive entity within the composite model. By carefully selecting the materials for establishing the GS model, a variety of properties could be acquired.

1.3.1 Gelatin microspheres as cellular anchors in GS system

In this system, microcarriers could be used as cellular ‘anchors’ to support cell adhesion and stretching on their spherical surfaces while the hydrogel served to immobilize the cells within the operation site. Settlement and commitment of osteo-progenitor cells were significantly enhanced in the GS model, as compared with those in plain agarose hydrogels, suggesting promising perspective of such composites for ADC delivery. However, fabrication of the microcarrier used in the previous GS system requires a series of highly complex reactions, accompanied with the use of strong chemical agents; and more importantly, the materials (polysaccharide) used for microsphere preparation was not recognized as bio-degradable, which would substantially limit the spatial growth / migration of cells within the hydrogel bulk. Therefore, new

microcarrier types with properties that can address these concerns are in great demand.

The greatly expedited understanding in matrix biology in the last decade has led to the development of bio-degradable and bio-responsive materials as tissue engineering scaffolds[75, 76]. For example, gelatin, one of the classical natural polymers used for tissue regeneration, has been found to contain both cell-affinitive and enzyme-cleavable domains[77]. It provides choices as the building blocks of novel microcarriers [77-79]. However, one disadvantage of gelatin is that it dissolves in water at physiological temperature and thus needs crosslinking treatment to maintain the demanded shape of scaffolds; but the most common crosslinking agents such as glutaraldehyde (GA) may lead to apparent cytotoxicity [80]. Therefore, aiming to harness the versatile advantages of gelatin without compromising on safety issues, researchers have developed natural, low-toxic crosslinking reagents such as genipin as a substitute for GA [80-84].

1.3.2 Genipin as a crosslinker for gelatin microspheres

Genipin is isolated from the fruits of *Gardenia jasminoides Ellis*, possessing some benign bio-active activities, and has shown encouraging potentials in crosslinking collagen or gelatin-based scaffolds [80]. The abundance of amino groups in the gelatin has enabled the crosslinking reaction between genipin and gelatin. This reaction results in a blue pigmentation which is due to the reactions between genipin and the amino acids on the gelatin. The suggested

mechanism by Touyama et al for the formation of the genipin-methylamine monomer is via a nucleophilic attack by methylamine on the olefinic carbon at C-3 in genipin. This is followed by the opening of the dihydropyran ring in the genipin and an attack by the secondary amino group on the resulting aldehyde group. The blue-pigment polymers are most likely formed through the oxygen radical-induced polymerization and dehydrogenation of many intermediary pigments [85-87].

In a study done by Liang and colleagues, they have prepared genipin crosslinked microspheres and transplanted them into a rat model[80]. It was found that compared to GA crosslinked microspheres, genipin crosslinked microspheres have significantly less inflammatory cells on the transplanted microspheres. This implies that genipin indeed possesses higher biocompatibility than glutaraldehyde.

1.3.3 Gelatin microspheres as porogens in GS system

Traditionally, degradable microspheres were used as a delivery vehicle for controlled release drugs [88, 89]. It is only recent years they were employed solely for the purpose of creating cavities within scaffolds. Other choices of material for porogen includes as soluble salts[90], microbubbles[91], ice crystals[92], and gelatin microspheres [69, 93, 94]were chosen as porogens in macroporous scaffold fabrications. The general idea is that the porogen dissolves while the scaffold material remains intact upon exposure to certain

conditions. It can be an introduction of a solvent to leach/dissolve the porogen or it could be as simple as a change in temperature, melting the porogen.

Given that gelatin microsphere readily dissolves in water at temperature of 37°C, it is a convenient porogen employed in GS composite system. By creating cavities within the hydrogel, it is proved that diffusion efficiency is enhanced because diffusion distance is drastically reduced[9]. In addition, our lab has also reported that the cavities in hydrogel bulk provided space for mediating neo-tissue islet formation [69, 74]. Compared to the plain hydrogel set up, higher cell density and ECM deposition were observed in the GS system.

1.4 Objective and organisation of this dissertation

The overall objective of this thesis project is to set up a GS composite model as a bioresponsive system for both delivery and culture of various cell types in tissue engineering. The major challenge in cell delivery is that different cell types demand different conditions for optimal cell growth and differentiation and generally a single scaffold system could not fulfill all requirements needed by the cells. For instance, in the delivery ADCs, presence of RGD moieties within the GS system is important for cell survival and it cannot be provided in simple hydrogel model. On the other hand, non-ADCs could be directly encapsulated within the hydrogel bulk to attain the desired rounded morphology. However, cell growth could be restricted both spatially and biochemically in the hydrogel bulk and long term culture of the cells in this system is not ideal.

Hence, the combination of two scaffold systems (hydrogel and microspheres) harnesses the advantages from each system and integrates them together to offer greater flexibilities for various cell type deliveries. Based on the target cell to be delivered, specific modifications of the GS system could be made to meet the demand of the cells to ensure maintenance of cell viability and phenotype. In this work, alginate was chosen as the gel component while gelatin microsphere was selected as spheres component in the GS system. Based on these materials, we aimed to develop three different GS systems for delivering of model cell lines, primary functional cells, and stem cells.

In the delivery of model cell lines, liver carcinoma cell, HepG2 and typical ADCs like osteoblast and mesenchymal stem cell (MSC) were chosen. Bioactive GS system is designed to use microspheres as a cell attachment site and a cell carrier. Given that gelatin microspheres readily dissolves in water at physiological temperature, crosslinking of gelatin microspheres was done to establish a stable cell attachments site and carrier for subsequent GS system. In the special case of HepG2 cell, since it possesses some features of typical liver cells, in particular, the spheroid formation properties, we designed an enzymatically degradable GS system where the microspheres played the role of a transient cell carrier and porogen so as to create space for cellular aggregate formation within the hydrogel.

We have also designed a third kind of GS system where microspheres were used solely as porogens to create cavities in the hydrogel bulk. This set up is thermally responsive as gelatin microspheres dissolve upon exposure to

physiological temperature. Unlike the previous set up, cells are encapsulated in the hydrogel instead of delivered via the microspheres. Hence, the target cells for delivery are primary cell - chondrocytes and pluripotent stem cell as they are well-known to prefer rounded morphology than the spreading out form. When compared with a simple hydrogel model, this cavitory GS system is proved to be better in promoting cell proliferation and cellular aggregate formation.

To summarize, we have devised three distinct GS systems to deliver different cell types for cell-based therapy purposes. This dissertation consists of the following 7 chapters.

Chapter 1, the current chapter, reviews the current progress on hydrogel scaffold, in particular, design of bioresponsive hydrogels for therapeutic purposes. It highlights the strategies for incorporation of bioactive molecules into hydrogels to increase survival of typical ADC cells and biodegradable hydrogels which degrades as a result changes in physical microenvironment or exposure to enzymes. The objective and the outline the entire dissertation is presented.

Chapter 2 begins the establishment of a bioactive GS system for delivery of model cells. The role of microspheres in this GS system is to present bioactive moieties for cell attachment. Optimization of crosslinking conditions of gelatin microspheres was carried out to achieve thermally stable microspheres.

Chapter 3 investigates the potential of the genipin crosslinked microspheres in mediating cellular aggregate formation in HepG2 cells. This

study elucidates the effect of substrate curvature on cell growth and proliferation in HepG2 cells.

Chapter 4 reports an enzymatically degradable GS composite system where the microspheres are both cell carriers and porogens in the system. In this chapter, cell-laden microspheres were encapsulated in alginate hydrogel and the microspheres were subsequently degraded via the introduction of collagenase, creating a microcavitary hydrogel (MCG) construct. The ability of the MCG construct in mediating the formation of cellular aggregates was investigated.

Chapter 5 discusses the third GS system for delivery of primary cell – chondrocyte. This GS system is designed to be physically responsive as gelatin microspheres dissolve upon exposure to physiological temperature, establishing a MCG construct. In contrast to all previous studies, cells were delivered in the gel phase rather than through microspheres. The favorable environment presented by this MCG construct allows great extent of neo-tissue formation and as a result, a scaffold-free living hyaline cartilaginous graft (LhCG) could be established. The focus of this chapter is placed on studying the potential application of the scaffold-free LhCG as an endochondral stem cell niche for bone formation.

Chapter 6 extends the application of the physically responsive GS system to pluripotent stem cells – embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). To determine the efficacy of this GS system, ESCs and iPSCs were encapsulated respectively and differentiated toward liver lineage.

Chapter 7 would draw conclusions from all the above findings and suggest possible future work.

Chapter 2 : Cell delivery with Genipin crosslinked gelatin microspheres in hydrogel/microcarrier composite

2.1. Introduction

As mentioned in chapter 1, hydrogels have been extensively used as cell-laden vehicles for engineered therapeutic transplantation in regenerative medicine [95, 96]. However, the frameworks of most common hydrogels do not support optimal cell adhesion and outstretching but confine the encapsulated cells into a spheroidal shape. Recently, a composite model, namely hydrogel / microspheres (GS), was developed to overcome this problem by providing cell-affinitive interfaces for cell attachment within hydrogels [8]. In this system, microcarriers were used as cellular ‘anchors’ to support cell adhesion and stretching on their spherical surfaces while the hydrogel served to immobilize the cells within the operation site. Generally, fabrication of the microcarrier involves a series of highly complex reactions, accompanied by the use of strong chemical agents; and more importantly, the materials (polysaccharide) used for microsphere preparation were not recognized as bio-degradable, which would substantially limit the spatial growth / migration of cells within the hydrogel bulk.

Therefore, in this chapter, we attempted to use naturally derived gelatin as a biodegradable material for fabrication of microspheres[77]. The biggest

disadvantage of gelatin is that it dissolves in water at physiological temperature and thus needs crosslinking treatment to maintain the demanded shape of scaffolds; but the most common crosslinking agents such as glutaraldehyde (GA) may lead to apparent cytotoxicity [80]. Therefore, aiming to harness the versatile advantages of gelatine without compromising on safety issues, researchers have developed natural, low-toxic crosslinking reagents such as genipin as a substitute for GA [80-84]. Genipin is isolated from the fruits of *Gardenia jasminoides Ellis*, possessing some benign bio-active activities, and has shown encouraging potentials in crosslinking gelatin-based scaffolds [80]. Nevertheless, genipin has seldom been used for preparing cell microcarriers, and these gelatin-based microcarriers have scarcely been employed to support ADC settlement in 3D hydrogel matrices. Hence, in this study, we aimed to develop a genipin crosslinked microcarrier, which preserves the well-acknowledged superiorities of gelatine, such as cytocompatibility and biodegradability, while minimizing the potential cytotoxicity of residual crosslinkers. We expect such a microcarrier, serving as cellular anchors, to be capable of delivering ADC like osteoblast and mesenchymal stem cell in an outstretched morphology and high viability within 3D hydrogel microenvironments.

2.2 Materials and Methods

2.2.1 Gelatin spheres fabrication

Unless stated otherwise, all reagents were purchased from Sigma Aldrich, Singapore. Gelatin microspheres were prepared by oil in water in oil (o/w/o) double emulsion method. 10wt % gelatin solution was made by dissolving gelatin in water and heating it to 70°C to fully dissolve all the solutes in water. 30 ml of the warm gelatin solution were added to 10ml of ethyl acetate and the mixture was stirred at 700 rpm for 1 minute to form the first emulsion. After which, 60ml of edible oil was added as the second oil phase and was stirred at 350rpm for 1.5 minute. The mixture was then transferred to a cool water bath and maintained at the same stirring rate for another 15 min before cooling in ice-cold ethanol for 10 min. The microspheres formed were washed with alternating dioxane and acetone solutions to remove the edible oil. The gelatin spheres collected were dried at 70°C in the oven before sieving them into various sizes.

2.2.2 *Preparation of Genipin crosslinked microspheres*

Gelatin spheres of size range (75- 160µm and 200-300µm) were selected after sieving. One gram of the dried gelatin spheres were dispersed in 5 ml of aqueous ethanol of various concentrations (100%, 90%, 80% and 50%) containing 0.5wt% of genipin. The microspheres were kept in the 4°C fridge for the first 24 hours and were either transferred to a 37 °C incubator or remained at 4°C for another 24 – 36 hours to investigate the effects of temperature and time on the degree of crosslinking in the gelatin spheres. The microspheres were

washed against ethanol thrice and dried in a 100°C oven for 3 hours. All microspheres were then hydrated in DMEM/F12 medium before use.

2.2.3 *Cell seeding on microspheres*

Human fetal osteoblasts (hFOB 1.19) were purchased from American Type culture Collections (ATCC, Manassas, VA, USA) and were cultured under proliferative condition[8, 97]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1, Invitrogen, Carlsbad, CA, USA) medium containing 1.5mM L-glutamine (ATCC), 0.3mg/ml G418 (PAA Laboratories, Pasching, Austria) and 10% (v/v) fetal bovine serum (FBS, 'Gold', PAA Laboratories) at 33.5 °C. Human mesenchymal stem cells (hMSCs) were purchased from Cambrex (East Rutherford, NJ, USA) and expanded over multiple passages in mesenchymal stem cell growth medium (MSCGM, Cambrex) to prevent unexpected differentiation before induction. The cells (passage 6) were then maintained in DMEM, 10% (v/v) FBS and 100units ml⁻¹ penicillin and 100µg ml⁻¹ streptomycin. These cell-related reagents were purchased from PAA laboratories. For cell seeding, each well of the 24-well tissue culture plates was coated with 300 µl of 1wt% of agarose gel. Approximately 0.4 g of genipin crosslinked big spheres (200-300µm) or small spheres (75-160 µm) were added onto the gel in each well of the tissue culture plate. With the cell having reached 70-80% confluence in the culture flask, they were trypsinized and re-suspended in media at a concentration of 5 x 10⁶ cells/ml. 300µl of this cell suspension were then added to each well were then

cultured in the incubator in proliferative condition of 33.5°C for osteoblasts [97] and 37°C for hMSCs.

2.2.4 Cell loading efficiency

Osteoblasts seeded onto the microspheres at Day 0 and were cultured for 2 days for maximum cell attachment. At Day 2, the medium or the supernatant was gently collected and unattached cells were counted using a hemocytometer.

The loading efficiency was calculated using the formula below:

$$\text{Cell loading efficiency} = \frac{C_t - C_n}{C_t} \times 100\%$$

where C_t is the total number of cells seeded and C_n is the number of cells that did not attach on the microspheres.

2.2.5 Cell viability testing

The osteoblasts were tested for viability and proliferation at Day 1, 3 and 7. For qualitative data, Live/Dead® cell viability kit (Invitrogen, Molecular Probes®) was used. For every milliliter of cell culture medium, 0.5µl of calcein-AM and 2µl of ethidium homodimer-1 were added. The live (green) and dead (red) cells were observed after 30 minutes of incubation at 37°C and sufficient PBS washing. For quantitative evaluation, WST-1 [8 Basel, Switzerland] colorimetric assay was used. The incubation lasted for 2 h and the absorbance at 450nm was measured with reference to 620nm in a microplate reader.

2.2.6 Encapsulation of cell-laden microspheres in hydrogel

To evaluate whether microcarriers can support cell adhesion and stretching in the 3D hydrogel bulk, the cell-laden microspheres were gently placed in a silica- rubber mold after 1-3 days of culture. 1.2 ml of liquefied low gelling-point agarose gel (w/v 1.25%. Type VII, Sigma-Aldrich) was equilibrated at 37.5°C and was added to the cell-laden microspheres in the mold (2cm × 2 cm). The agarose precursor was allowed to cool down at room temperature in which gelation would take place, encapsulating the cell laden microspheres. After gelation, the hydrogel was transferred to tissue culture plate and cultured with the appropriate medium.

2.3 Results and Discussion

The abundance of amino groups in the gelatin has enabled the crosslinking reaction between genipin and gelatin. This reaction results in a blue pigmentation which is due by Touyama et al for the formation of the genipin-methylamine monomer is via a nucleophilic attack by methylamine on the olefinic carbon at C-3 in genipin. This is followed by to the reactions between genipin and the amino acids on the gelatin. The suggested mechanism the opening of the dihydropyran ring in the genipin and an attack by the secondary amino group on the resulting aldehyde group. The blue-pigment polymers are most likely formed through the oxygen radical-induced polymerization and dehydrogenation of many intermediary pigments [85-87].

