Injectable hydrogel/micro-composite systems for in situ therapeutic cell delivery

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**INJECTABLE HYDROGEL / MICRO-COMPOSITE
SYSTEMS FOR IN SITU THERAPEUTIC CELL DELIVERY**

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SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING

2009

**Injectable Hydrogel / Micro-Composite Systems for *in situ*
Therapeutic Cell Delivery**

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	I
TABLE OF CONTENTS	III
LIST OF TABLES	VII
LIST OF FIGURES	VIII
LIST OF ABBREVIATIONS	X
SUMMARY	XIII
 Chapter 1 Introduction	 1
1.1 Hydrogel – a promising vehicle for cell delivery	1
1.2 Anchorage-dependent cells (ADCs) and focal adhesion (FA)	5
1.3 Hydrogels for ADC delivery: a substantial challenge	10
1.3.1 Common, ‘non-adhesive’ hydrogels	10
1.3.2 ‘Adhesive’ hydrogels	12
1.4 Hydrogel modification for ADC delivery	14
1.4.1 Specific modification: with cell-adhesive ECM peptides / proteins	14
1.4.1.1 Specific modifications to 2D substrates	14
1.4.1.2 Specific modifications to 3D matrices	19
1.4.2 Non-specific modification: with chemical groups and physical hybridisation	22
1.5 Objective and organisation of this dissertation	25
 Chapter 2 Hydrogel / nano-aggregate composite: attempts to enhance cell affinity of hydrogels	 29
2.1 Introduction	29

2.2 Materials and methods	30
2.2.1 Silica-hybridised gel: 2D evaluation	30
2.2.1.1 Hydrogel-silica hybridisation	30
2.2.1.2 Protein adsorption and cell attachment	31
2.2.1.3 Gene expression	32
2.2.1.4 Immunofluorescent staining	33
2.2.2 Hydrogel / nano-aggregate composite: 3D exploration	34
2.2.2.1 Direct hybridisation	34
2.2.2.2 Functionalised silica aggregate	34
2.2.2.3 Cell-laden silica aggregate	35
2.2.2.4 Evaluation of cytotoxicity	36
2.2.3 Statistical analysis	37
2.3 Results and discussion	38
2.3.1 The 2D hybridised gel: the role of silica	39
2.3.2 The 3D exploration	43
2.3.2.1 Direct hybridisation	43
2.3.2.2 Functionalised silica aggregates	44
2.3.2.3 Cell adhesion on functionalised silica	46
2.3.2.4 Cytotoxicity	48
2.4 Summary	51

Chapter 3 Hydrogel / microcarrier (GC) composite: the control of ADC behaviour

3.1 Introduction	53
3.2 Materials and methods	55
3.2.1 Cell culture	55
3.2.2 Fabrication of cell microcarrier	56

3.2.3 TriG Microcarrier culture of ADCs	58
3.2.4 Settlement of ADC in GC: investigation in an osteogenic model	60
3.2.5 Biochemical analysis for GC system	62
3.2.5.1 Gene expression: at transcriptional level	62
3.2.5.2 Gene expression: at proteinic level	63
3.2.6 Animal experiments	66
3.2.7 Statistical analysis	67
3.3 Results and discussion	68
3.3.1 The TriG cell microcarriers	68
3.3.1.1 Gellan gel-based microspheres	68
3.3.1.2 Gelatin grafting	69
3.3.2 Microcarrier culture of HDFs	73
3.3.3 Microcarrier culture of hFOB: proliferation and differentiation	77
3.3.4 GC model: osteoblastic viability and apoptosis	79
3.3.5 GC model: integrin expressions, focal adhesion and cytoskeleton	81
3.3.6 GC model: insight into biochemical mechanisms	83
3.3.7 GC model: osteoblastic functionalities	86
3.3.8 Engineering explorations of GC: (1) biochemical study	88
3.3.9 Engineering explorations of GC: (2) <i>in vitro</i> and <i>in vivo</i> histology	91
3.4 Summary	96

Chapter 4 Hydrogel / microsphere composite: Injectable immunoconstruct for *in situ* tumour therapy 98

4.1 Introduction	98
4.2 Materials and methods	100
4.2.1 Cell culture	100
4.2.2 Materials system	100

4.2.3 Gene expression	102
4.2.4 Statistical analysis	104
4.3 Results and discussion	104
4.3.1 Dual-layer hydrogel composite	104
4.3.2 Activation of the GS system	106
4.3.3 Efficacy of GS system on carcinoma growth	114
4.3.4 Long-term fates of therapeutic cells	118
4.4 Summary	121
Chapter 5 Conclusions and future work	123
5.1 Conclusions	123
5.2 Future outlook	125
REFERENCES	128
PUBLICATION LIST	152