Figure 2.1: Gelatin microspheres after 48 hours in 4°C (A) 100% EtOH, (B) 90% EtOH (C) 80%

Temperature and solvent concentrations are critical elements in the crosslinking of gelatin microspheres. As shown in Figure 2.1, all microspheres kept at 4°C for 48 hours have no change in the color of the microspheres indicating that no observable crosslinking was initiated. In addition, at 80% ethanol concentration, small aggregations of microspheres are formed. Aggregation is more prominent in 50% ethanol concentration where microspheres formed one large aggregate demonstrating the significance of different water content in the solvent on sphere aggregation.

As presented in **Figure 2.2**, some of gelatin microspheres kept in the 37°C incubator for 24h have their color changed, from yellow to blue. However, the microspheres immersed in 100% ethanol solution containing genipin remained yellow. For 90% aqueous ethanol, there was a change in the color of microspheres to blue color and in 80% aqueous ethanol, the aggregations of microspheres turned dark purple in color. In **Figure 2.2**, there is a progressive color change of the gelatin microspheres from light blue to dark purple as the time increases indicating the increasing of crosslinking degree.

The importance of temperature for crosslinking to occur is illustrated in **Figure 2.1** where no cross linking is initiated at all concentrations of ethanol at 4°C. Other than temperature, the concentration of the solvent (ethanol concentration) also plays an essential role. The high aggregations seen at 80% ethanol concentration in **Figure 2.1C and 2.2D** suggest that the water content in the solvent would determine the level of aggregations. We hypothesized that since gelatin is hydrophilic, it attracts the water molecules in the solvent which acts as an adhering molecule between microspheres and causes the microspheres to aggregate. As a result, higher water content leads to greater aggregation (as observed from severe aggregations at 50% ethanol concentration) and this is unfavorable as this reduces the effective surface area of the microspheres for cell attachment.

Figure 2.2: (A) Gelatin microspheres after 24 h cross-linking with genipin at 37 °C in various concentration of ethanol (Left to right: 100%, 90%, 80% and 50%) (B-D) Photomicrographs of gelatin spheres in 100%, 90% and 80% ethanol concentration respectively. No picture is available at 50% ethanol concentration due to heavy aggregation. Scale bar applies to B, C and D.

Water content determines not only the degree of aggregation but also degree of crosslinking. Gelatin is hydrophilic; water is needed to bring the

cross-linker, genipin, into contact with the gelatin microspheres. In the absence of water like in the case of pure ethanol as solvent, genipin was not able to come in contact with the gelatin therefore no cross-linking occurred. At 90% or 80% ethanol concentrations, crosslinking took place to various degrees due to the presence of water. Higher water content means gelatin microspheres could be exposed to genipin at a faster rate allowing a greater degree of crosslinking to happen in a given time duration.

Since 50% and 80% aqueous ethanol solutions give rise to fast crosslinking rates but promote aggregations; while pure ethanol prevents both aggregation and crosslinking to take place, 90% aqueous ethanol concentration was chosen to fabricate genipin crosslinked microspheres. At this concentration, it provides a reasonable cross linking rate with insignificant aggregations. To investigate the different degrees of crosslinking as a function of time, the microspheres are observed at different time points in the 37°C incubator as shown in **Figure 2.3**. The progressive change in color intensities illustrates crosslinking is taking place and the maximum cross linking was achieved at 36 hours since no further change in color intensity was observed between 36 to 48 hours. Therefore, the microspheres cross linked for 36 hours were chosen for further study.

Figure 2.3: Small gelatin spheres after (A) 14 h (B) 24 h (C) 36 h and (D) large spheres after 36 h (E) Small spheres at 48 hours. Images showed that no further change in color intensity after 36 hours.

Figure 2.4: Cell loading efficiency onto microspheres after 2 days

Figure 2.5: Live/dead staining of osteoblast attached on (A) small (B) large microspheres after 1 day

Figure 2.6: Cell proliferation of osteoblast seeded on Genipin crosslinked microspheres using WST-1 colorimetric assay

Osteoblasts were used as model cell in the examining of the genipin cross linked microspheres as a potential microcarrier. Cell loading efficiencies and cell viabilities are some of the fundamental criteria in the evaluation of cell microcarriers. It is presented in **Figure 2.4** that the genipin crosslinked microspheres were able to achieve high cell loading efficiency of at least 98% regardless of their sizes and both spheres sizes have relatively the same loading efficiency. As shown in **Figure 2.5**, all the cells attached on the microspheres of both sizes are viable. There is no dead cell present which coincides with the results obtained from the WST-1 assay in **Figure 2.6**. The assay illustrates the stable viable cell number even after 7 days compared to day 3, indicating the conducive environment provided by the microspheres. These results showed that both microsphere sizes could be used as an injectable cell microcarrier delivering highly viable cells in a stretched out morphology. It is also noted that in **Figure 2.5**, there is a difference in cell morphology on different microspheres sizes. The cells on the large microspheres (200-300 μ m) adopted a more spreading out morphology compared to those on the small microspheres (75-160 μ m). Consequently, this makes the cell-cell interactions in the small microspheres more significant and hence favorable in the delivery of cellular aggregates [98]. On the other hand, that cells exhibit the desired stretched morphology on large microspheres suggests the presence of a conducive environment for cell anchorage and growth.

Since hMSCs are multipotent and have potential therapeutic effects, they are used as the target delivery cell to investigate the maintenance of cells

in the stretched out morphology using microspheres encapsulated within the 3D hydrogel bulk. As shown in **Figure 2.7**, the cells are highly viable on both small and large microspheres and the encapsulation of hMSCs-laden microspheres into the agarose gel resulted in a clear maintenance of the stretched out cell morphologies within the hydrogel after 2 days of culturing. Comparing to encapsulating mono-dispersed cells in the hydrogel which results in poor cell survival [8], our microcarrier-hydrogel system proves to be superior over this pure gel encapsulation model. Moreover, we have demonstrated the potential of the genipin cross linked microspheres in providing ‘anchors’ for cell attachment in the 3D hydrogel environment.

Figure 2.7: Live/dead staining of hMSC on small microspheres (A) 40X (B) 100X and Large microspheres (C) 40X (D) 100X 2 days after encapsulated in agarose hydrogel

2.4 Summary

In summary, our study has shown that genipin crosslinked microspheres are capable of maintaining high cell viability in the hydrogel scaffold by posing the encapsulated cells in fully focal adhesive morphology. Various important parameters affecting the crosslinking treatment for microspheres fabrication have been optimized. In this newly developed GC system, we have demonstrated that the crosslinked gelatin microspheres could provide ‘anchors’ within the hydrophilic 3D hydrogel environment and hence maintaining the high cell viability and morphology of the target cell. In the future, we expect to investigate the cell-cell and cell-matrix interactions in this microcarrier-hydrogel system so as to explore the possibility of extending this GC composite system onto other cell types such as hepatocytes and adipocytes.

Chapter 3 : Genipin crosslinked microcarriers mediating hepatocellular aggregates formation and functionalities

3.1 Introduction

In Chapter 2, we have successfully fabricated genipin crosslinked gelatin microspheres for delivery of typical ADCs. In this chapter, we will make use of the same microspheres for culturing of another model cell, HepG2. In the area of liver therapeutic research, several research has shown that hepatocytes exhibit superior functionality and maintenance of high viability in cellular aggregates as compared to two dimensional (2-D) monolayer or mono-dispersed cells[99-102]. In liver cell culture, hepatocytes require substrate for attachment to avoid apoptosis. When this condition is satisfied, they could survive and proliferate and when cell number is sufficiently high, functional cell aggregates would be formed. Hence, we proposed to use microcarriers, which are capable of forming three-dimensional (3D) matrices with inter-spherical space, to provide cell adhering substrate and promote the formation of cell aggregates in liver tissue engineering.

Microcarriers of various materials such as dextran, gelatin and collagen are commercially available and have been proved to be useful in various tissue engineering applications[79, 103-105]. The size of microcarriers is limited to 150-400 μm as it is the optimal size range for cell delivery. Owing to their size

range, this platform still enjoys the convenience of injectability and therefore greatly facilitates implantation. Microcarriers could also provide cellular anchors for cell attachment: given the high surface area to volume ratio, large scale cell expansion could be possible. Most importantly, unlike those encapsulated in hydrogel, cells attached on microcarriers are not spatially constrained, which implies the ample room for growth.

In this study, we have chosen genipin crosslinked gelatin microspheres within optimal size range to culture human liver carcinoma cell line (HepG2). The abundance of cell adhesive moieties on gelatin chain makes it a superb candidate for cell attachment; however, due to the water soluble nature of gelatin, pre-treatment with crosslinking agents is necessary before it could be employed in engineering applications. In comparison with most commonly used crosslinkers such as glutaraldehyde and formaldehyde that are not ideal in terms of biocompatibility, genipin - a natural crosslinker derived from the fruits of *Gardenia jasminosides Ellis* - has been reported as a suitable replacement due to its low cytotoxicity proven by its long history of use in herbal medicine[80, 106]. Collectively, the genipin crosslinked gelatin microcarrier presented in this study could promisingly promote scaffold-free liver cell aggregate formations.

3.2 Materials and Methods

3.2.1 Gelatin spheres fabrication

Unless stated otherwise, all reagents were purchased from Sigma Aldrich, Singapore. Gelatin microspheres were prepared by oil in water in oil (o/w/o) double emulsion method that was mentioned in section 2.2.1.

3.2.2 Preparation of Genipin crosslinked microspheres

Genipin crosslinked microspheres were fabricated based on the same method described in our previous work[107]. Briefly, one gram of the dried big (BS, 200-300 μ m) and small (SS, 74-165 μ m) gelatin spheres were dispersed in 5 ml of 90% aqueous ethanol containing 0.5wt% of genipin (Wako, Japan). The microspheres were kept in the 4°C fridge for the first 24 hours and were then transferred to a 37 °C incubator for another 36 hours. The microspheres were washed against ethanol thrice and dried in a 100°C oven for 3 hours. All microspheres were then hydrated in Dulbecco's Modified Eagle Medium (DMEM) cell culture medium before use.

3.2.3 Cell seeding on microspheres

HepG2 cell was purchased from American Type culture Collections (ATCC, Manassas, VA, USA) and were maintained in DMEM containing 1.5mM L-glutamine (ATCC), 10% (v/v) fetal bovine serum (FBS, 'Gold') and 100 units-mg/ml penicillin–streptomycin at 37 °C. Unless stated otherwise, all cell related reagents were purchased from PAA Laboratories. For cell seeding, each well of the 24-well tissue culture plates was coated with 300 μ l of 1wt% of agarose gel. Approximately 0.4 g of genipin crosslinked big spheres (200-300 μ m) or small

spheres (75-160 μm) were added onto the gel in each well of the tissue culture plate. With the HepG2 cells having reached 70-80% confluence in the culture flask, they were trypsinized and resuspended at a concentration 5×10^6 cells/ml of media. 300 μl of this cell suspension were then added to each well and cultured together with the microspheres in the incubator at 37 °C for 24 hours. After which, the cell laden microspheres were transferred onto the strainer of mesh size 40 μm at Day 0 (**Figure 3.1**).

3.2.4 Cell loading efficiency

After transferring the cell-laden microspheres onto the strainer, any unattached cells would fall through the strainer. The medium or the supernatant was gently collected and unattached cells were counted using a hemocytometer. The cell loading efficiency was calculated using the formula below:

$$\text{Cell loading efficiency} = (C_t - C_n) / C_t * 100\%$$

where C_t is the total number of cells seeded and C_n is the number of cells that did not attach on the microspheres.

Figure 3.1: Schematic illustration of cell-laden genipin crosslinked gelatin microspheres. In Step 1, genipin is dissolved in 90% aqueous ethanol solution and gelatin microspheres are added. After 24h in 4°C fridge and subsequently 36h at 37°C in the incubation, genipin crosslinked microspheres were obtained via oxygen radical-induced polymerization and dimerization forming intramolecularly and intermolecularly crosslinked products with a heterocyclic structure [108]. In Step 2, HepG2 cell suspension were added to genipin crosslinked microspheres in a agarose pre-treated well and incubate overnight for maximum cell attachment. It is then transferred to a strainer of mesh size 40µm where non-attached cells would fall to the bottom of the well.

3.2.5 Morphology of HepG2 on microcarriers

Scanning electron microscopy (SEM, JSM 6390LA, JEOL Ltd., Tokyo, Japan) was used to examine the cell-laden microcarriers. Briefly, the microcarriers were harvested and preserved in glutaraldehyde (2.5%) for at least 2 hours. They were then dehydrated in gradient ethanol solutions and further dried in vacuum overnight. Platinum was sputtered for SEM contrast.

3.2.6 Liver-Specific Functions

HepG2 cells on the microcarriers were also tested for liver-specific functions such as albumin secretion. The culture supernatant of both big and small microcarriers were collected at various time points and were determined colorimetrically using commercial albumin test kit (Bio-Quant).

3.2.7 Cytotoxicity of genipin microspheres degradation product

Matrix metalloproteinases 9 (MMP-9), also known as type IV collagenase were dissolved in 6ml of culturing medium to obtain a concentration of 100µg/ml or 50µg/ml. The solutions were filtered with before adding 0.1g genipin crosslinked small spheres respectively. The mixture was left overnight in the incubator at 37°C to degrade the microspheres and release genipin from the microspheres. The supernatant were then collected respectively and incubated with HepG2 cells to test for any cytotoxicity effect. The viability of the cells were assess on day 1, 3 and 7 using WST-1 assay.

3.2.8 Statistical analysis

Where appropriate, ANOVA was performed to analyze results and $P < 0.05$ was considered to indicate a statistically significant difference. Data presented as mean $\pm$ SD.

3.3 Results

3.3.1 Cell loading efficiency

After 24 hours of incubation of HepG2 cell with the gelatin microspheres, the cell loading efficiency was calculated. It is presented in **Figure 3.2** that SS have higher cells loading efficiency (64%) compared to BS (52%).

Figure 3.2: Graph of HepG2 cell loading efficiency on microcarriers

3.3.2 Cell viability and proliferation

There is no significant difference in cell proliferation between BS and SS for the first 7 days as illustrated in both **Figure 3.3 and 3.4A-D**. On day 17, WST-1 assay revealed differences between the SS and the BS samples. The cells cultured on SS have relatively lower proliferation rate compared to those on the

BS. At the same time, the live/dead staining of the cells on the microcarriers depicts formation of cell aggregates on day 17 (**Figure 3.4E and F**). As presented in **Figure 3.4E and F**, the cells cultured on both BS and SS have proliferated rapidly and there were formation of inter-spheric HepG2 aggregates. It is also notable that most of the microspheres were fully embedded within the cell aggregate in BS while in SS, the cell aggregate were smaller and less dense with microspheres still visible.

Figure 3.3: Cell proliferation profile of cells on microcarriers using WST-1 assay.

*p<0.05 between BS and SS

3.3.3 Morphology of HepG2 on microcarriers

The morphology of HepG2 on the genipin crosslinked microspheres was assessed using SEM. As presented in **Figure 3.5**, numerous cells attached on the microcarriers regardless of sphere sizes and cell density on the microcarriers

increased significantly on day 20 (**Figure 3.5B and D**) when compared to day 7 (**Figure 3.5A, C**). In particular, large pure cell aggregates were observed in the BS sample as shown **Figure 3.5D** while no such aggregates was seen in SS.

Figure 3.4: Live/dead staining of the cells on small (A,C,E) and big (B,D, F) microspheres on Day 3 (A-B), Day 7 (C-D) and Day 17 (E-F)

Figure 3.5: SEM micrographs of small microspheres (A-B) and big microspheres (C-D) on day 7 (A, C) and Day 20 (B, D)

3.3.4 *Liver-Specific Functions*

The percentage increase in albumin secretion in the cells on microcarriers was presented in **Figure 3.6** respectively. The percentage increase displayed a gradual rise with a peak at around day 7 and then decreased from day 10 onwards. The percentage increase in albumin secretion was higher in SS on day 3 but from day 7 onward; BS consistently had a slightly higher percentage increase than SS.

Figure 3.6: Relative percentage increase in albumin secretion of HepG2 on microcarriers at various time points. * indicates $p < 0.05$ between BS and SS

3.3.5 Cytotoxicity of genipin microspheres degradation product

As shown in the WST-1 assay in **Figure 3.7**, the supernatant from the degradation of genipin microspheres using MMP-9 have no negative effects on the cell viability and proliferation rate as compared to the control. There is

maintenance of high cell viability in cells cultured in supernatant from genipin microspheres degradation using MMP-9 at 100 μ g/ml and 50 μ g/ml throughout 7 days of the experiment as illustrated in **Figure 3.7**.

Figure 3.7: Cell proliferation profile of HepG2 cultured in supernatant of microspheres/MM-9 using WST-1 assay. * $p < 0.05$ compared with the control group

3.4 Discussions

Most commercial microcarriers are fabricated with an optimal size range of 150-400 μ m to favor cell proliferation[103] and also to facilitate injection. In this study, gelatin microcarriers selected in our experiment are within this size range: SS are in range of 74-165 μ m diameter and the BS are in the range of 200-300 μ m. Amino groups present or pendent on gelatin backbone react with

genipin and thus crosslink altogether. The blue pigmentation showed in **Figure 3.1** is a result of oxygen radical-induced polymerization and dehydrogenation of many intermediary pigments as proposed by Touyama et al.[85, 86], which indicates the success in crosslinking.

Cell loading efficiencies and cell viabilities are key criteria in assessment of cell microcarriers. The observed higher cell loading efficiency in SS is believed to be due to the greater number of microspheres - thus the greater surface area - present in the same mass of microspheres compared to BS counterparts. The results of WST-1 assay as indicated in **Figure 3.3** and the live/dead staining as indicated in **Figure 3.4** clearly showed that the both microspherical sizes are suitable for HepG2 cell survival and growth. In particular, spontaneous formation of viable inter-spherical cell aggregates are observed from both BS and SS samples on Day 17. General increase in cell number, also as illustrated in both WST-1 assay and live/dead staining, again confirms the favorable environment presented by the genipin-crosslinked microcarriers of both sizes. The cell aggregates on BS are comparably bigger and denser than those on SS.

The morphology of cells cultured on microcarriers was monitored with time. During the first few days of cell culture, the HepG2 cells settled and attached on the microcarriers. From Day 3 through Day 10, the cells proliferated and increased in number on both microspheres. After approximately 12 days, cell aggregates began to form on both types of

microspheres. Further culture of the cell aggregates on these microspheres leads to variations in observations. Large and dense cell aggregations are formed on BS, as indicated in **Figure 3.5E and F**; but the same scaled phenomenon did not take place on SS (**Figure 3.5B**) after the same (17) days of culture. With regards to the liver-specific functions, the cells cultured on both BS and SS showed fine maintenance in albumin secretion which the performance of BS samples was again appreciably better than that of SS samples. Urea synthesis being one of another important feature of hepatocytes is believed to be deficient in this cell line[109], albeit from a number of studies, trace amount of urea production and variation had been detected [110-114]. In this study, minute amount of urea was found in both BS and SS samples (data not shown) and is insignificant compared to the amount found in hepatocytes.

Although both BS and SS have shown to mediate cell aggregate formations, there are dissimilarities in cell proliferation rate and liver-specific functions. These differences are believed to be due to different substrate curvatures provided by each microcarrier. Substrate curvature is one of the topographical cues presented by the microcarriers and research have shown that curvature plays a significant role in cellular processes such as neurite outgrowth[115, 116] and apoptosis[117]. In the studies reported by Smeal et al.[115, 116], the neurite outgrowth direction highly favors a direction with minimal curvature. This observation coincides with our results where growth was reduced in cells attached on small spheres where there is high substrate curvature. In particular, high curvature surface requires cells to bend the

microtubules to a larger extent during cell adhesion which may lead to high potential microtubule breaking. By using the relation between free energy and curvature in Odde et al's paper [118],

$$F = \frac{1}{2} EI (d\theta/ds)^2,$$

where EI is flexural rigidity with the value of $6.9 \times 10^{-24} \text{ Nm}^2$ for microtubules in the absence of MAPs and the curvature of the microspheres ($d\theta/ds$), we are able to calculate the free energy in terms of kT/dimer in the HepG2 cells on BS and SS microcarriers as shown in **Figure 3.8** [119, 120].

Figure 3.8 Graph of Free energy in relation to microsphere diameter. Indicated in the graph, the range of free energy gives the size range of the BS and SS.

The size ranges of small and big microspheres give rise to free energies of 1.7-8.4 kT/dimer and 0.51-1.15 kT/dimer respectively. Comparing these values

with literature where the mean energy for microtubule distortion is $8kT/\text{dimer}$ [118], the free energy in cells cultured on the SS is sufficient to substantially disturb the microtubules and consequently interfere with various cell processes such as proliferation rate. On the other hand, the amount of free energy in cells on BS is relatively smaller and has limited effect on cell processes. This implies that BS provides a modestly more favorable substrate condition for faster cells settlement and proliferation compared to SS even though both microsphere sizes could essentially support cells growth and aggregates formation.

Lastly, a major concern arises about the longer-term fate of the microcarriers since it could be expected that this system were to be applied in *in vivo* culture, various types of collagenases present in the body would degrade the microcarriers and release the crosslinker -- genipin. Hence, to address the issue of potential cytotoxicity from local release of genipin from such degradations, cytotoxicity effects of genipin released was evaluated. High maintenance of cell viability as displayed in **Figure 3.7** indicates that the practical release of genipin has negligible cytotoxicity on the HepG2 cells.

It is believed that this “detoxication” effect yields by conjugation of genipin to gelatin macromolecules. Once toxic small molecules such as genipin are conjugated to the backbone of non-toxic water soluble macromolecules, typically polyethylene glycol (PEG) or polypeptides, they would be substantially detoxicated[121, 122]. Similarly, since collagenase is unable to cleave the covalent bond between genipin and gelatin, but only cuts the gelatin

backbone shorter, genipin released from the degrading microspheres remains in the form of a genipin-gelatin conjugated (smaller) macromolecule that has much lower toxicity from the pure genipin compound.

3.5 Summary

In conclusion, this work has successfully demonstrated the use of genipin crosslinked microspheres to support the growth of highly viable HepG2 cells and further mediate the spontaneous formation of functional hepatocellular aggregates on different sized microcarriers. There is a detectable variation in cell proliferation rate, aggregation degree and liver specific functionalities on these differently sized microcarriers, which are believed to be a result of different substrate curvatures presented by different microcarrier sizes. Degradation products of the genipin crosslinked microcarriers have indicated negligible cytotoxicity effects on cell growth. All results from this work prospect for the use of these microcarriers in generating scaffold-free cellular aggregates for therapeutic purposes.

Chapter 4 : Formation of model hepatocellular aggregates in hydrogel scaffold using degradable Genipin crosslinked gelatin microspheres as cell carriers

4.1 *Introduction*

In the previous chapter, chapter 3, we noticed that the hepatic cellular aggregates formed on the microspheres were of irregular shapes and sizes. Irregularities would mean that cellular performances can be inhomogeneous and thus it is necessary to develop a strategy to control the cell aggregates. In this chapter, we designed a second GS system which employs microspheres as cell carriers and porogens to deliver cells and create micro-cavities within the hydrogel bulk. The hydrogel layer plays an important role to restrict the size and the shape of the cell aggregates formed; in addition, it also immobilized the microspheres at target site. Using HepG2 as the model cell, this GS system is aimed to provide prospects for future liver cell therapeutics.

Liver cell transplantation relies on introduction of mature hepatocytes[123, 124] or liver stem cells[125] into host to restore, maintain, or improve defective liver functions. Mature hepatocytes have suboptimal proliferation capacity in vitro and they rapidly lose their phenotype in two dimensional monolayer cultures. Although hepatocytes transplantation may possess immediate therapeutic effect, its clinical application is limited by the availability and quality of the cells [126]. Studies have reported maintenance of liver specific functionalities in three-dimensional culture when hepatocellular

aggregates or spheroids were formed [127-129]. In this sense, generating liver cell spheroids with controllable size and shape poses one of the key challenges in liver tissue engineering research and development. Various methodologies have been explored to aid the formation of these spheroids. Common approaches include using bioreactors[124], photolithography[130] or micropatterning [131-133]to create molds of appropriate sizes. Nonetheless, these approaches require specialized equipment in order to generate spheroids of controllable size and faced considerable difficulties in scaling-up.

In our previous study, we have demonstrated that genipin crosslinked gelatin microspheres were able to mediate the formation of HepG2 cell based model hepatocellular aggregates[114]. In addition, the aggregates generated displayed good maintenance of liver-specific functionalities. However, the previous set-up does not encompass any facility for cell/aggregates delivery and immobilization. Accordingly, we developed a new platform that makes use of degradable microspheres as cell carriers and porogens to create cavities for controllable sized hepatocellular spheroid development within the bulk of alginate hydrogel constructs. By this approach, cells are first seeded onto genipin crosslinked microspheres and these cell-laden microspheres are encapsulated in alginate hydrogel. After gelation, microspheres are disintegrated via introduction of collagenase (MMP9) so that cavities are made in gel bulk; the loaded cells are suspended and entrapped in these cavities providing space for spheroid formation while the alginate bulk serves as a confinement to restrict the size of spheroids generated within range. Since the

entire set up is simple and does not require any specialized equipment, it is economic and provides convenience in scaling up. Another advantage of this system is that by employing alginate as the hydrogel bulk, cellular spheroids could be recovered from the alginate gel bulk by sodium citrate treatment [70]. Among the polysaccharide hydrogel, alginate is the only gel system that possesses this unique property for cell recovery. Hence, scaffold-free cellular aggregates could be harvested for further analysis or subsequent investigations.

4.2 *Materials and Methods*

4.2.1 *Preparation of Genipin crosslinked gelatin microspheres*

Unless stated otherwise, all chemicals and reagents were purchased from Sigma Aldrich, Singapore. Gelatin microspheres were fabricated by oil in water in oil (o/w/o) double emulsion method as described in our previous work and in section 2.2.1 [74, 114].

To crosslink the microspheres with genipin, we adopted a similar method from our previous work[107]. Briefly, one gram of gelatin spheres (74-165 μ m) was dispersed in 5 ml of 90% aqueous ethanol containing various concentrations (0.1wt%, 0.25wt% and 0.5wt %) of genipin (Wako, Japan). The microspheres were kept in 37 °C incubator for 16 hours to allow crosslinking. The genipin crosslinked microspheres were then washed with pure ethanol three times before drying at 100°C in the oven for 3 hours. All microspheres were sterilized with 10X penicillin/streptomycin (P/S) before hydrating in Dulbecco's

Modified Eagle Medium (DMEM) cell culture medium at 4°C for long term storage.

4.2.2 *Characterization of the crosslinked microspheres*

0.05g of genipin crosslinked microspheres crosslinked to various degrees was immersed in either 5ml of Phosphate Buffer Solution (PBS) at 4°C or PBS containing matrix metalloproteinases 9 (MMP-9) at the concentration of 100µg/ml and were incubated in 37°C for 30min and 4 hours respectively to determine their stability. The integrity of the microspheres was checked under light microscope (Olympus IX71).

4.2.3 *Preparation of cell-laden microspheres*

Unless stated otherwise, all cell related reagents were purchased from PAA Laboratories. HepG2 cell was purchased from American Type culture Collections (ATCC, Manassas, VA, USA) and were maintained in standard DMEM containing 1.5mM L-glutamine, 10% (v/v) fetal bovine serum (FBS, 'Gold') and 100 units-µg/ml P/S at 37 °C. When HepG2 cells reached 70-80% confluence in the culture flask, they were detached by trypsin and re-suspended at a concentration 5×10^6 cells/ml with media. For the preparation of cell-laden microspheres, only those crosslinked in 0.25wt% genipin solution was used. For every milliliter of cell suspension, 0.13g of microspheres was added. 300 µl of the entire mixture was then added to each well of agarose gel pre-coated 24-well culture plate and kept in the incubator at 37 °C for 24 hours to allow cell

attachment to occur. The cell laden microspheres were then transferred onto the strainer of mesh size 40 μ m (BD Falcon TM) to facilitate media change [114].

4.2.4 *Fabrication of cell-laden microspheres hydrogel composite with or without MMP-9*

After cell-laden microspheres were cultured on the strainer for 3 days, they were encapsulated into alginate hydrogel to form the microcarrier-gel composite. As shown in the schematic illustration in **Figure 4.1a**, three different groups were set up, namely, control, MMP-9 in media (MM) and MMP-9 in gel (MG). For control and MM group, each milliliter of alginate solution (1.5 wt.% in 0.15 M NaCl) was gently mixed with 0.1g of cell-laden microspheres. 85 μ l of the mixture was injected into a circular mold (ϕ = 5mm) and 102mM of aqueous calcium chloride was added to facilitate gelation. The control constructs were cultured in standard DMEM as mentioned while MM group constructs were incubated in standard DMEM containing 100 μ g/ml of matrix MMP-9. This concentration of MMP-9 is chosen based on our previous study where no acute toxicity was observed in HepG2 cells[114]. As for the MG group, instead of normal alginate gel precursor solution, cell-laden microspheres were suspended in the same concentration of alginate precursor solution containing 100 μ g/ml MMP-9 during fabrication of the constructs. MG constructs were culture in standard DMEM just like the control. The day that the constructs were fabricated was noted as Day 0. On Day 1, culture media in

all groups were changed and refreshed with standard DMEM without any MMP-9 and shown in **Figure 4.1b**.

Figure 4.1:(a) Schematic illustration of fabrication steps for cell-laden microsphere hydrogel composite construct (control, MM and MG); insert picture is of 10× magnification (b) Phase contrast picture of control, MM and MG constructs with or without MMP-9 treatment

4.2.5 *In vivo subcutaneous implantation into nude mice*

A total of 6 male Balb/c nude mice (4-week-old, mutant BALB/C, i-DNA Biotechnology Singapore) were used in this experiment. They were sedated using a combination of Ketamine (40 mg/kg) and Diazepam (5 mg/kg). Each mouse had three incisions made to create subcutaneous pockets for implantation and every mouse received one Day 1 construct from each group (control, MM and MG). The constructs were kept in the mice for 13 days and before retrieving them for histological examinations. All nude mice experiments were carried out in accordance with regulations of the Institutional Animal Care and Use Committees (IACUC), Nanyang Technological University (NTU), Singapore.

4.2.6 Cell viability and HepG2 spheroid morphology

All samples were tested for viability and proliferation at various time points throughout the 14 days' of culture. Live/dead fluorescent staining (Invitrogen) was used where the cells were observed after 30 min incubation in calcein AM and ethidium homodimer-1. A total of three samples were collected from each sample group at each time point for WST-1 (Roche, Singapore) colorimetric assay. The samples were incubated at 37 °C for 1.5h and the absorbance at 450nm was measured with reference to 620nm using a microplate reader.

4.2.7 Albumin and cytochrome P450 gene expressions

For each sample group, three constructs were pooled together and RNA was extracted using TRIzol® (Invitrogen). 500ng of RNA from each group were

used to convert to cDNA using reverse transcription kit (Promega), adhering strictly to protocol given by the supplier. Subsequently, they were analyzed quantitatively using real-time polymerase chain reaction (RT-PCR). SYBR green iQ Buffer (Bio-Rad) was used to perform RT-PCR and the relative gene expressions was normalized to the housekeeping gene (β -actin) using $2^{-\Delta C_T}$. Here, C_T represents the cycle number when an arbitrarily placed threshold was reached and $\Delta C_T = (C_{T, \text{target gene}} - C_{T, \beta\text{-actin}})$. Primers with annealing temperatures and product sizes are listed in **Table 4.1**.

Table 4.1. List of primer sequence used for real-time PCR. A.T denotes annealing temperature while P.S denotes product size.

<i>Genes</i>	<i>Primer sequence (both 5'- 3')</i>	<i>A.T (°C)</i>	<i>P.S (bp)</i>	<i>Ref.</i>
hβ-Actin	Forward: GTGGGGCGCCCCAGGCACCA Reverse: CTCCTTAATGTCACGCACGATTTC	58	540	[134]
hCYP1A1	Forward: TCTTTCTCTTCCTGGCTATC Reverse: CTGTCTCTTCCCTTCACTCT	58	596	[135]
hALB	Forward: GTGGGCAGCAAATGTTGTAA Reverse: TCATCGACTTCCAGAGCTGA	58	188	[136]

4.2.8 Albumin quantification

Culture medium from each construct (n=3) was collected and replaced at various time points throughout the 14 days' of culture. Media collected were stored at -20°C before testing. Albumin concentrations in the media supernatant were determined colorimetrically by commercial albumin test kit (Bio-Quant). The values obtained were averaged and normalized against the control on Day 1.

4.2.9 Histology staining

All samples were fixed in 4% aqueous paraformaldehyde overnight before dehydrating in increasing concentrations of ethanol. The samples were embedded in paraffin and sectioned to a thickness of 8µm. Subsequently, they were immersed in xylene to remove the paraffin and separately stained for H & E and 4',6-diamidino-2-phenylindole (DAPI) before viewing in fluorescent microscope (Olympus IX71).