LIST OF TABLES

Table 1.1 Some representative hydrogel types for biomedical usage	3
Table 2.1 Primers used for real-time PCR analysis in Chapter 2	33
Table 3.1 Primers used for PCR analysis in Chapter 3	63
Table 3.2 Antibodies and related immunofluorescent dyes used in Chapter 3	66
Table 3.3 Oxidation degree of gellan microspheres	72
Table 4.1 Primers used for PCR analysis in Chapter 4	103
Table 4.2 PCR Array analysis of $M\Phi$ gene expression	108

LIST OF FIGURES

Figure 2.1 Cell adhesion and protein adsorption to silica-blended agarose gel	39
Figure 2.2 Integrin expression on silica-blended agarose gel	41
Figure 2.3 Focal adhesion and cytoskeleton reorganisation on silica-blended gel	42
Figure 2.4 Live-specific staining of cells in silica-incorporated agarose gels	44
Figure 2.5 Functionalisation and characterisation of silica nanoparticles	45
Figure 2.6 Cell adhesion to the functionalised silica aggregates	47
Figure 2.7 Integrin expression of hMSCs in SiC and SiF	48
Figure 2.8 Cell viability of hMSCs on SiC and SiF	49
Figure 2.9 Cytotoxicity and cellular intake of SiC	50
Figure 3.1 Methodology of GC set-up and hypothesis of cellular fate	55
Figure 3.2 Morphological observation of gellan gel-based microspheres	69
Figure 3.3 Schematic diagrams of fabrication of TriG microspheres	71
Figure 3.4 Extent of gelatination on gellan microspheres	73
Figure 3.5 Proliferation of HDFs on TriG microspheres	74
Figure 3.6 Cell attachment and focal adhesion on TriG microspheres	76
Figure 3.7 Proliferation and maturation of hFOB on TriG microspheres	79
Figure 3.8 Osteoblastic viability / apoptosis on TP, in Gel and GC	80
Figure 3.9 Integrin expression and focal adhesion of hFOB on TP, in Gel and GC ...	82
Figure 3.10 Expression of osteogenic markers of hFOBs on TP, in Gel and GC	87
Figure 3.11 Analysis of hMSCs viability and apoptosis in Gel and GC	89
Figure 3.12 Osteogenic markers (protein level) of hMSCs in GC and Gel	90
Figure 3.13 Osteogenic markers (transcriptional level) of hMSCs in GC and Gel ...	91
Figure 3.14 Histological evaluation of <i>in vitro</i> hMSC-laden GC and Gel	92

Figure 3.15 Histological evaluation of <i>in vivo</i> hMSC-laden GC and Gel	93
Figure 4.1 Schematic diagram of immunocyte-laden GS system	102
Figure 4.2 Examination of the activation of GS system	111
Figure 4.3 Examination of the effects of GS system on carcinoma growth	116
Figure 4.4 Long-term monitoring on cell viability of GS constructs	121

LIST OF ABBREVIATIONS

2D	two dimensional
3D	three dimensional
ADC	anchorage-dependent cells
ALP	alkaline phosphatase
AO gel	plain agarose hydrogel
APS	3-aminopropyltriethoxysilane
AS gel	silica-hybridised agarose hydrogel
BAEC	bovine aorta endothelial cells
BSA	bovine serum albumin
Col I	type I collagen
DC	differentiative condition
DI-H₂O / DI-water	deionised water
DMEM	Dulbecco's modified eagle's medium
ECM	extracellular matrix
EDC	<i>N</i> -Ethyl- <i>N'</i> -(3-dimethylaminopropyl) carbodiimide
ESC	embryonic stem cells
FA	focal adhesion
FAK	focal adhesion kinase
FBS	foetal bovine serum
FESEM	field emission scanning electron microscopy
FITC	fluorescein isothiocyanate
FN	fibronectin
FT-IR	Fourier Transform Infrared Spectroscopy
GAG	glycosaminoglycan

GC	hydrogel / microcarrier
HA	hyaluronan
HDF	human dermal fibroblast
hMSCs	human mesenchymal stem cells
KBr	potassium bromide
LPA	lysophosphatidic acid
MMEP	mono(2-methacryloyloxyethyl)
MSC	mesenchymal stem cell
MSCGM	mesenchymal stem cell growth medium
OC	osteocalcin
OPN	osteopontin
PBS	phosphate buffer saline
PC	proliferative condition
PCR	polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	poly(ethylene glycol)
PEGDA	poly(ethylene glycol) acrylate
PEGDM	poly(ethylene glycol) methacrylate
pFAK	phosphorylated FAK
PHEMA	poly(hydroxyethyl methacrylate)
PHPMA	poly(N-(2-hydroxypropyl) methacrylamide)
PI3K	phosphatidylinositol 3-kinase
PNaAMPS	poly(2-acrylamido-2-methyl-1-propanesulfonic sodium)
PNaSS	poly(sodium p-styrene sulfonate)
pNIPA	poly(N-isopropylacrylamide)
PVA	poly(vinyl alcohol)
p(VPA-co-AM)	poly(vinyl phosphonic acid-co-acrylamide)

RDG	Arg-Asp-Gly
RGD	Arg-Gly-Asp
SDS	sodium dodecyl sulfate
SiA	aldehyde-generated SiR
SiB	BSA-modified SiR
SiC	Col I-modified SiR
SiF	fibronectin-modified SiR
SiR	raw and unmodified fumed silica nanoparticles
SPAK	sulphopropyl acrylate potassium-acrylamide
TCPS	tissue culture polystyrene
TGF-β	transforming growth factor- β
TRITC	tetramethylrhodamine isothiocyanate
VAPG	Val-Ala-Pro-Gly
VN	vitronectin
VPA	vinyl phosphonic acid
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio] -1,3-benzene disulfonate
YIGSR	Tyr-Ile-Gly-Ser-Arg

SUMMARY

Hydrogels are promising cell delivery vehicles due to their remarkable advantages, especially injectability that enables *in situ* delivery and localisation. However, expanding applications of hydrogels into wider fields of cell therapeutics has been hindered by a substantial challenge, that is, anchorage-dependent cells (ADCs) show poor settlement when encapsulated into 3D hydrogel frameworks. Knowledge from cell biology and endeavours made by pioneer researchers suggested that the problem should lie in the absence of ‘adhesive anchors’ for ADCs within hydrogel bulk. Accordingly in this PhD study, such ‘anchors’ were proposed to be set up, with the aim to activate cellular focal adhesion (FA) and promote cell growth within a bioinert hydrogel. Furthermore, these ‘anchors’ were assumed to be at micro-scale, serving as cell-adhesive interface in the aqueous and bio-inert 3D matrices.

Based on such hypothesis, a hydrogel / micro-composite systems was devised for delivering ADCs with tissue regenerative prospect with nano-aggregates (in micron-size) or polysaccharide-based microcarriers as the ‘adhesive anchors’. Typical ADC species (and their progenitor cells) such as fibroblasts, osteoblasts and / or mesenchymal stem cells were first cultured on the ‘adhesive anchors’ to evaluate their FA-promoting efficacies. Eventually, the hydrogel / microcarrier composite with ADC-laden microspheres encapsulated inside hydrogel was considered as a

competitive platform for ADC delivery, according to its encouraging performance in supporting ADC spreading, proliferation, differentiation, as well as tissue formation in *in vivo* osteogenic models. Meanwhile, the biochemical mechanisms behind cell survival / death within 3D hydrogels were elicited, which confirmed the necessity of integrin ligation in avoiding apoptosis and promoting differentiation / proliferation of ADC species.

Provision of ‘adhesive anchors’ was shown to be essential in regulating ADC fate and behaviours in hydrogels, which illuminated us to take advantage of the contradiction between ADCs and common hydrogels without ‘anchors’. In the context of cell-based tumour immunotherapy, immunocytes were expected to be delivered to the target site for exerting tumouricidal activities. Accordingly, an ‘anchor-free’ hydrogel / microsphere system – with macrophages doubly encapsulated in microspheres and hydrogel – was contrived for the conveyance of activated macrophages. Results indicated that this model preserved all the advantages of hydrogel and presented effective elimination of carcinoma cells. Notably, the delivered macrophages were heavily confined from freely moving or proliferating, and most of them underwent apoptosis within a few months due to the lack of ‘anchors’. This strategy enables potential treatment modalities to be initiated at a desired location and implemented in a controlled manner.

In summary, these hydrogel / micro-composites are promising platform to deliver

living cells for tissue engineering and other therapeutic purposes. The settlement and commitment of delivered cells in such 3D matrices can be desirably controlled.

Chapter 1 Introduction

1.1 Hydrogel – a promising vehicle for cell delivery

The rapid development of biomedical materials has greatly expedited the progress of modern biotechnology and regenerative medicine. From the early acellular surgical implants and woven wound dressing, to the established polymeric cell-laden scaffolds and polycationic gene-delivery vectors, to the emerging cell-free protein expression system and nucleic-acid biosensor microchips – the fascinating exhibit of biomedical material collections through the past decades has deeply attracted the attention of both scientific and public communities. Polymer chemists, cell biologists, orthopaedic surgeons, clinical oncologists, computer-aided designers, mechanical engineers and many other researchers and practitioners ally from bench to bedside to develop and evaluate innovative materials, devices, as well as novel concepts of regeneration, towards a healthier life for all.