4.2.10 Statistical analysis

Where appropriate, ANOVA was performed to analyze results and $P < 0.05$ was considered to indicate a statistically significant difference. Data presented as mean with $\pm$ SD.

4.3 Results

4.3.1 Characterization of Genipin crosslinked gelatin microspheres

To determine a suitable degree of crosslinking for the microspheres, three different concentrations of genipin solutions (0.1wt %, 0.25wt % and 0.5wt %)

were used to crosslink the gelatin microspheres. As shown in **Figure 4.2**, spheres crosslinked in higher concentration of genipin resulted in greater intensity of the blue pigment formation, indicating a higher degree of crosslinking. After 30 minutes incubation at 37 °C, microspheres crosslinked in 0.1wt% genipin solution dissolves while those crosslinked in 0.25wt% and 0.5wt% genipin remained intact. In the presence of MMP-9, microspheres crosslinked in 0.1wt% and 0.25wt% degraded after 4 hours but those in 0.5wt% could not degrade completely. The desired properties of the microspheres in this study are present in 0.25wt% crosslinked microspheres where they maintain their integrity under normal culture condition but readily degrade upon introduction of MMP-9. Therefore, only microspheres crosslinked in 0.25wt% genipin were used for subsequent experiments.

Figure 4.2: Overview of gelatin microspheres after crosslinking in different concentrations of genipin (0.1wt%, 0.25wt% and 0.5wt %). First column: Genipin crosslinked microspheres after swelling in PBS. Approximation of crosslink degree is indicated by the intensity of blue pigment formed. Second column: genipin crosslinked microspheres in PBS incubated at 37°C for 30min. Third column: GM incubated in 100μg/ml MMP-9 containing media at 37°C for 4 hours

4.3.2 Cell viability and HepG2 spheroid morphology in constructs

HepG2 cells were cultured on 0.25wt% genipin microspheres for 3 days before encapsulating them in alginate hydrogel. As mentioned, three different set ups were made, namely the control where it did not receive any MMP-9 treatment, MM where MMP-9 was added to the culture medium and lastly MG where

MMP-9 was introduced via the alginate hydrogel (**Figure 4.1a**). As shown in **Figure 4.1b**, 6 hours after MMP-9 treatment, the microspheres within MM and MG constructs degraded, creating cavities of corresponding sizes. The cells which were originally attached on the microsphere now reside at the edge of the cavities created.

Figure 4.3: Cell proliferation profile of cells in control, MM and MG constructs using WST-1 assay. * represents $p < 0.05$ when compared to control sample of that day

Cell viability is an important indicator of the compatibility of the platform with the cells. As illustrated in **Figure 4.3**, WST-1 assay of the various construct groups showed a significant increase in absorbance from Day 1 to Day 4 but a gradual decrease in the subsequent days. This may be due to a decrease in cell proliferation rate from the spatial constraint in the hydrogel.

When the cell spheroid fully occupies the space in the cavity, proliferation rate decreases since there is no more room for further cell expansion. In all time points other than Day 1, cells in MG group had a much a higher absorbance than the control group and MM group. For qualitative analysis, live/dead staining was carried out on Day 4, 7 and 14 as shown in **Figure 4.4**. Give the fluorescent properties of microspheres after crosslinking [111, 137], the microspheres could be easily identified in red and could be clearly observed in the control group. Most cells in the control were stained green but no HepG2 cell spheroids were observed in all the time points tested. On the other hand, viable HepG2 cells aggregates were observed on Day 4 in both MM and MG and these aggregates developed into spheroids on Day 14. The spheroids were approximately 100 ± 20 μm in size which were of the similar size to the microspheres used.

Figure 4.4: Live/dead staining and phase contrast images of the cells in control, MM and MG constructs on Day 4, 7 and Day 14.

4.3.3 *Liver-Specific Functions of cell-laden microspheres hydrogel composite*

To investigate the efficacy of spheroid generation with a focus on this novel biomaterial based cell-delivery platform, human hepatocellular carcinoma cell line, HepG2, is conveniently adopted as a model cell as it fulfills many of the hepatocytes characteristics like albumin secretion and cytochrome P450 (CYP1A1) activity [132, 136] and they are examined as representing hepato-functional parameters in this study. Urea synthesis being one of another important feature of hepatocytes is believed to be deficient in this cell line[109], albeit from a number of studies, trace amount of urea production and variation had been detected [110-114]; in this study it was not tested.

Albumin and CYP1A1 are two important markers of liver-specific functions and were assessed on the gene expression and protein levels. In **Figure 4.5**, albumin expression level in MM and MG were higher than the control group at all the time points and all groups had the highest albumin expression level on Day 14. The gene expression level of CYP1A1 on Day 4 were 0.15 and 0.13 in MM and MG group respectively and were approximately 5 folds higher compared to the control on the same day. Although CYP1A1 level showed a decreasing trend over the following days, in general, the expression level of MM and MG group were higher than the control at most time points.

Figure 4.5: Gene expression of albumin and cytochrome P4501A1 (CYP1A1) of control, MM and MG constructs at various time points. * represents $p < 0.05$ when compared to control sample of that day

On the protein level, albumin secretion was evaluated. The values obtained from each construct were normalized to the control sample on Day 1 in **Figure 4.6**. There was an increasing trend in albumin secretion from Day 1 to 7 followed by a slight decrease from Day 7 to 14. Similar to those observed in gene expression, both MM and MG samples had higher albumin secretion than the control at all the time points.

Figure 4.6: Albumin secretion from control, MM and MG constructs at various time points. * represents $p < 0.05$ when compared to control sample of that day

4.3.4 *In vivo nude mice subcutaneous implantation*

To investigate spheroid formation capability in the *in vivo* environment, the constructs were implanted subcutaneously into nude mice on Day 1. After 13 days, the samples were retrieved for histological evaluations by H & E and DAPI stainings. The H & E staining in **Figure 4.7** showed cavities within the construct and there were cells adjacent to the cavities. Furthermore, DAPI staining showed distinct cell aggregates within MM and MG constructs (highlighted with arrows) but these aggregates were absent in the control

groups. In addition, genipin crosslinked microspheres in the control group did not degrade as shown in the positive red fluorescence in **Figure 4.7**.

Figure 4.7: Histochemical staining of control, MM and MG constructs 14 days after subcutaneous implantation in nude mice. Red dotted lines outline the cavities while arrows indicate HepG2 cell aggregates within the constructs.

4.4 Discussions

Generation of hepatocellular spheroids to preserve its phenotypic properties is one of the major focuses in liver tissue engineering in recent years. Many

approaches have been developed but it often requires sophisticated and high precision equipment to facilitate well-defined size spheroids. This inevitably increases the cost of fabrication and makes scaling up challenging. In view of this issue, we have developed a simple set up that is able to initiate HepG2 model cell spheroids generation within alginate hydrogel bulk with the aid of genipin crosslinked gelatin microcarrier and MMP-9.

In this study, genipin crosslinked microspheres are dual-functioning as they serve as transient cell carriers and also templates for cavity creation. Since typical gelatin microspheres dissolve readily at physiological temperature of 37°C, genipin, a natural crosslinker is chosen to crosslink the microspheres to ensure maintenance of the microspheres' structural integrity during cell delivery. We have determined that 0.25wt% concentration of genipin solution would give rise to desired crosslinking degree after 16 hours of incubation. At this crosslinking degree, stability of the microspheres at 37°C is achieved and at the same time they could be completely degraded via introduction of MMP-9.

In plain hydrogel cell encapsulation model, strict confinement imposed by the hydrogel limits aggregate formation in most cases; or results in development of irregular aggregate structures that could not be consistently replicated[74]. The results presented here have highlighted the importance of cavities in generating HepG2 spheroids. A control group was set up where microspheres remained intact throughout the culture period. Although cells remained viable, no spheroid was formed at any of the observed time points.

Cells were able to proliferate within the system but the presence of spheres poses spatial constraint and therefore prohibited spheroid formation. On the contrary, the cell carriers were degraded by MMP-9 in MM and MG samples. MMP-9 was added in the media in the MM group while MMP-9 was introduced via the alginate bulk in MG group in order to maintain the advantage of injectability in the system. Cavities created not only bring space for better cell proliferation and spheroid generation; in addition, they marked the boundary for spheroid expansion. As cell proliferation takes place, the spheroids generated expand in size within the cavities, having an outward expansion force on the surrounding gel bulk. When this outward force is ultimately balanced by the mechanical force from the gel, equilibrium is reached and the spheroid size could no longer expand. Since the cavity size was determined by the microspheres size and the selected spheres size did not exceed 200 μ m, the size of spheroids generated were within diffusion limits therefore no necrotic core was observed in them. The liver specific functionalities of the generated cell spheroids were assessed on transcriptional and protein level. The higher albumin and CYP1A1 gene expressions together with higher albumin secretion levels in MM and MG compared to the control demonstrated better liver specific functions' performance in the spheroids formed.

To investigate the feasibility of spheroid formation within this system in an in vivo environment, Day 1 constructs from all groups were subcutaneously implanted into nude mice. All samples were retrieved on Day 14 for analysis and successful spheroid formation was confirmed as illustrated in **Figure 4.7**.

The DAPI staining highlighted that cell spheroids were only observed in MM and MG samples whereas a uniform single cell distribution of possibly fibroblasts were seen in the control. As mentioned in the results, gelatin microspheres possessed red fluorescent properties after crosslinking with genipin, these microspheres remained detectable in the control samples after 13 days of implantation.

Accordingly, the optimized crosslinking degrees in the gelatin microspheres achieved ideal properties of a cell carrier where it is able to deliver cells to target site yet it could easily degrade so as not to interfere with further cell expansion. The importance of cavities is highlighted in this study where they are essential for creating space for spheroid formation and also regulation of spheroid size. The positive hepatocellular traits found in these MM and MG constructs suggested that spheroids generated in this platform could enhance and preserve the liver cell phenotype. Based on the similar outcome obtained between MM and MG groups; it also proved that the introduction of MMP-9 by mixing it with alginate precursor solution does not affect cell viability and subsequent cell functionalities. Hence, this system is injectable and has minimal invasiveness.

In addition, many studies have proved better functionality of liver cells spheroid by co-culturing them with endothelial cells[128], fibroblasts[138, 139], epithelial cells[129] and hepatic stellate cells[140]. The platform established in this study is customizable as it could be also easily be modified to set up a co-culture system by simply adding a second cell type into the alginate precursor

solution. The hepatocellular spheroids would reside in the cavities while the second cell type would be in the gel phase of the construct.

4.5 Summary

In conclusion, we have set up a platform which does not require sophisticated equipment yet able to generate hepatocellular spheroids with controllable size and shape. The genipin crosslinked gelatin microspheres employed in this study were dual-functioning as they were both cell carriers and porogens for cavities creation within the alginate bulk. HepG2 was used as a model cell to demonstrate the feasibility of hepatocellular spheroid formation and maintenance of liver-specific functionalities in this system. Upon the success, this technology could be rendered to the use of hepatocytes or progenitors. We believed that by this translation, it may ultimately benefit clinical applications.

Chapter 5 : Cells in hydrogel: Endochondral ossification in scaffold-free LhCG

5.1. Introduction

All previous chapters focus on delivering cells via microspheres in GS system. In the upcoming chapters, cells were delivered in the hydrogel phase of the GS system. As the cells would be delivered in their rounded morphology, this GS system is particularly suitable for non-ADCs like primary chondrocytes.

The unique property of this GS system lies in its responsive property to temperature changes of the physical environment. When exposed to physiological temperature, gelatin microspheres spontaneously dissolve in the GS system, creating micro-cavities of corresponding sizes. Besides providing better nutrient diffusion and greater space for the cells, the cavities also created multiple gel edges which in turn imparted an asymmetrical mechanical stimulation on encapsulated chondrocytes to induce directional outgrowth into the cavities. As a result, this directional outgrowth from gel phase into the cavity would lead to formation of scaffold-free isogenous group within the cavities. This entire cell culture strategy is termed “phase transfer cell culture (PTCC)”.

With prolonged culture period, the continuous expansion and active secretion of ECM proteins from these chondrocyte isogenous groups give rise to a cellular construct consisting of cells and interpenetrating network of ECM

proteins and hydrogel. Given that alginate was employed as the gel phase in the GS system, it was easily removed through citrate leaching. The resultant product is a macroscopic scaffold-free cartilaginous construct made up of only cells and their ECM protein, named “living hyaline cartilaginous graft” (LhCG)[69]. As a cartilaginous graft, its efficacy in chondral defect repair was evaluated. Full thickness repair with good integration with native tissue was achieved in rabbit model by 100 days.

Based on the encouraging result from cartilaginous repair, we believed that this LhCG construct could be a suitable platform for mimicking in vitro endochondral ossification for bone regeneration. Hence, in this chapter, we employed the GS system derived scaffold-free LhCG construct as a cartilaginous template for bone formation.

Bone development and formation occur in two distinct pathways: intramembranous ossification and endochondral ossification. Intramembranous ossification which typically takes place in craniofacial skeleton development involves direct recruitment of osteo-progenitors, such as mesenchymal stem cells (MSCs) and osteoblasts, to initiate osteogenesis without the need for a cartilaginous template. On the other hand, endochondral ossification requires cartilaginous precursors from which the formation and elongation of bony tissues are initiated. In endochondral ossification, cartilaginous extra cellular matrices (ECMs), typically collagen type II (Col II) and glycosaminoglycan (GAG), are secreted in abundance during early stage of the process, the

majority of which, however, will be eventually degraded by matrix metalloproteinases (MMPs) or mineralized as the hypertrophic chondrocytes apoptose [141, 142]. The breakdown of ECMs is vital for vascular invasion that would facilitate the recruitment of osteo-progenitors consisting of both osteoblasts and MSCs. In addition, vascularization is also essential for transporting calcium ions for subsequent mineralization process [143, 144].

However, neither endochondral nor intramembranous ossification process described could effectively heal large and complex bone defects or voids. These defects require bone grafts such as autografts, allografts or engineered grafts made of osteoconductive biomaterials to bridge and unite the gap[145-147]. Autografts is the most ideal graft available since it is immune-compatible but have the constraint of availability and donor site morbidity. Allografts and xenografts on the other hand could be more attainable from various sources but carry dangerous risks for potential immune response[148, 149]. The use of engineering graft could possibly overcome the limitations mentioned in autografts and allografts since its performance and properties could be customized from proper engineering design and choice of material. Synthetic biomaterials such as ceramics may offer osteoinductive properties for MSC survival and differentiation but are unable to fully synchronize scaffold resorption kinetics with bone formation rate[148]. Furthermore, their degradation product may be harmful to the body[150, 151]. In recent years, scaffold-free tissue constructs developed[152, 153] may have offer a possible solution in circumventing problem in synthetic material-based, engineered

grafts and have demonstrated success in myocardial [153, 154], corneal[155, 156] and cartilage[69, 157] tissue engineering. We have previously designed and fabricated a 3D porous macroscopic LhCG for cartilage regeneration[69]. LhCG construct is purely made up of viable chondrocytes and their own cartilaginous ECMs; specifically, it is free of man-made materials. In this study, we employ this scaffold-free LhCG construct as a 3D macroscopic cell-based platform for seeding and culturing osteo-progenitors for osteogenesis. The use of LhCG as a cartilaginous template for bone formation is essentially bio-inspired from natural endochondral ossification process.

Since many monolayer co-culture[158, 159] and mixed pellet culture[160-162] studies have proved that co-culturing chondrocytes with osteo-progenitors could benefit osteogenesis, we believed that this cell-based scaffold-free LhCG platform could bring about similar benefits in differentiating the seeded progenitors towards osteogenesis. In contrast to the mixed pellet system (pellet size ~1mm) [160-162], LhCG employed in this study is tangible and macroscopic in size (approximately 5mm × 5mm) which make this platform practically useful in the clinical setting. Furthermore, being a scaffold-free construct, it avoids any complications as a result of undesirable cell-material interactions and hence provides a closer mimic of natural endochondral microenvironment for bone formation compared to synthetic polymer scaffolds.