Among the numerous types of biomaterials, hydrogels have shown great promises in a variety of biomedical applications [1-3]. In nature, hydrogels are hydrated, water-insoluble polymeric networks cross-linked by water-soluble precursors. The composition of hydrogels can be natural polysaccharides [4] (agarose, alginate, gellan, etc.), proteins [5] (e.g. collagen and gelatin), or synthetic macromers such as poly(ethylene glycol) (PEG) and its derivatives [6], poly(vinyl alcohol) (PVA) [7] and

poly(hydroxyethyl methacrylate) (PHEMA) [8]. The crosslinking, i.e. gelation, is triggered via different mechanisms (e.g., thermo-, ion- or photo-initiated), and converts precursor solutions into three-dimensional (3D) gel matrices. The period of time for this transition can be adjusted, but is usually as short as several seconds to a few minutes [3]. For applications of drug or cell delivery, therapeutic agents or living cells are first suspended into the fluid precursors, and then encapsulated within the gel bulk upon gelation. The hydrogel framework is filled with fluid and the range of its pore size is limited. Taking PEG hydrogels as example, their typical pore sizes are 40 – 200 Å. Therefore, the movement of encapsulated cells is restrained and the release of drugs is controlled [2, 3, 9, 10]. Moreover, these drug- or cell-laden hydrogels are often fabricated into different shapes and sizes, from nano-scale particles [11] to centimetre sheets [12], to meet various practical requirements. In a recent work published in *Nature*, French researchers contrived a multi-layer, onion-like hydrogel with highly controlled physicochemical properties and cell-laden capability. It provides a brand new, interesting architecture for hydrogel design [13]. Some of the representative hydrogels are illustrated in **Table 1.1**.

Hydrogels are particularly suitable for cell delivery-based therapeutics, due to their unique and versatile advantages [10, 14-16]. First, the liquid precursor of hydrogels containing therapeutic agents / cells is injectable, which can ensure the spatial fit between the gelatinised implants and any randomly shaped cavities of defects – as spe-

Table 1.1 Some representative hydrogel types for biomedical usage.

Types of hydrogel	Gelling mechanism
<u>Poly (ethylene glycol)</u> : PEG; Synthetic polymer with several derivatives; very widely used as hydrogel models	Covalent: modified with acrylate and photo-polymerised Example: PhosPEG molded into mm-cm scale [15]
<u>Poly (vinyl alcohol)</u> : PEG; Synthetic polymer; good property to form films	Covalent/physical: with gluteraldehyde or by freeze-thaw Example: PVA/ferritin nanofibres [17]
<u>Agarose & Gellan</u> : Natural polysaccharides easily forming hydrogels; widely used in molecular / micro- biology	Physical/ionic: Thermo- (or with ion-) induced gelation Example: Gellan cylinders in cm-size [18]
<u>Alginate & Chitosan</u> : Natural polysaccharides widely used in bio-encapsulation and drug delivery	Ionic/covalent: Ion-induced (chitosan needs crosslinker) Example: Alginate/chitosan capsule [19]

-cifically required for *in situ* therapy. Second, the cell-encapsulating hydrogel can be rapidly constructed by gelatinising a cell-suspended macromer solution via thermo-, ion-, or photo-cures, leading to a uniform distribution of the transplanted cells in their 3D matrices. Third, the water content and physical properties of hydrogels can be manipulated to precisely mimic their native tissue counterparts. Last but not least, the structure of hydrogels with limited pore size allows free diffusion of water / metabolites and exchange of nutrients / wastes, but confines the overall migration of individual cells.

In a large number of *in vitro* and *in vivo* practices, hydrogels have been utilised to deliver and accommodate therapeutic cells, aiming for the regeneration of cartilage [20], cornea [21], liver [22], islet [23], nerve [24] and other tissue / organs. It is not surprising that cartilage engineers especially favour hydrogels. On the one hand, hyaline cartilage is avascular; once injured, it can not self-regenerate in adults – so cell delivery-based therapeutics is highly demanded. On the other hand, hydrogels are ideal scaffolding choice for cartilage engineering, because hydrogel bulk is similar to native cartilage in many aspects, and can maintain the encapsulated chondrocytes in their native round shape. Since Elisseeff and Anseth *et al.* employed PEG-based hydrogel to photo-encapsulate chondrocytes [20], their two independent groups have kept exploring the potentials of photopolymerised hydrogels for cartilage regeneration. Bryant and Anseth *et al.* serially elucidated the effects of various material parameters

including scaffold thickness [25], photoinitiating systems [26], gel properties [10, 27], degradation [28], crosslinking density [29, 30] on the encapsulated chondrocytes; further, they incorporated tissue-specific molecules and applied spatial patterning to hydrogels, analysing the gene expression and cell metabolism of chondrocytes [31]. Elisseff's group carried out explorations of inducing chondrogenesis of mesenchymal stem cells (MSCs) [32], embryonic stem cells (ESCs) [33, 34] and ESC-derived MSCs [35] within hydrogel matrices, as well as developing novel biodegradable hydrogels for chondrocyte encapsulation [15, 36]. Notably, in their two research works [37, 38], Wang and Elisseff *et al.* addressed the opinion that the integration of hydrogels with surrounding native tissue is important, and demonstrated that such integration facilitated both immediate functionalities and long-period stability of engineered tissues. In tandem with these contributions by materialists, Tuan and colleagues investigated chondrogenesis and its related signal pathways within a variety of 3D matrices, ranging from agarose or alginate hydrogels [39-41], fibrous / porous scaffolds [42-44], to vehicle-free micromass [45]. These comprehensive findings at cellular and molecular level have provided biological insights to the understanding of cell behaviour in engineered hydrogels [46, 47].

1.2 Anchorage-dependent cells (ADCs) and focal adhesion (FA)

The term of anchorage dependence refers to the phenomenon that the survival of

cells requires attachment to a substrate; cells having no contact with a matrix may easily go through apoptosis [48, 49]. Most cell types used in tissue engineering are anchorage-dependent, including fibroblasts, osteoblasts, endothelial, epithelial and smooth muscle cells. The anchorage-dependent nature of these cells is a fundamental topic of cell-material interaction; its fulfilment determines the survival / apoptosis, adhesion / migration, proliferation / differentiation and many other important behaviours of therapeutic cells [49, 50]. Over the years it has been realised that the biology of ADCs is of great importance to the design of cell-delivery vehicles.

A successful settlement of ADCs begins with the binding of their integrin receptors and specific motifs on extracellular matrix (ECM). Integrins are transmembrane proteins, in the form of α - β dimers; and those peptide motifs, such as Arg-Gly-Asp (RGD), locate on the chains of fibronectin, vitronectin, collagen, and etc. Integrins can recognise and bind to RGD or other specific domains, and subsequently initiates a series of intracellular events – one of which is focal adhesion (FA) [49]. It starts from the phosphorylation of several amino-acid sites of the focal adhesion kinase (FAK) [51, 52]. The phosphorylated FAK (pFAK) can recruit Src kinase that further phosphorylates other FA components like paxillin and tension, and is also linked to various signal pathways such as the Ras – MAPK cascade. As a result, integrin, FA and related downstream events act in a complicated but precisely controlled manner to regulate cell fate and behaviour.

Cell survival. Cell survival is the prerequisite to all of other cellular behaviours: proliferation, differentiation, secretion, migration, etc. FA plays a key role in transmitting survival signals from ECM proteins [53, 54]. Established pFAK provides binding domains to Grb2-Sos and phosphatidylinositol 3-kinase (PI3K), through which the Akt pathway is activated. Consequently, several pro-survival and anti-apoptosis events are stimulated, including the inactivation of Caspase-9, blockage of certain pro-apoptotic proteins, maintenance of mitochondrial membrane potential and inhibition of mitochondrial-mediated apoptosis. Further, FAK is also involved in the prevention of p53-mediated apoptosis.

Cell growth. Cell proliferation requires integrin-mediated adhesion, which stimulates at least two pathways promoting cell proliferation [54, 55]. First, the growth factor-mediated Ras-ERK cascade is blocked when cells are not well adhering to substrates; once cells are anchored to ECM, integrins remove the block by activating Rac or PI3K. Second, the association of FAK with Src is required to activate MAPK-JNK pathway, which subsequently transmit important signals into nucleus for cell proliferation.

Cell migration. FA is determinant to the dynamic process of cell migration [53, 55]. First, members of FA components such as paxillin, talin, vinculin and FAK are necessary to stabilise the membrane protrusion; then, both FAK and Src are required to realise the ‘turnover’ of FA – cells continuously reduce and re-establish FA to migrate;

also, some FA-associated proteins, e.g. the universal FA marker vinculin, are re-localised to the lamellipodium especially during cell spreading, as a regulatory mechanism for more stable cell migration.