The performance of LhCG as a cell-based platform to support proliferation and differentiation of osteogenic progenitors is examined in this study. As a porous, engineered living cartilage template, LhCG is rich in cartilaginous ECMs, it can serve as a viable platform for cell seeding without the need for any surface/interface treatment. It also allows direct co-culture of existing chondrocytes and osteo-progenitor cells to take place in a macroscopic 3D engineered environment. This permits the establishment of dynamic interactions between viable chondrocytes and the newly seeded osteogenic progenitors through direct cell-cell contact, which in turn facilitates coordinated cartilaginous ECM replacement with bone matrix during osteogenesis. Both osteoblast and human mesenchymal stem cell (hMSC) were seeded onto LhCG and stimulated towards osteoblast maturation and osteogenic differentiation respectively. At designated time points, the co-culture constructs were evaluated for cell viability and any indication of osteogenesis. An “empty” LhCG template without any cell seeding was also set up in parallel for comparison. To assess biocompatibility and possible osteogenic maturation and matrix replacement in the in vivo environment, subcutaneous implantation of the co-culture constructs into nude mice was also carried out

5.2. *Materials and methods*

5.2.1 *Preparation of LhCG prototype*

The establishment and full characterization of LhCG was done in our previous work and the protocol for deriving LhCG prototype was adapted[69]. Unless stated otherwise, all chemicals were purchased from Sigma Aldrich, Singapore and all cell culture related reagents were purchased from Invitrogen. Double emulsion method of oil/water/oil emulsion was used to fabricate gelatin microspheres that were sieved into spheres size in the range of 150-180 μm . 1000 units/ml penicillin and 1000 $\mu\text{g/ml}$ streptomycin solution was used to sterilize the spheres overnight before storing in phosphate buffer saline (PBS) at 4°C. Porcine chondrocytes were extracted from 5-month old pig articular cartilage and expanded in cultured flask. Passage one chondrocytes (10 million cells/ml) were suspended together with the microspheres in 1.5 ml of alginate solution (1.5% w/v in 0.15 M NaCl, 4°C). The mixture was added onto a 30 mm petri dish. Gelation occurred within a few minutes by gentle introduction of 102 mM aqueous calcium chloride [9, 69, 74]. The construct was cultured in chondrocytes culture (CC) medium that comprises of Dulbecco's Modified Eagle Medium (DMEM) containing 20% (v/v) fetal bovine serum (FBS), 0.1 mM nonessential amino acids (NEAA), 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 0.05 mg/ml vitamin C, 0.4 mM proline and 100 units/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. The constructs were kept on orbital shaker with shaking speed at 50 rpm in a humidified incubator at 37°C with 5% CO_2 . At this temperature, gelatin microspheres within the construct would readily dissolve and create cavities of corresponding sizes in the alginate bulk (within 2 days). The constructs were culture for 35 days to reach

maturation and alginate bulk would then be removed by immersing in sodium citrate (55 mM in 0.15 M NaCl) solution for 10 minutes at room temperature [70]. The clearance of gel bulk establishes LhCG construct (prototype) which is made of chondrocytes and their ECM only.

5.2.2 Cell seeding to LhCG

The day for alginate removal is denoted as “Day 0” and LhCG constructs were maintained in CC medium for 7 days until Day 7, to allow further chondrocyte growth and development. Agarose-coated tissue culture plates were prepared by adding 300µl of warm 1.2wt% agarose solution into each well of the 24-wells plate (Iwaki, Japan). The culture plate was then cooled in 4°C fridge for 30min before usage. On Day 7, LhCG samples were transferred to agarose-coated 24-wells tissue culture plate. Following which, osteoblast or hMSCs were seeded onto the LhCG constructs respectively. Henceforth, the co-culture system was established as shown in **Figure 5.1a**.

5.2.2.1 Human fetal osteoblast (hFOB) seeding (LhCG+FOB)

Human fetal osteoblasts (hFOB 1.19, American Type Culture Collection (ATCC)), were used to seed onto LhCG samples (4 million cells/ml, 300µl per construct) to obtain the LhCG+FOB co-culture construct. LhCG+FOB was then cultured in osteoblast proliferation (OP) medium made up of Dulbecco’s Modified Eagle’s medium (DMEM)/Ham’s F12 medium containing 20% (v/v) FBS, 2.5 mM L-glutamine (PAA Laboratories) and 0.3 mg/ml G418 (PAA

Laboratories, Pasching, Austria). It was maintained at OP condition of 33.5°C for 7 days until Day 14. To distinguish the seeded hFOB from existing ones within LhCG construct, green fluorescent protein (GFP)-labelled hFOB were used during the first time seeding. Plasmids containing GFP gene were introduced into hFOB by adenovirus transfection. Osteoblast differentiation (OD) conditions were introduced on Day 14 where the OP medium were supplemented with 10^{-8} M menadione, 100 µg/ml ascorbic acid and 10^{-8} M 1,25-dihydroxy vitamin D₃ as well as an elevation of incubation temperature to 39.5°C for 3 days until Day 17. The conditions and procedures for osteoblasts culture and differentiation were according to the protocol given by ATCC and have been widely practiced by others previously [163]. The temperature and medium were returned to those of proliferative conditions for further culturing till Day 30 to investigate long term effect of the co-culture system. Concurrently, a control was set up in parallel using LhCG without osteoblast seeding (LhCG-FOB) to investigate how chondrocytes in LhCG would be influenced under the same conditions. In addition, hFOB monolayer control was set up for gene expression analysis purposes.

5.2.2.2 Human mesenchymal stem cell (hMSC) seeding (LhCG+MSC)

For hMSC seeding, a similar protocol was adopted. hMSCs were purchased from Cambrex (East Rutherford, NJ, USA). Only passage 5-6 hMSCs were used in the experiment. hMSCs were suspended in CC medium at a density of 1 million cells/ml and 300 µl of the suspension was added to each Day 7 LhCG

construct. This construct was denoted as 'LhCG+MSC'. LhCG+MSC were maintained in CC medium at 37°C until Day 14 before switching to osteogenic (OG) medium where the CC medium was supplemented with 100 nM dexamethasone, 50 µM ascorbic acid 2 phosphate (Invitrogen) and 10 mM β-glycerophosphate (Sigma). For LhCG+MSC samples, a control LhCG without hMSCs addition (denoted as 'LhCG-MSC') undergoing the same OG conditions was set up in parallel for comparison purposes. The detail timeline for the various conditions applied to different co-culture samples were described in **Figure 5.1b**.

5.2.3 *Animal Study*

All animal experiments were performed under guidelines approved by the Institutional Animal Care and Use Committees, SingHealth, Singapore. Day 35 LhCG-MSC and LhCG+MSC were implanted subcutaneously into Balb/c nude mice. Each mouse had two LhCG-MSC and two LhCG+MSC constructs and a total of 6 mice were used for this experiment. The specimens were retrieved 10 days (Day 45) or 21 days (Day 56) after implantation for calcium quantification and histological evaluation.

5.2.4 *Analytical Methods*

5.2.4.1 *Cell viability test*

Cell viability in the construct was determined using Live/Dead dye staining (Molecular Probes, Invitrogen). 0.5 µl of calcein and 2 µl ethidium homodimer

were added to 1 ml of medium containing the sample. After 30 minutes of incubation at 37°C, the sample was observed under fluorescence microscope.

5.2.4.2 Gene expression

RNA from the sample was extracted using TRIzol[®] (Invitrogen). The acquired RNA samples were converted to cDNA by reverse transcription before the subsequent polymerase chain reaction (PCR). For semi-quantitative PCR, cDNA were amplified by respective primers and analyzed in 1.2% agarose gel. The gel was then stained ethidium bromide (EB) solution before viewing. All the reverse transcription and PCR reagents used were purchased from Promega. The follow PCR parameters were utilized: 94°C for 60s followed by 35 cycles of 94°C for 30s, 58°C for 30s and 72°C for 30s and finally 72°C for 5min. Real-time quantitative PCR (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) and gene expression was calculated with the comparative $2^{-\Delta\Delta C_T}$ method, using β -actin as the reference gene and normalized to the value from Day 14 sample. All the primers used in PCR and qPCR were engineered by AIT Biotech (Singapore) and their sequences were presented in **Table 5.1** and **5.2**.

Figure 5.1: (a) Schematic illustration of the establishing in vitro endochondral osteogenesis niche using LhCG prototype (b) Timeline showing various conditions applied to different co-culture constructs on different days

Table 5.1. Human osteogenic primers used for qPCR. A.T, P.S and Ref stand for annealing temperature, product size and reference respectively

Genes	Ref.	Primer Sequence (both 5'-3')	A.T (°C)	P.S (bp)
β-actin	[164]	Forward: CCTGGCACCCAGCACAAT Reverse: GGGCCGGACTCGTCATACT	60	540
Human Osteopontin (hOPN)	[165]	Forward: ACCTGAACGCGCCTTCTG Reverse: CATCCAGCTGACTCGTTTCATAA	53	66
Human Osteonectin (hON)	[166]	Forward : ACATGGGTGGACACGG Reverse : CCAACAGCCTAATGTGAA	58	408
Human Alkaline phosphatase (hALP)	[165]	Forward: GGGAACGAGGTACCTCCAT Reverse: TGGTCACAATGCCACAGAT	53	67
Type I Collagen (Col I)	[165]	Forward: CCTGCGTGTACCCCACTCA Reverse: ACCAGACATGCCTCTTGTCCTT	53	84
Human Cbfa-1/Runx2	[165]	Forward: AGTGATTTAGGGCGCATTCCT Reverse: GGAGGGCGGCGTGGGTTCT	50	71
Human Osteocalcin (hOCN)	[165]	Forward: AGCAAAGGTGCAGCCTTTGT Reverse: GCGCCTGGGTCTCTTCACT	50	63

Table 5.2: Primers used in polymerase chain reaction (PCR). A.T, P.S and Ref stand for annealing temperature, product size and reference respectively

Genes	Accession number/ Ref	Primer Sequence (both 5'-3')	A.T (°C)	P.S (bp)
Porcine Osteonectin (pON)	AM490166.1	Forward: AGACCTTTGACTCTTCCTGCCACT Reverse : TCTTCACTCGCAGCTTCTGCTTCT	58	243
Porcine/Human Alkaline phosphatase (p/hALP)	[167]	Forward: ATGAGCTCAACCGGAACAA Reverse: GTGCCCATGGTCAATCCT	56	131
Porcine/human Type I Collagen (p/hCol I)	AK223175.1	Forward: ATACGCGGACTTTGTTGCTGCTTG Reverse: TGTCCCTTCAATCCATCCAGACCA	58	556
Porcine Col II	AF201724.1	Forward: TGTTCTGAGAGGTCTTCCTGGCAA Reverse: AGTCAGACCTCTTCCGCCGTCTTT	58	487
Porcine/human Osteocalcin (p/hOCN)	NM_001164004.1	Forward: AAGGTGCAGCCTTTGTGTCCAA Reverse: GAGTTTATTTGGGAGCAGCTGGGA	58	353
Porcine Cbfa-1/Runx2	XM_001924306.1	Forward: TTACTIONACACCCCGCCAGTC Reverse: ACGCCATCATTCTGGTTAGG	55	289
Porcine Type X Collagen (pCol X)	AF222861.1	Forward: TGCCAACCAGGGAGTAACAGGAAT Reverse: AAGCCTGATCCAGGTAGCCTTTGA	58	287
Human β -actin	[168]	Forward: GTGGGGCGCCCCAGGCACCA Reverse: CTCCTTAATGTCACGCACGATTTC	60	540
Human Osteopontin (hOPN)	[166]	Forward: AGCCGTGGGAAGGACAGTTATG Reverse: GGAGTTTCCATGAAGCCACAAAC	58	473
Human Osteonectin (hON)	[166]	Forward : ACATGGGTGGACACGG Reverse : CCAACAGCCTAATGTGAA	58	408
Human Cbfa-1/Runx2	AF053952.1	Forward: TTCTGTGGCATGCACTTTGACCAC Reverse: GGCCGTTAGGGTTTGAAGGCAAAT	58	202
Porcine Aggrecan (pAgg)	[169]	Forward: CGAGGAGCAGGAGTTTGTCAAC Reverse: ATCATCACCACGCAGTCCTCTC	58	177
Porcine GAPDH	[169]	Forward: ACCCCTTCATTGACCTCCAC Reverse: ATACTCAGCACCAGCATCGC	58	179

5.2.4.3 Calcium quantification

Calcium quantity in the constructs was determined by Calcium-O-Cresolphthalein complexone method[145]. Briefly, the constructs were collected and rinsed with PBS to remove any calcium ions from the medium. They were then homogenized in 3 M aqueous hydrochloric acid and left overnight to ensure that all calcium content was extracted. Colour reagent containing O-cresolphthalein complexone and 8-hydroxyl-quinline was mixed with 2-amino, 2-methyl, 1-propanol (AMP) buffer in the ratio of 1:1 to form the working solution for calcium quantification. 25 µl of the sample was then added to 1 ml of working solution and incubate at room temperature for 5min. The intensity of the colour was then measured at 575 nm. The calcium quantity was calculated based on the absorbance value of calcium standard set up.

5.2.4.4 Enzyme-linked immunosorbent assay (ELISA)

To determine the presence of osteocalcin (OCN), the medium was removed 24 hours before each specific time point and replaced with an equal volume of medium (1 ml per construct). The medium collected 24 hours later was stored at -20°C until analysis. ELISA was conducted according to the manufacturer's instruction (Quidel, USA).

5.2.4.5 Immuno- histology

Samples from each time point were fixed in 4% (w/v) paraformaldehyde for 1 day. The fixed specimens were then embedded in paraffin and sectioned to a

thickness of 10 μm using a microtome. Sections from all groups were subsequently stained with Alizarin Red. For immunofluorescent staining, the sectioned specimens were incubated with 10% goat blocking serum (w/v, in PBS) for 20 minutes to avoid any non-specific binding of IgG. To test for chondrogenic/osteogenic makers, the specimens were incubated with Col II (2 $\mu\text{g}/\text{ml}$ in PBS, Chemicon), collagen I (2 $\mu\text{g}/\text{ml}$, mouse monoclonal IgG, Santa Cruz Biotechnology) or osteocalcin (2 $\mu\text{g}/\text{ml}$, mouse monoclonal IgG, Santa Cruz Biotechnology) primary antibody respectively at 4°C overnight and washed with PBS. Subsequently, the specimens were incubated with Anti-IgG (5 $\mu\text{g}/\text{ml}$ in PBS, Invitrogen AlexaFluor, 488) or Anti-IgG (5 $\mu\text{g}/\text{ml}$ in PBS, Alexa Fluor 543, Invitrogen) at room temperature for 1 hour in the dark followed by PBS washing for 3 times. Nuclei were then counterstained by 4',6-diamidino-2-phenylindole (DAPI).

5.2.5 *Statistical analysis*

Where appropriate, ANOVA is used to analyze results and a $P < 0.05$ with 3 specimens in each group is considered to indicate significant difference statistically. Data are presented as mean $\pm$ SD and any statistical significance is denoted in the figures.

5.3. ***Results and discussion***

The use of biomaterial-based scaffolds as engineered bone grafts for large bone defect repair encounters a bottleneck in synchronizing degradation rate with

bone formation rate. Early degradation of scaffold would lead to premature loss of structural integrity for cell and tissue growth while delay degradation or any remnant biomaterials would interfere with tissue repair and formation of neotissue[170, 171]. The development of scaffold-free constructs may have offered a possible solution in circumventing the challenges with biomaterial scaffolds. Our previous work has successfully derived a macroscopic 3D cartilaginous graft (LhCG) for cartilage regeneration. Based on the knowledge derived from natural endochondral bone development process, we are inspired to employ this platform for bone formation.

In this study, macroscopic LhCG construct was used as a naturally derived in vitro cartilaginous based cell seeding platform for direct co-culture of osteo-progenitors, namely hFOB and hMSCs, for bone formation. Not only it is scaffold-free, LhCG also provides a favorable microenvironment with direct chondrocytes exposure and rich cartilaginous ECM content that is comparable to the natural endochondral environment. The involvement of blood vessels during natural in vivo endochondral ossification is critical and is essential to bring in nutrients and calcium to initiate bone formation. Comparably, high serum culture media (20% FBS) is used in this in vitro study to provide necessary nutrients. Given close proximity between the chondrocytes and the newly seeded progenitors in this LhCG co-culture system, dynamic interactions and mutual influences between these two types of cells could be established which would ultimately benefit the osteogenesis process in terms of cell differentiation and matrix replacement.