Cell shape. Integrins and FA highly regulate cell spreading and shape [56, 57]. Briefly, integrins activate the Rho-family of small guanine nucleotide-binding proteins. These proteins, via multiple downstream effectors, induce FA and control the actin cytoskeleton. Consequently, integrin-located filopodia are extended, actin-rich lamellipodia are generated; actin monomers are assembled into a filamentous network (F-actin) that promotes the orientation of cytoskeleton and spreads out the whole cell body.

It should be noted that the spreading shape, led by successful cytoskeleton reorganisation, is extremely important to the survival and functions of ADCs [58]. A study by Colotta group demonstrated that even in the presence of the integrin-binding RGD peptides, the endothelial cells could not be rescued from apoptosis if the cells were not permitted to spread [59]. The authors suggested that, aside from the cell-matrix interaction mediated by integrins, cell spreading is required and obligatory to rescue cells from apoptosis. Similarly, the famous work by Chen *et al.* also indicated that round cells were prone to apoptosis but stretching cells survived and proliferated [46].

The concept of anchorage-dependence has been increasingly emphasised in the

design and modification of cell-delivery vehicles. Therapeutic ADCs should adhere to their ECM anchors and exert a spreading morphology, so as to survive and retain their phenotype. Taking bone engineering as example: researchers deliver osteoblasts or their progenitor cells like MSCs with vehicles to repair and regenerate bone loss and major bony defects prevalently inflicted by injuries, diseases, ageing, or congenital absence. For osteoblasts, it is known that among the 22 heterodimers of α and β integrin sub-units, $\alpha 1$ and $\alpha 5$ are expressed by all types of bone cells on human tissue section, while high levels of $\alpha 1\beta 1$, $\alpha 3\beta 1$ and $\alpha 5\beta 1$ are expressed by cultured osteoblasts [60-62]. It has been well known that FA mediated by these integrins directly promotes survival of osteoblasts – just like other ADC species [63]. Using RGD moieties as a model, Grigoriou and co-workers confirmed that osteoblast was rescued from apoptosis via FA-induced PI3K activation and the mitochondrial pathway [64]. Integrin-FA plays a key role in osteoblast differentiation / maturation as well, based on the investigations conducted in both *in vitro* matrices and transgenic mice models [65, 66]. For progenitor cells like MSCs, their commitment into the ADC lineage, e.g. bone-related cells, is also significantly regulated by the integrin binding and FA. By employing human MSCs together with NIH3T3 fibroblasts (typical ADC) and MC3T3E1 osteoblasts (typical bone-cell model and ADC), Jadowiec *et al.* found that the integrin-RGD binding could initiate, via the MAPK pathway, the expression of differentiative genes and lead to the formation of highly-organised mineralised tissues

[67]. In a series of studies, Salaszyk and Plopper *et al.* emphasized the importance of FA during the osteogenic commitment of MSCs, demonstrating that the activation of FAK is essential and one of the earliest decisive events that direct MSC commitment into the osteogenic lineage [36, 68-70].

1.3 Hydrogels for ADC delivery: a substantial challenge

1.3.1 Common, ‘non-adhesive’ hydrogels

Most hydrogels, composed of synthetic polymers or natural polysaccharides, have poor cell affinity. They do not support adhesion and spreading of ADCs, which has been one of the biggest challenges in broadening their cell-delivery applications. This is generally owing to the lack of appropriate interface between cells and substrates [6, 71].

As discussed in the previous section, the attachment of ADCs requires the binding between cellular receptors and ECM proteins like fibronectin and collagen. However, common (synthetic or polysaccharide) hydrogels possess an extremely hydrophilic nature, which resists not only the adsorption of proteins, but also the unfolding of protein structure and exposure of specific domains (such as RGD). As a result, once ADCs are seeded onto the gel surface, their integrin receptors cannot find the peptide ligands and cell adhesion is consequently denied.

Cells in 3D hydrogel matrices are constrained into a round shape – regardless of cell types or native morphology, due to a unique 3D-enlacing mechanism of hydrogel encapsulation. The bulk of hydrogels, like their surface, provides hydrophilic and bio-inert microenvironment in which integrin-protein communication cannot be initiated. In addition, cells are suspended in the fluid surrounding of gel that is highly aqueous polymeric frameworks with nano-scale pores. Without strong biochemical signals, cells are restricted from spreading or freely moving into any direction. This would be beneficial towards certain aims, such as chondrogenesis, in that the native cartilage cells are spheroidal and their outstretching may easily lead them into dedifferentiation; however, it makes osteogenesis or some other morphogenesis scarcely achievable because the likes of osteoblasts belong to ADCs, which require attachment to substrate and prefer spreading to maintain their normal phenotype. These ADCs lose their nature and survive poorly in such inappropriate environment, reflecting in practice that the conveyance of ADCs with hydrogels has always been accompanied with mass depletion of cell amounts and/or failure of differentiation into desired lineages.

One typical case may well represent the dilemma of using hydrogel encapsulation for cell delivery: the identical agarose gels that reportedly could preserve the chondrocytic phenotype (and could even induce the dedifferentiated cells to redifferentiate) [72], were evidenced to inhibit osteoblastic proliferation and the

expression of typical bone markers such as type I collagen (Col I) and alkaline phosphatase (ALP) [73]. Other attempts to conduct osteogenesis in a polymer-based hydrogel have also encountered similar problem, that the viability of hMSCs (progenitor cells of osteoblasts) was heavily impaired during osteogenic induction [74, 75]. Therefore, much effort needs to be devoted to create a favourable interface between ADCs and gel matrices, so as to ensure an expected settlement and commitment of therapeutic cells in hydrogels.

1.3.2 ‘Adhesive’ hydrogels

Some types of hydrogels are arguably considered as ‘adhesive’ hydrogels because they are made of ECM proteins that comprise cell-adhesive domains. Examples include collagen / gelatin-based hydrogels, which can support ADC adhesion on their surfaces. However, substantial challenges still remain when these hydrogels are used for ADC delivery.

Many reports have used collagen / gelatin-based hydrogels for drug / gene delivery and cultured cells to their sponges. Their good biochemical properties and particularly, cell-recognised sites and degradability, make them attractive as cell scaffolds [3]. However, unmodified collagen or gelatin is not suitable for cell encapsulation. Gelatin forms thermo-reversible hydrogel but its gelling point is far below the temperature range that is too low for living cells. Cell-suspended gelatin solution at around 37.5 °C

cannot convert to gel. To load cells into gelatin, some researchers seeded cell suspension to the pre-prepared gelatin sponges, and the cells could intrude into the hydrated, hydrogel-like scaffolds. However in this way, many key advantages of hydrogels for cell delivery would be lost: i) the system is not injectable; ii) the cells are lower in density and less homogenised than expected; iii) the gels cannot be formed *in situ*, and thus fail to spatially fit the defective shapes. The use of collagen faces the same problem. To overcome this drawback, Hoshikawa et al. invented a photopolymerised gelatin hydrogel. This attempt successfully achieved encapsulation of living cells in gelatin hydrogel, but posed a new problem: the crosslinking caused a marked decrease in cell growth, the mechanism of which remains further study [76]. Further, gelatin hydrogels are also considered unstable as cell matrices and limited to combined use and/or crosslinking; while the wide use of collagens is limited due to ‘qualitative batch variations and loss of shape and consistency through shrinkage’ [3].

In addition, it has also been found that the cell morphology and phenotype in collagen sponges / hydrogels are different from those of ADCs on 2D substrates. As mentioned above, 3D encapsulation and enlacing by hydrogel matrices and associated physical factors play important roles in cell shaping. Using typically anchorage-dependent fibroblasts as model cells, Grinnell and colleagues conducted a series of studies on cell behaviour within 3D collagen matrices [77-80]. It was observed that fibroblasts were in a special dendritic morphology with retracted

cytoskeleton within 3D collagen, in contrast to their spreading shape with extended F-actin on collagen-coated coverslips [78, 79]; in terms of cell function, fibroblasts were surprisingly found in a quiescent, resting state in floating collagen matrices, instead of a proliferative, activated phenotype [80]. The authors attributed this abnormality to the unaccomplished Ras coupling to its downstream molecules, but more detailed study is required for further use of collagen as 3D cell vehicles.

In brief summary, to simply rely on using collagen or gelatin-composed hydrogels cannot utterly solve the problem that ADCs settle poorly in hydrogel matrices. Strategies to modify common, ‘non-adhesive’ hydrogels are in great demand.

1.4 Hydrogel modification for ADC delivery: from ‘non-adhesive’ to ‘adhesive’

1.4.1 Specific modification: with cell-adhesive ECM peptides / proteins

1.4.1.1 Specific modifications to 2D substrates

Specific modification refers to the chemical incorporation of peptides / proteins that comprise specific cell-adhesive domains. The idea rises from the biological discoveries on the interactions between ADCs and ECM – as described in the previous sections. In both physiological and artificial (e.g., foreign substrate) environments, it is well understood that cell adhesion and subsequent settlement is triggered by cellular transmembrane receptors that are occupied and activated by specific binding ligands – some substantially functional domains located on certain ECM proteins. One of the

well studied and widely used pairs is the cellular integrin and cell-adhesive peptide RGD.