Accordingly, osteoblast being responsible for general bone formation process[146, 147, 172], is used as a model cell for seeding onto LhCG for osteogenesis. Osteoblasts were seeded on Day 7 (LhCG+FOB) and proliferated on LhCG in OP conditions for another 7 days until Day 14 so that there is sufficient cells on LhCG before subjecting them to three days of OD conditions[97]. After maturation of osteoblasts, the constructs were maintained for another 13 days (until Day 30) to investigate the long term effect of the co-culture system. The first batch of osteoblasts used was transfected with GFP gene to express green fluorescent for identification from existing chondrocytes in LhCG construct. Strong green fluorescent from the cells on LhCG+FOB (**Figure 5.2a**) suggested that LhCG could provide a favorable environment for cell attachment. To examine the cell viability, live/dead staining was carried out and it revealed the presence of high cell viability in both chondrocytes and the newly seeded osteoblasts in LhCG+FOB constructs. The typical stretched morphology of osteoblasts on LhCG+FOB was clearly observed as well, which again indicated that LhCG's rich ECM highly favored additional cell seeding (**Figure 5.2b**) while the gross overview of the constructs from various time points showed that all constructs remained translucent and did not revealed any significant differences among them (**Figure 5.2c**).

Figure 5.2: Assessment of LhCG as a possible open platform for cell seeding. (a) Green fluorescent protein (GFP) labeled osteoblasts were used to illustrate cell seeding was feasible in LhCG+FOB (Day 14). The image on the left is at 40× magnification while the one on the right is at 100× magnification. (b) Live/dead staining of cells in LhCG+FOB showed both chondrocytes and osteoblasts were viable in this co-culture system on Day 14. Furthermore, osteoblasts, being typical anchorage dependent cell, were able to maintain the desired stretched morphology. (c) Gross overview of LhCG-FOB and LhCG+FOB constructs at various time points

Figure 5.3: qPCR of osteogenic markers of LhCG+FOB before and after treatment with differentiation conditions compared to monolayer osteoblasts. Differentiation conditions were introduced from Day 14 to Day 17 and proliferation conditions resumed on Day 17 till Day 30. The results indicated up regulation of osteogenic markers in osteoblasts within LhCG+FOB construct after osteoblast differentiation conditions. The obtained values represent the means $\pm$ standard deviations (N = 3). *Relative gene expression of sample LhCG+FOB on that day compared to Day 14 is significant ($p < 0.05$)

Figure 5.4: Calcium assay of LhCG-FOB and LhCG+FOB construct at various time points. The obtained values represent the means $\pm$ standard deviations (N = 3).
 *Differences between different constructs are significant ($p < 0.05$)

To evaluate the osteogenic potential of osteoblasts on LhCG, both plain LhCG-FOB (no further cell seeding) and LhCG+FOB constructs were examined for calcification and osteogenic markers expression. On the gene expression level, there was a general increasing trend in various osteogenic markers such as hALP and Col I in LhCG+FOB after exposure to OD conditions (**Figure 5.3**). Compared to the monolayer osteoblasts that also underwent similar treatment, osteoblasts in LhCG+FOB construct had much higher expression of the osteogenic markers (data not shown) suggesting a more favorable osteogenic environment offered by the LhCG construct. Although there was no observable calcification from the translucent gross overview of the construct, a detailed calcium assay indicated that LhCG+FOB constructs had higher calcium content compared to LhCG-FOB constructs (**Figure 5.4**). This difference was due to the osteoblast maturation under OD

conditions and with longer term culture, calcification was achieved by the mature osteoblasts. This result coincided with observations from Alizarin Red S staining showing positive bright red staining from gradual calcification in LhCG+FOB samples (**Figure 5.5a**). In addition, positive immunostaining indicated steady osteocalcin (OCN) and Col I secretions in the constructs after introduction of osteoblasts and subjecting LhCG+FOB to OD conditions with long term culture(**Figure 5.5b-d**). Conversely, no observable calcification, little OCN or Col I was present in LhCG-FOB samples undergoing the same treatment (**Figure 5.5**). This suggested that the differences observed were solely due to the introduction of osteoblasts. The continuous positive staining of Col II in both LhCG-FOB and LhCG+FOB implied that there was conservation of the construct's cartilaginous nature and integrity throughout the process.

Figure 5.5: Histochemical and immune-fluorescent staining of LhCG-FOB and LhCG+FOB at various time points. Clockwise beginning from top left corner: Alizarin Red S staining; immunostaining of OCN; Col II and Col I. Nuclei were stained blue by DAPI.

Due to the limited cell sources of osteoblast in adults, the use of this model cell in clinical setting is not realistic. The success from LhCG+FOB motivated further investigations on seeding of other stem cells onto LhCG to evaluate practical usage of this platform. In this regard, hMSCs were employed and seeded to LhCG on Day 7 (LhCG+MSC) and directed toward osteogenic differentiation later on. The culture medium for LhCG system was switched from CC medium to OG medium on Day 14 for both LhCG-MSC and LhCG+MSC. The samples were analyzed for traits of osteogenic differentiation after 3 weeks (Day 35) and 6 weeks (Day 56) of exposure to OG medium.

Figure 5.6: (a) Assessment of LhCG for cell seeding of hMSCs. LhCG+MSC refers to LhCG with seeded hMSC while LhCG-MSC refers to a plain LhCG construct undergoing the same OG condition with LhCG+MSC. Live/dead staining of cells in LhCG-MSC and LhCG+MSC revealed that cells were viable in the both constructs. Chondrocytes were rounded in morphology while hMSCs had typical stretched morphology. (b) Gross overview of LhCG-OG and LhCG+MSC constructs at various

time points indicating the gradual whitening of the constructs. 200µm scale bar is applied to all 100× images while 100µm scale bar is applied to all 200× images.

Figure 5.7: Gel electrophoresis osteogenic and chondrocytic marker expressions in LhCG-OG and LhCG+MSC using of species specific primers. ‘-’ represents LhCG-MSC while ‘+’ represents LhCG+MSC. There were co-expression of both chondrocytic and osteogenic markers in both constructs.

Similar to the observation in LhCG+FOB, the typical spreading out morphology of hMSCs in the LhCG+MSC construct could be clearly observed from the live/dead images and it also revealed high viability of both chondrocytes and hMSCs in the construct even after osteogenic differentiation (**Figure 5.6a**), This provided evidence that favorable environment was presented by LhCG for hMSCs survival and proliferation. In contrast to the

gross overview of LhCG-FOB and LhCG+FOB from the previous set up, both LhCG-MSC and LhCG+MSC constructs progressively whitened and became opaque (**Figure 5.6b**), possibly as a result of strong calcification. This interesting phenomenon suggested that LhCG-MSC construct was probably influenced by osteogenic medium and led to similar calcium mineralization as LhCG+MSC. Hence, species-specific osteogenic primers were used respectively to distinguish the expressional contributions from the porcine chondrocytes of LhCG or the added hMSCs (**Table 5.2**). From the gel electrophoresis result, it showed that there were presence of typical osteogenic markers in hMSCs of LhCG+MSC samples and more importantly, it showed up regulation of ALP and OCN in both LhCG-MSC and LhCG+MSC constructs (**Figure 5.7**). The porcine cells within these LhCG constructs, despite the presence of osteogenic phenotype, cartilaginous markers were not totally lost but concurrently expressed in both LhCG-MSC and LhCG+MSC constructs (**Figure 5.7**). As hMSC was the target cell of interest, real time PCR was also performed on LhCG+MSC constructs to further quantify the expression of the human osteogenic markers. It was shown that there were up-regulations of the osteogenic markers such as hALP and hOCN in the hMSCs within LhCG+MSC constructs (**Figure 5.8**).

Figure 5.8: qPCR of human osteogenic markers in hMSCs of LhCG+MSC. It showed a general up-regulation of the osteogenic markers with OG condition treatment. The obtained values represent the means $\pm$ standard deviations (N = 3). *Relative gene expression of sample L LhCG+MSC on that day compared to Day 14 is significant ($p < 0.05$)

Figure 5.9: (a) Calcium assay of LhCG-MSC and LhCG+MSC constructs at various time points. *Differences between different constructs are significant ($p < 0.05$) (b) ELISA assay of osteocalcin (OCN) secretion in LhCG and LhCG+MSC. The obtained values represent the mean $\pm$ standard deviations (N = 3). *Differences between different constructs are significant ($p < 0.05$)

The calcium content in LhCG-MSC and LhCG+MSC showed a drastic increase from Day 35 to Day 56 and there was no significant difference in calcium content between these two sample groups at any time point (**Figure 5.9a**). OCN was also quantified by ELISA and the result showed that OCN production was similar between LhCG-MSC and LhCG+MSC constructs throughout 49 days' of culture (**Figure 5.9b**). The production of OCN was relatively stable with LhCG-MSC having higher OCN than LhCG+MSC sample at Day 56. There was also strong Alizarin Red S staining as well as OCN and Col I immunostainings in LhCG+MSC sample with increasing duration in OG medium (**Figure 5.10a-c**). Similar positive staining for calcification, OCN and Col I were observed in LhCG-MSC construct after 3 weeks' culture in OG medium. Even in the presence of calcification and OCN expression, Col II still remained abundant in the ECM of both constructs (**Figure 5.10d**) and this coincided with the result from gene expression (**Figure 5.8**).

Figure 5.10: hMSCs was seeded onto LhCG (LhCG+MSC) on Day 7. Both LhCG and LhCG+MSC were cultured in chondrocyte culture (CC) medium was switched to osteogenic (OG) medium on Day 14. Samples were evaluated for evidence of osteogenic differentiation after 3 weeks (Day 35) and 6 weeks (Day 56) in OG medium respectively using histochemical and immunostaining of LhCG and LhCG+MSC. (a) Alizarin Red S for calcification; immunostaining of (b) OCN ; (c) Col I and (d) Col II. Nuclei were stained blue by DAPI.

Figure 5.11: After implantation of constructs in nude mice, the samples were retrieved on Day 45 and Day 56 respectively for evaluations. (a) Gross overview of LhCG-MSC and LhCG+MSC constructs at various time points (b) Calcium assay of LhCG-MSC and LhCG+MSC. (N=3) *Differences between different constructs are significant ($p<0.05$)

Both LhCG-MSC and LhCG+MSC were implanted into subcutaneous pockets of the nude mice on Day 35 and they were retrieved on Day 45 and Day 56 respectively (**Figure 5.11a**). Calcification of both constructs was high as shown in the calcium assay (**Figure 5.11b**) and the intense alizarin red S staining (**Figure 5.12a**). In addition, OCN, Col II and Col I were all brightly staining in the immunostainings indicating OG maturation has occurred in the in vivo environment too (**Figure 5.12 b-d**).

Figure 5.12: Histochemical and immune-fluorescent staining of LhCG-OG and LhCG+MSC. (a) Alizarin Red S staining; immunostaining of (b) OCN; (c) Col I and (d) Col II. Nuclei were stained blue by DAPI.

Collectively, the results obtained from osteoblasts and hMSCs seeding onto LhCG suggested that LhCG could support the survival of these two cell types without loss in phenotype. Most importantly, both osteoblasts and hMSCs were able to mature and differentiate respectively to express osteogenic markers and achieve significant mineralization within LhCG construct. This suggests that the cartilaginous ECM in LhCG allowed mineralization process to take place in a fashion where the cartilaginous template was gradually replaced by calcified matrix similar to that in native endochondral ossification. We believed such organized mineralization process is a result of established direct cell-cell contact between chondrocytes and osteo-progenitors so that dynamic interactions and communications synchronized the cartilaginous resorption with bone formation rate in LhCG.

In addition, an interesting point to highlight from the hMSCs experiment is that in contrast to our previous study where long term culture of LhCG in CC medium [69] or exposure to OD conditions (LhCG-FOB) did not alter the intrinsic hyaline cartilage phenotype; LhCG-MSC exhibited significant osteogenic properties albeit the maintenance of abundant Col II after OG treatment. Other studies have also shown that implantation of chondrocytes have positive outcomes in bone healing and repair[173, 174]. Collectively, it may suggest that a subpopulation of LhCG chondrocytes possesses abilities to differentiation toward osteogenic lineages. We hypothesize that there is some degrees of phenotypic flexibility in this population of cells, namely there is a population of resident osteoprogenitors, within LhCG, which permits such

osteogenic differentiation and commitment. This hypothesis could be supported by several studies in which mesenchymal progenitor cells that are CD44+, CD54+, CD90+, CD166+ and Notch 1+ have been identified and isolated from human adult articular cartilage[175-179]. Hence, we believed that this population of progenitors is extracted together with the mature chondrocytes during cell isolation process from porcine articular cartilage in this study. Consequently, they were used in the fabrication of LhCG and after exposure to the right condition (OG condition), these progenitor cells in LhCG-MSC differentiate to the designated lineage, in this case, osteogenic lineage. Based on the observation that a higher OCN was secreted in LhCG-MSC, it is possible that the residential population of MSCs has a faster rate of osteogenic differentiation compared to the seeded hMSCs in LhCG+MSC construct. The cross-species interaction between human MSCs and porcine chondrocytes in LhCG+MSC might have consequently attributed to the difference in the rate of osteogenic differentiation. Though the hypothesis of progenitors existing within LhCG and whether the cross-species interaction indeed caused difference in OCN secretion require further validation, the presence of osteogenic markers in LhCG-MSC has raised the possibilities that LhCG, itself as a cell-based platform, could be employed as a versatile platform for other tissue regeneration purposes.

5.4. *Summary*

As a porous, man-made living cartilage template, the establishment of LhCG has provided a scaffold-free cell-based platform for maturation and differentiation of osteo-progenitor cells. The positive outcomes from seeding of osteoblasts and hMSCs have illustrated that LhCG provided a favorable environment for cell attachment and bone formation. The LhCG platform permits direct cell-cell contact between the chondrocytes and osteo-progenitors which in turn facilitated mineralization of the cartilaginous template. Additionally, when LhCG undergoes similar OG treatment (LhCG-MS) with LhCG+MSCs, osteogenesis was observed in both constructs in vitro and in subcutaneous implantation in nude mice. From this result and together with other research groups' work, we hypothesized that phenotypic flexibility exhibited in LhCG is likely contributed by a resident subpopulation of progenitor cells that is extracted together with mature chondrocytes during cell isolation process from porcine articular cartilage. In conclusion, the LhCG platform have proved to support osteogenesis in the progenitors used in this study and the phenotypic flexibility in LhCG suggests that it holds great potential as a versatile platform for various applications in other research areas.

Chapter 6 : Hepatogenesis of murine induced pluripotent stem cells (iPSC) in 3D microcavitary hydrogel system for liver regeneration

6.1 Introduction

It has been demonstrated that the MCG system provides a favorable environment for isogenous group formation in primary chondrocytes. This inspired us to explore the potential of MCG system on other aggregate forming cells. It is well-known that embryoid body (EB) is the functional unit of pluripotent stem cells for differentiation. Hence, two pluripotent stem cells – induced pluripotent stem cell (iPSC) and embryonic stem cells (ESCs) were encapsulated in this MCG system and sequentially differentiated toward hepatic lineage. Their ability to form EB in MCG system is investigated, as well as, the differentiation potential to mature hepatocytes is discussed in this chapter.

The use of hepatocytes in cell-based therapy may provide a promising alternative in replacement of the existing orthotopic liver transplant. However, the efficacy of hepatocytes transplantation in restoring liver functions is constraint by cell availability, cell quality as well as potential immuno-complications from non-autogenic cell sources [124, 180, 181]. The successful development of induced pluripotent stem cell (iPSC) technology [180, 182, 183] provides a potential solution to circumvent such limitations in the current cell-based therapies by generating patient-specific stem cells and associated

progeny [181, 184] for tissue replacement. Unlike embryonic stem cells (ESCs) where derivation requires early embryos – blastocysts [185-187], iPSCs can be derived from the patients' own somatic cells without invoking significant immuno-rejections or ethical concerns.

Many studies have demonstrated the capacity of iPSCs and ESCs to differentiate into hepatic lineage by recapitulating the natural in vivo microenvironment of liver development [188-191]. In natural liver development, high level of Nodal protein, a member of the TGF β superfamily, specifies endoderm differentiation during gastrulation [188, 192]. Subsequently, fibroblast growth factors (FGFs) and bone morphogenic proteins (BMPs) are up-regulated which then further direct endodermal cells toward hepatic lineage [193]. Final maturation of hepatic progenitor cells to hepatocytes requires a combination of hepatocyte growth factor (HGF), oncostatin M and dexamethasone [194, 195]. In vitro, hepatic lineage differentiation of pluripotent stem cells follows a similar three-step process as the in vivo environment: endodermal induction, hepatic cells specification and hepatic maturation [191, 196, 197]. Since activin A shares the same receptor as Nodal, it is commonly used at high dosage for endoderm induction of iPSCs and ESCs in vitro [188-190]. However, for hepatic lineage specification, various combinations of FGFs, BMPs and HGF have been investigated, and all of which showed successful hepatic differentiation [181, 196-198]. In particular, Takata et al., demonstrated the use of only activin A and HGF for endodermal and hepatic differentiation respectively [199]. Not only does this protocol minimise the use

of complex cytokine cocktails, it also shortens the typical differentiation process by 7 days making this approach highly efficient and thus practical. However, most iPSCs or ESCs differentiation studies are conducted in suspension culture of embryoid bodies (EBs) which limits translational medicine applications due to a lack of proper cell delivery systems.

To date, few studies have reported the use of three-dimensional (3D) hydrogel based scaffolding system to culture iPSCs for hepatogenesis. Moreover, the formation of EB is often carried out separately before they are incorporated into the final cell delivery system. In this study, murine iPSCs (miPSCs) are encapsulated in a 3D micro-cavitary hydrogel (MCG) system for both EB formation and subsequent hepatic lineage differentiation. Murine ESCs (mESCs), a model type of pluripotent stem cells, are included as controls. MCG system is designed and applied as a continuous system to facilitate better medium diffusion and also provide much larger living space for encapsulated pluripotent stem cells; it enables rapid growth of cells into colonies and/or EBs to substantially execute desired functionalities. Through this study, we endeavour to pursue better understanding of iPSC technologies and explore translational applications into tissue engineering and regenerative medicine.

6.2 Materials and Methods

6.2.1 Cell Culture

Unless stated otherwise, all cell culture related reagents were purchased from invitrogen. Murine iPSC (APS0003) [182] was obtained from RIKEN Bio Resource centre (Ibaraki, Japan) and murine ESC (E14tg2A) was purchased from American Type Culture Collection (ATCC). Both murine iPSCs and ESCs were maintained in 0.1% gelatin coated tissue culture dish in feeder-free conditions. The media used was Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum ('Gold' Standard, PAA Laboratories), 0.1mM NEAA, 0.1mM 2-Mercaptoethanol (2-ME, Sigma) and 1000U/ml leukemia inhibitory factor (LIF, Millipore). This medium is referred to as "LIF medium". Upon reaching 50-60% confluence, the cells were trypsinized and used for subsequent experiment. Only cells of passage number 5-8 were employed in this study.

6.2.2 Setting up of cell-laden micro-cavitary hydrogel (MCG)

Double emulsion method (oil/water/oil emulsion) was used to fabricate gelatin microspheres as previously described [69, 74, 200]. The microspheres were sieved into selected spheres size in the range of 150-180 μm and washed with 10 $\times$ concentration of penicillin/streptomycin solution for sterilization. Murine iPSCs or murine ESCs were suspended in cold alginate solution (1.5% w/v in 0.15 M NaCl, 4°C) at a concentration of 10 million cells/mL. The cells suspension was then mixed with gelatin microspheres at a ratio of 0.3g of sphere per mL of alginate solution. 100 μL of the mixture was injected into a circular mold diameter 5 mm. Gelation was achieved within a few minutes by

introduction of 102 mM of aqueous calcium chloride, during which no dissolution of gelatin microspheres was observed [9, 69, 74]. The constructs formed were in shape of pellets and were maintained in a 37°C incubator containing 5% CO₂. When exposed to the temperature of 37°C, the gelatin microspheres within the construct would melt; forming cavities in corresponding size and the cell-laden MCG system was set up as illustrated in **Figure 6.1a**. The medium used for the culture was LIF medium mentioned above for up to 10 days for cell proliferation and formation of colonies and/or EBs. Cell-laden non-MCG constructs were also set up in parallel as controls; using the same cell seeding density but no gelatin microsphere was co-encapsulated.

Figure 6.1: Overview of the MCG establishment and cell culture conditions (a) Schematic illustration of the protocol for cell encapsulation and various stages of cell growth in MCG construct. (b) Timeline of cell culture conditions at various stages. Day 0 marks the establishment of cell-laden MCG construct. The samples were cultured in LIF DMEM, which contains DMEM media, 10% FBS, 10^3 unit/ml of LIF, 0.1mM 2-ME and 0.1M NEAA, for 10 days. Subsequently, media was changed to RPMI+ActA. It is made up of RPMI 1640 medium supplemented with B27 and 100ng/ml of activin A for 4 days to induce endoderm formation. For hepatic lineage specification, RPMI+HGF is used. It is made up of RPMI 1640 medium supplemented with B27 and 20ng/ml of HGF. Hepatic lineage specification was carried out for 6 days until Day 20. For final hepatic maturation, cells were cultured in HCM media

supplemented with 10ng/ml of oncostatin (HCM+OCM) for 5 days and subsequently maintained in HCM media till Day 30. (c) Phase contrast pictures of cell-laden MCG construct at various stages of growth and differentiation showing the formation and maintenance of EBs within the construct.

6.2.3 Hepatic differentiation of murine iPSC and ESCs in the hydrogel construct

To drive the cells (both iPSCs and ESCs) in the constructs to differentiate towards endoderm lineage, the medium was changed on Day 10 to serum-free RPMI 1640 medium (PAA Laboratories) supplemented with B27 supplement (Invitrogen, Singapore) and activin A (100ng/mL, R & D systems) for 4 days[199]. This medium is subsequently referred to as “RPMI+ActA”. Then, the medium was changed to serum free RPMI 1640 medium containing B27 and HGF (20ng/mL, Invitrogen) for another 6 days to generate hepatic cells[199]. This medium is subsequently referred to as “RPMI+HGF”. For hepatic maturation, hepatocyte culture medium (HCM, Lonza, Singapore) was supplemented with oncostatin M (OCM, 10ng/ml, R & D systems) and was used to culture the samples for 5 days. This medium is subsequently referred to as “HCM+OCM”. HCM was used to maintain the derived hepatocytes subsequently until Day 30. The entire cell culture and differentiation process is summarised in **Figure 6.1b**.

6.2.4 Cell viability

All samples were tested for viability and proliferation at various time points throughout 30 days' of culture. Live/dead fluorescent staining (Invitrogen) was used where the cells were observed after 30 min incubation in calcein AM and ethidium homodimer-1. A total of 3 constructs were collected from each sample group for WST-1 (Roche, Singapore) colorimetric assay. Each construct was immersed in 300µl of DMEM with 30µl of WST-1 reagent and they were incubated at 37 °C for 1.5h. The supernatant was collected and added into the wells of 96-well plate for reading of absorbance at 450nm with reference at 620nm using a microplate reader.

6.2.5 Gene expressions

6.2.5.1 Polymerize Chain Reaction (PCR) and Gel electrophoresis

At each designated time points, three constructs from each sample group were pooled together for RNA extraction. RNA from the sample was extracted using TRIzol[®] (Invitrogen) following the protocol given by the manufacturer. The acquired RNA concentration was measured and a total 500ng of RNA from each sample group were reverse transcribed to cDNA before the subsequent polymerase chain reaction (PCR) experiments. All the reverse transcription and PCR reagents used were purchased from Promega (Madison, MI, USA) and were used according to the instruction given in the kit. For conventional semi-quantitative PCR, cDNA were amplified by respective primers using GoTaq polymerase. The thermal cycling conditions included 94°C for 5min followed by 35 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec, 72°C for 30 sec.

The reaction was completed with a final incubation at 72°C for 5 min. The PCR products were analyzed in 1.2% agarose gel followed by ethidium bromide (EB) staining.

6.2.5.2 Quantitative PCR

For real-time quantitative PCR (qPCR), iQ™ qPCR system (Bio-Rad) was used. The thermal cycling conditions for qPCR were exactly the same as the one describe for PCR except a total of 40 cycles were ran instead of 35. The relative gene expression values were obtained and calculated using the comparative ΔC_T (threshold cycle) method, using β -actin as the reference gene. The result were then further normalised to Day 0 of their respective sample group. All the primers used in PCR and qPCR were engineered by AIT Biotech (Singapore) and their sequences were presented in **Table 6.1** and **6.2** respectively.

Table 6.1. Primers used for PCR. P.S stands for product size

Genes	Accession Number	Primer Sequence (both 5'-3')	P.S (bp)
β-actin	NM007393.3	Forward: TCATGAAGTGTGACGTTGACATCCGT Reverse: CCTAGAAGCATTGCGGTGCACGATG	285
OCT4	X52437.1	Forward: AAAGCCCTGCAGAAGGAGCTAGAA Reverse: TCTCATTGTTGTCGGCTTCCTCCA	229
Nanog	AF507043.1	Forward: TCGAATTCTGGGAACGCCTCATCA Reverse: CCGCTTGCACTTCATCCTTTGGTT	408
Sox 17	AK004781.1	Forward: TGCCCTTTGTGTATAAGCCCGAGA Reverse: TTGGCAGGTGTCGAGATGACTGAA	789
Foxa2	L10409.1	Forward: AATGGACCTCAAGGCCTACGAACA Reverse: ATGACAGATCACTGTGGCCCATCT	353
GATA4	BC137824.1	Forward: ATGCCTGTGGCCTCTATCACAAGA Reverse: AGGACCTGCTGGCGTCTTAGATTT	287
HNF4α	BC039220.1	Forward: AATGGCGACATTCGGGCAAAGAAG Reverse: ACTGCCGGTCGTTGATGTAATCCT	466
AFP	BC066206.1	Forward: TGACAACAAGGAGGAGTGCTTCCA Reverse: ATACCAGAGTTCACAGGGCTTGCT	906

Table 6.2. Primers used for qPCR. P.S stands for product size

Genes	Accession Number	Primer Sequence (both 5'-3')	P.S (bp)
β-actin	NM007393.3	Forward: TCATGAAGTGTGACGTTGACATCCGT Reverse: CCTAGAAGCATTGCGGTGCACGATG	285
OCT4	X52437.1	Forward: AAAGCAACTCAGAGGGAACCTCCT Reverse: TAGCTCCTTCTGCAGGGCTTTCAT	122
Nanog	AF507043.1	Forward TGGTGTCTTGCTCTTTCTGTGGGA Reverse: ACACTCATGTGTCAGTGTGATGGCGA	180
Sox 17	BC060612.1	Forward: TGTAAGGTGAAAGGCGAGGTGGT Reverse: GCATCTTGCTTAGCTCTGCGTTGT	182
GATA4	BC137824.1	Forward: TCAAATTCCTGCTCGGACTTGGGA Reverse: GTTTGAACAACCCGGAACACCCAT	163
FoxA2	L10409.1	Forward: AAGGGAAATGAGAGGCTGAGTGGGA Reverse: ATGACAGATCACTGTGGCCCATCT	130
HNF4α	BC039220.1	Forward: TGGAAGCTACAGTCAAGGTGCAT Reverse: TCTCACAGCCCATTCTTCCACTT	177
AFP	BC066206.1	Forward: ACAGGAGGCTATGCATCACCAGTT Reverse: TGCTCCTCTGTCAGTTCAGGCTTT	181

6.2.6 Immuno- Histochemistry

Samples from each time point were fixed in 4% (w/v) paraformaldehyde overnight. The fixed specimens were dehydrated before embedded in paraffin. They were sectioned to a thickness of 10 μ m using a microtome. The sections from all groups were subsequently stained for Nanog, Foxa2/hepatocyte nuclear factor 3 beta (HNF3 β), AFP and HNF4 α respectively. The sectioned specimens were first immersed in xylene for 4 hours to remove the paraffin followed by fixation in 2.5% glutaraldehyde for 30min. They were then incubated with 10% horse serum (w/v, in PBS) for 20 minutes to avoid any non-specific binding of IgG. All primary antibodies were purchased from Santa Cruz Biotechnology and used at 2 μ g/mL in PBS. To test for pluripotent, endoderm and hepatic

makers, the specimens were incubated with rabbit polyclonal IgG Nanog, goat polyclonal IgG Sox17, goat polyclonal IgG HNF3 β (Foxa2), rabbit polyclonal IgG HNF4 α antibodies, goat polyclonal IgG albumin (ALB) and goat polyclonal IgG CYP1A1 (cytochrome P450) respectively at 4°C overnight followed by washing with PBS three times the next day. Subsequently, specimens incubated with Nanog and HNF4 α antibodies were incubated with goat anti-rabbit IgG-FITC(2 μ g/ml in PBS, Santa Cruz). Sox 17 and CYP1A1 samples were incubated with rabbit anti-goat IgG (5 μ g/ ml in PBS, AlexaFluor 543, Invitrogen). For Foxa2 and ALB samples, they were incubated with rabbit anti-goat IgG (5 μ g/ml in PBS, AlexaFluor 488, Invitrogen). All incubation with secondary antibodies was carried out at room temperature for 1 hour in the dark. This was also followed by PBS washing for 3 times before the nuclei were counterstained by 4',6-diamidino-2-phenylindole (DAPI).

6.2.7 *Quantification of urea and albumin*

At designated time points, cell culture media in every construct (n = 3) were replaced with 1ml of fresh media and incubated for 24-hours for collection. A 2D control was set up to analyse the urea and albumin production. Cells were seeded in 12-well plate at a density of 500 000 cells/well and undergo the same proliferation and differentiation stages described in **Figure 6.1b**. Media were collected in the similar manner as mentioned and analysed for urea and albumin production. Urea production was determined using colorimetric assay purchased from Biovision. Mouse albumin enzyme-linked immunosorbent

assay (ELISA) kit (ICL lab) was used following strictly to the manufacturer's protocol to quantify albumin secretion.

6.2.8 *Statistical analysis*

Where appropriate, one way ANOVA was used to analyze results using Tukey post hoc test with 3 specimens from each group. Results with $p < 0.05$ were considered statistically significant. Data were presented as mean value $\pm$ SD.

6.3 *Results*

6.3.1 *Cell morphology and viability*

To establish the MCG system, gelatin microspheres were employed as porogen and were co-encapsulated along with cells within the alginate hydrogel bulk. Upon exposure to higher (physiological) temperature in cell culture, gelatin microspheres dissolve and create micro-cavities of corresponding sizes within the gel bulk. The day of establishment of the cell-laden constructs is marked as Day 0. All constructs were cultured in proliferation media for 10 days to generate cell aggregates before undergoing through various stages of differentiation as stated in **Figure 6.1b**. The encapsulated cells in hydrogel adopted the typical rounded morphology on Day 1 as showed in **Figure 6.1c**. EBs were observed in both mESC and miPSC samples on Day 5 but they are relatively small ($<50\mu\text{m}$) and therefore continued to be maintained in proliferative medium till Day 10 when the EB achieved approximately $150\mu\text{m}$ in size. Endoderm induction was carried out from Day 10 to Day 14 while

hepatic lineage specification was carried out on Day 14 and last till Day 20. Final hepatic maturation was implemented on Day 20, for 5 days and the derived hepatocytes were maintained in hepatocyte culture media till Day 30. The EBs observed throughout the differentiation period remained relatively similar in size at approximately 150µm.

Figure 6.2: Comparison between MCG and non-MCG constructs. Phase contrast and live/dead images revealing the ability of MCG in facilitating viable EB formation compared to non-MCG constructs. Insert images are of 10× magnification.

Cell viability throughout the culture period is a key assessment in the evaluation of 3D scaffolds cell culture. It has to be first fulfilled before we proceed to differentiating them to any specific lineages. The live/dead staining in **Figure 6.2** highlights the importance of MCG system in improving cell viability and mediating EB formation. Comparing non-MCG (typical hydrogel cell encapsulation) system with MCG system on Day 10, the absence of cavities within the hydrogel bulk significantly impedes the formation of EBs in both mESCs and miPSCs. In addition, there were more dead cells in non-MCG constructs compared to MCG ones. This suggests that nutrient exchange is considerably improved through the introduction of cavities in MCG constructs. Based on this observation, MCG system is employed in all subsequent experiments.

Figure 6.3: Assessment of cell viability in MCG constructs. (a) WST-1 assay indicating general increase in absorbance. (b) Live/dead staining of ESCs and iPSCs in the microcavitary construct. Cells remained highly viable throughout the culture period and EBs formation was observed on Day 10. * indicates $p < 0.05$

WST-1 assay was used to evaluate cell viability and proliferation in both mESC and miPSC samples. As shown in **Figure 6.3a**, a steady increase in absorbance is observed in the first 10 days when cells were cultured in proliferative medium. This suggests a steady increase in number of viable cells in both mESC and miPSC constructs. Subsequently, absorbance value is decreased to a lower value on Day 14 and maintained approximately at that level for the rest of the culture period. In **Figure 6.3b**, live/dead staining confirmed that the EBs generated from mESCs and miPSCs remained largely viable throughout 30 days' of culture and were mainly rounded with typical size of 100-250µm in both cell types. In the first 10 days, the constructs were maintained in proliferative medium to generate sufficient EBs of appropriate size for differentiation. After introduction of differentiation media on Day 14, some dead cells were observed in both mESCs and miPSCs samples.

6.3.2 *Gene expression*

The evaluation of hepatic differentiation efficacy was done by assessing gene expression of the pluripotent markers, endoderm markers and hepatic markers respectively. As presented in **Figure 6.4**, pluripotent marker Nanog showed a more obvious down-regulation trend than Oct-4 in both mESC and miPSC samples. Most of the endoderm markers were strongly expressed from Day 14 till Day 25 while hepatic markers were highly expressed on Day 20 to Day 30. GATA4, one of the endoderm markers were detected only on Day 14 but diminished from Day 20 onwards in mESC samples. In miPSC samples, most

of the endoderm markers tested were expressed from Day 14 to Day 25. Alpha-fetoprotein (AFP) and Hepatocyte nuclear factor 4 (HNF4 α) are early hepatic markers and were detected from Day 14 onwards.

For higher sensitivity detection and a more detailed evaluation of gene profile, qPCR analysis was carried out. **Figure 6.5** showed that both Nanog and Oct4 were indeed progressively down-regulated after induction conditions were introduced in both mESC and miPSC samples. It is also noted that these pluripotent markers were down-regulated to a greater extent in miPSC samples than mESC samples. Endoderm markers were all up-regulated by Day 20 and down-regulated on Day 30. The qPCR analysis of AFP and HNF4 α expression followed a similar trend observed in gel electrophoresis in **Figure 6.4** where they are expressed at high levels on Day 20 and Day 25.

Figure 6.4: Gel electrophoresis of pluripotent markers, endoderm markers and hepatic progenitor markers from Day 0- 30 in both mESCs and miPSCs samples.

6.3.3 *Histology and immunohistochemistry*

All samples were evaluated for histologically and for presence of pluripotent marker, Nanog, endodermal markers, Sox 17 and Foxa2, and hepatic markers HNF4 α , ALB and CYP1A1 as shown in **Figure 6.6 and 6.7**. From the H & E staining, cell aggregates/EBs were formed by Day 10 and size were approximately ~150 μ m in both mESC and miPSC constructs. Occasionally, the aggregates were not round in shape but oval. This is probably as a result of aggregate formation within the gel bulk rather than at the cavities.

Figure 6.5: Gene expression of mESCs and miPSCs samples at different stages of differentiation by qPCR. All data are normalized to Day 0 of their respective group (N = 3) where Day 0 is represented as 1 unit. * indicates $p < 0.05$ when compared to its Day 0 counterpart.

a

Figure 6.6: (a) Histochemical (H&E) and immuno-histochemical staining of pluripotent marker (Nanog), endoderm markers (Sox17 and Foxa2) and (b) hepatic lineage marker (HNF4 α) and mature hepatic markers (ALB and CYP1A1) in murine ESC samples at from Day 5 to 30.

For the various marker expressions, Nanog was brightly stained in mESC constructs on Day 5 and 10 indicating the preservation of pluripotency in the system before differentiation. The level of Nanog decreased over time as induction media were introduced this data is in line with the data from the gene expression analysis in **Figure 6.4 and 6.5**. Sox 17 was detected mainly on Day 14 and generally decreased in amount at later time points but remained positively stained in both samples. The other endoderm marker, Foxa2, had a different staining trend in compared to Sox 17. Foxa2 was almost undetectable except on Day 30 as shown in **Figure 6.6a**. To evaluate hepatic lineage specificity, HNF4 α expression was evaluated. The brightly stained cell aggregates on Day 20 suggest successful differentiation to hepatic progenitors and they remained positively stained till Day 30. Hepatic maturation was confirmed by the exclusive positive staining of ALB and CYP1A1 on Day 25 and 30 (**Figure 6.6b**).

Murine iPSCs samples were stained in parallel for the same markers as shown in **Figure 6.7**. Nanog was brightly stained on Day 5 and 10 and diminished significantly from Day 14 onwards indicating that miPSC pluripotency was conserved until differentiation conditions were introduced. Sox 17 and Foxa2 were observed on Day 14 but mainly restricted to the edge of the EBs. By Day 25, the distribution of these two markers was more even throughout the entire EB. Notably, the protein expressions of these two markers in miPSC samples on Day 30 decreased as compared to previous two time points (**Figure 6.7a**). In contrast to the observation in ESC samples, HNF4 α

was detected from Day 20 instead of from Day 14 suggesting a more controlled differentiation than ESCs. Albumin and CYP1A1 were expressed exclusively on Day 25 and Day 30 indicating successful hepatic maturation (**Figure 6.7b**).

6.3.1 Urea and Albumin quantification

Both urea and albumin productions represent key traits of mature functional hepatocytes. Both urea and albumin are quantified from the culture media collected throughout the experimental period. Generally before maturation was carried out on Day 20, urea production was either negative or present at trace amount in both miPSC and mESC systems (data not shown). From **Figure 6.8a**, urea was produced at significant quantity on Day 25 and 30 in both mESC and miPSC 3D MCG constructs after maturation was induced. Compared to their respective 2D monolayer counterpart, miPSC encapsulated in 3D MCG constructs produced significantly higher amount of urea on Day 30.

Albumin production also remained negative from both Day 0 to Day 14 before maturation commenced on Day 20 (data not shown) and was only detected afterwards with highest albumin concentration in 3D MCG miPSC constructs (15.8ng/ml) as observed in **Figure 6.8b**. Similar to the observation in urea production, 3D MCG constructs have higher albumin secreted than 2D monolayer cells.

a

Figure 6.7: (a) Histochemical (H&E) and immuno-histochemical staining of pluripotent marker (Nanog), endoderm markers (Sox17 and Foxa2) and (b) hepatic lineage marker (HNF4α) and mature hepatic markers (ALB and CYP1A1) in miPSCs samples from Day 5 to 30.

Figure 6.8: Assessment of liver-specific functionalities. Quantification of (a) urea and (b) albumin produced in mESCs and miPSCs after hepatic lineage induction. * indicates $p < 0.05$

6.4 Discussion

The discovery of iPSCs brings great hopes in the field of biomedical research and translational practice. The ability to generate cells with comparable pluripotency to that of ESCs from the patients' own somatic cells creates a very valuable cell source alternative for tissue regeneration and provide a very useful platform for physiopathological studies. At present, most of the research work

on iPSCs remain in scientific exploratory stage and has yet to progress into therapeutic or biomedical engineering applications [181, 193, 196]. In this study, we attempted to combine knowledge on iPSCs differentiation with hydrogel-based 3D scaffold platform to fabricate pertinent hepatic constructs for possible liver tissue engineering purposes.

Hydrogels have been extensively used for cell delivery due to its biocompatibility and injectability. The high hydrophilicity and the lack of cell adhesive moieties in (non-protein made) hydrogels discourage cellular attachment (typically via focal adhesion) within gel scaffolds [201, 202] and influence the encapsulated cells to adopt a rounded morphology, which naturally facilitates cell aggregate formation [74, 100, 203]. In the case of pluripotent stem cells, it aids in colony/EB formation. The high proliferative capacity of ESCs and iPSCs demands huge nutrients supply and efficient exchange of metabolites. Though hydrogel is well-known for its good permeability, current work showed that typical hydrogel encapsulation (non-MCG) system is not permeable enough to fulfill the nutrient demand of ESC and iPSCs. The creation of cavities within the hydrogel system is required to further enhance permeation by decreasing distance of the diffusion paths and in turn increases the overall viability of the encapsulated cells. In addition, our previous studies [69, 74] together with the data presented here confirmed that MCG system could better facilitate cell aggregate formation within hydrogel bulk compared to non-MCG hydrogel system. In non-MCG model, the encapsulated cells or cell colonies have restricted proliferation and expansion

due to the mechanical constraint of (non-degradable) hydrogel bulk materials. On the contrary, the cavities created in MCG system provided living space for cells for much greater cell growth and expansion. Collectively, MCG system not only improved nutrients diffusion efficiency but more importantly, created space for cell aggregate formation.

Being a pilot study, the focus of this project was placed on investigating possible 3D cell delivery system that is capable of providing a favorable microenvironment for both miPSC growth and differentiation towards hepatic lineage. Unlike existing methods where EB formation and cell differentiation were done in 2D monolayer culture prior to seeding into a 3D biomaterial platform for cell delivery[204], the data presented here suggests that both EB formation and subsequent differentiation processes could be done within the cell delivery platform – MCG system per se.

The results demonstrated that MCG system is a continuous system capable of supporting good cell viability and spontaneous colonies/EBs formation in both mESCs and miPSCs. Oct-4 and Nanog expressions remained positive on Day 10 in both miPSC and mESC constructs. This suggests that the hydrogel system itself has minimum effect in inducing any uncontrolled differentiation in the colonies/EBs formed as long as leukemia inhibitory factor (LIF) remained present in the medium. When LIF was removed and differentiation conditions were applied, gradual down-regulation in pluripotent markers and up-regulation of endoderm markers, or even later - hepatic markers, were observed.

Moreover, both ESC and iPSC –derived hepatocytes in this MCG system were positively stained for ALB and CYP1A1. They were also capable of producing urea and albumin showing that they possessed hepatocytes functionalities. This result indicates that the entire process of colony/EB formation, endoderm induction, hepatic lineage specification and hepatic maturation in mESCs or miPSCs could be done continuously in single MCG system – only through a switch of culture conditions. Therefore, MCG system is both a favorable cell culture construct and a cell delivery vehicle where implantation could be done right after hepatic maturation is complete.

Interestingly, it was also observed that there were concurrent protein expressions of endoderm markers (Sox 17 and Foxa2) and hepatic makers (HNF4 α , ALB, CYP1A1) from Day 20-30. Although there is significant down-regulation of the endoderm markers by Day 30, Sox 17 and Foxa2 remained detectable on Day 30. This finding suggests that uncommitted endoderm cells were still present within the MCG construct after hepatic induction and maturation were carried out. Similar observation was also reported in a recent paper by Cheng et al, where the endoderm progenitor cells persists in culture even after prolonged hepatic induction[205]. In that study, activin A was used for endoderm induction followed by hepatic lineage specification using BMP4 and basic FGF. Hepatic cells was detected after 2 weeks but even after prolonged culture for up till 4 weeks, the cultures still contained a mixed population of Sox 17+ uncommitted endoderm cells and AFP+ hepatic cells. It was hypothesized that the endoderm progenitor cells might undergo both self-

renewal and differentiation into hepatocytes simultaneously in the culture[205]. The data reported in our current study also supported this hypothesis but further characterization of the encapsulated cells is required to verify the presence of these two distinct populations of cells within this MCG system. This future work may potentially provide further valuable information on differentiation efficiency of pluripotent stem cells in hydrogel based 3D cultures compared to that in EB suspension cultures which could eventually help to improve existing differentiation protocols.

6.5 Summary

Collectively, we have illustrated that both EB formation and subsequent differentiations could be carried out within the 3D MCG system. Compared to the monolayer counterparts, cells encapsulated in MCG system proved to have higher urea and albumin productions highlighting the beneficial effects of 3D microenvironment. Given that the MCG system is implantable, surgical procedure could be done right after generation of ESC/iPSC-derived hepatocytes. As the focus of this pioneer study lies in the establishment and development of a 3D engineered system for both colony/EB formation and hepatic differentiation, the findings in this work would serve as a good exploring reference in building foundations for translating iPSC technology into practical applications. The *in vivo* efficacy of iPSC-derived hepatocytes encapsulated in hydrogel system in restoration of liver-specific functions will be investigated on basis of the *in vitro* outcomes from this work.

Chapter 7 : Conclusion and Future work

7.1 Conclusion

In these studies, three different GS systems were proposed and evaluated for their potentials in delivery of various cell types. Importantly, the dual functionalities of the microspheres – as cell carrier and porogen – in establishing a bioresponsive GS composite system was highlighted. In the first system for instance, gelatin microspheres provided attachment sites for encapsulated cells and thereby utilized as cell carriers within the hydrogel. They were crosslinked with genipin to achieve long-term thermal stability for delivery of ADCs. In addition, this crosslinked microsphere is also found to facilitate the formation of cellular aggregates in another model cell, HepG2.

Given that size and shape control are essential criteria in cellular aggregate formation, the second GS system was designed to attain such control. It utilizes partial crosslinked microspheres as cell carriers as well as porogens to deliver cells and to restrict the aggregate size and shape within the hydrogel bulk. The microspheres in this system are responsive to enzymatic degradation and hence, through introduction of collagenase, microspheres were degraded, creating cavities within the gel bulk and the cells were delivered directly into the cavity. The dual functionality property in microspheres enables formation of cell spheroids that are of corresponding sizes to the cavities. The role of hydrogel in this GS system is a physical barrier to confine the cell spheroid size and to immobilize the cells at target site.

Finally, the third GS system is proposed for delivery of non-ADCs. Similar to the second system, it too generates a MCG system for neo-tissue regeneration. However, the distinctive feature of this third GS system is that cells were delivered in the hydrogel instead of through microspheres. As a result, this system is particularly fit for delivering non-ADCs like chondrocytes. The microspheres were introduced solely for the purpose of generating cavities within the gel bulk. This in turn enhances the overall permeability of the entire construct and at the time, free up space for ECM protein deposition. Consequently, a tangible construct, LhCG, made up of a dense intricate network of ECM proteins and isogenous group of chondrocytes was fabricated based on this GS system. LhCG, being a scaffold-free construct, is a very close resemblance of the natural cartilage. Therefore, it is investigated as a possible cartilaginous template to mimic the endochondral ossification pathway for bone regeneration.

Additionally, the application of the third GS system extends to pluripotent stem cells delivery. It is demonstrated that this GS system supports both EB formation and hepatic differentiation of mESC and miPSC. The MCG model derived from GS system provides high diffusion rate and ample space for cell growth and EB generation.

In conclusion, the studies in this dissertation proved that GS composite system is an easily customizable platform for delivery of various cell types – model cell lines, primary cells, and pluripotent stem cells. As a standalone

delivery system, hydrogel is not suitable for delivery of ADCs as it lacks in cell adhesive moieties and the microspheres could not be immobilized at target site after delivery. Through integration, the systems compensated for each other – the incorporation of microspheres introduces cell attachment sites for ADCs while the hydrogel serves as an immobilization agent for the microspheres. Depending on application, we believed that GS composite system could be further improved and customized to deliver a wider range of cell types.

7.2 Future work

Towards providing a closer niche for tissue regeneration, continuous exploration in cell delivery system is expected. In particular, future research directions could be focused on the following three aspects: i) combining strategies to co-deliver growth factors/genes with cells; ii) delivery of multiple cell types within GS system; iii) comprehensive study on the degradation profile of the scaffold.

To assist and direct the differentiation of the cells in the composite system, growth factors could be incorporated. For instance, activin A could be encapsulated within the microspheres of the GS system; upon temperature changes, dissolution of gelatin microspheres occur and thus releasing large dosage of activin A for endodermal differentiation of the pluripotent stem cells. Similarly, plasmids coding proteins could be introduced by incorporating viral or non-viral vectors within the GS system for long-term delivery of proteins.

Secondly, many studies have demonstrated the importance of crosstalks among various cell types in regenerating an organized tissue construct. Therefore, the establishment of a co-culture system is necessary. GS system, consisting of two separate delivery systems, is an ideal candidate for simultaneous delivery of multiple cell types. For instance, ADCs can be delivered on the microcarriers while the non-ADC cells can be encapsulated in the hydrogel bulk. Alternatively, the genipin crosslinked microspheres employed for HepG2 cells could also be used for delivery of endothelial cells. As a result, two types of cell-laden microspheres – each carrying a unique type of cell are encapsulated in the hydrogel, creating a co-culture system.

Apart from focusing on cell growth and differentiation within the scaffold system, subsequent remodeling of tissue is one of the final stage in tissue regeneration. Remodeling process includes both structural reorganization and integration of the construct with surrounding tissue. During this process, the supportive role of scaffold material is no longer necessary and may even be a physical barrier for remodeling. Hence, the degradation profile of hydrogel scaffold in GS system would significantly affect the remodeling process and should be characterized and optimized.

Publications

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