Jeffrey A. Hubbell and colleagues were among the pioneers who deployed RGD to improve cell affinity of the hydrogel surface. Based on the group's previous findings on RGD-modulated cell-material interaction [81, 82], Hern and Hubbell covalently conjugated RGD peptides to the photopolymerised poly(ethylene glycol) diacrylate (PEGDA, MW 8000), and demonstrated that human foreskin fibroblasts could attach to and spread on the crosslinked RGD-PEGDA gel [6]. Stretched and well-reorganised F-actin cytoskeleton was visualised on the substrate with RGD spacer at a density of 1.0 pmol/cm^2 . In contrast, no cell adhesion was observed on unmodified or Arg-Asp-Gly (RDG)-modified gels, suggesting that the specific binding between cellular receptors and corresponding ligands is essential in modulating cell-surface interactions. Almost during the same period, Mooney's group grafted RGD onto the sugar chain of the alginate polysaccharide – one of the commonly-used natural hydrogels. Mouse C2C12 myoblasts cultured on the RGD-modified alginate gel exhibited a spreading morphology and rapidly proliferated to confluence within 72 hours, while no adhesive cells was found on the unmodified gel [83]. These works had resonated with numerous laboratories in the past decade. For instance, Zhu and Marchant *et al.* contrived new ways of chemistry to couple linear or cyclic RGD to the non-adhesive surface [84, 85]; and several other groups have successfully modified the

peptides to a variety of both synthetic and natural hydrogels including PVA [86], poly (*N*-(2-hydroxypropyl) methacrylamide, PHPMA) [87], poly (propylene fumarate-*co*-ethylene glycol) [88] and hyaluronan (HA) [89]. Also, aiming for specific therapeutic purposes, people cultured cells of different origins / lineages such as smooth muscle cells [14], osteoblasts [90], chondrocytes [91], corneal epithelial cells [92] and MSCs [75] onto such RGD-gel substrates and found the cell adhesion / functions were correspondingly enhanced.

Since these cell-adhesive, amino-acid sequences were initially discovered from some ECM proteins, these ECM components should be of equal potential as those short peptides in improving cell affinity of hydrogels. One such candidate is fibronectin (FN), a glycoprotein that contains integrin-binding domain and plays pivotal roles in regulating cell motion and behaviours in native tissue. Nuttleman and Anseth *et al.* conjugated FN to the polymer chain of PVA, producing a glutaraldehyde-crosslinked PVA (FN-PVA) hydrogel substrate on which the NIH3T3 skin fibroblasts could attach well and spread. They also observed that proteins did not adsorb strongly into the gel bulk, which indicated that bioactive proteins like FN could not be physically arrested to general hydrogels for initiating cell attachment [93]. Covalent immobilisation of FN is necessary. Zajackowski *et al.* compared such an engineered FN-PVA substrate with two theoretical substrates of FN-adsorbed and FN-conjugated coverslips, and pointed out that the formation of cell adhesion structures on the engineered FN-PVA gel,

including the co-localisation of integrin / paxilin markers and the extent of fibrillogenesis, was in an intermediate manner between the two controls [94]. Due to its inherent and stable cell-adhesive property, FN has also become a popular choice in theoretical studies such as micro-patterning of substrates. Using microcontact printing techniques, Brock and colleagues fabricated cell-adhesive FN-coated islands on the cell-non-adhesive substrate. Cells only attached onto these islands in different shapes, and extended their filopodia / lamellipodia to sample the terrain. This interesting work offered an approach to geometrically control cell adhesion and spreading [95].

Collagen is another ECM protein that has been utilised for bridging cells to the hydrogel surfaces. Collagen family are major components of ECM in a variety of tissues, and their primary structures comprise both cellular integrin- and fibronectin-binding sites. Collagen (mostly type I) and its denatured form, gelatin, are widely fabricated into cell scaffolds with favourable biochemical and physical properties. Sosnik *et al.* synthesised a collagen-poloxamine hydrogel, which supported the adhesion and spreading of both human HepG2 hepatocarcinoma and bovine aorta endothelial cells (BAEC) [96]. Another group covalently incorporated collagen into HA precursor, and crosslinked a HA-Col hydrogel that not only promoted cell attachment but was also controlled cell orientation on its surface. Additionally, it was proved degradable to collagenase [97]. Gelatin is denatured from collagen with identical amino acids; it is considered equally effective and more economical. In a

number of cases it was coupled with polysaccharide hydrogels. Prestwich's laboratories did a series of work grafting gelatin onto the sugar chain of HA and chondroitin sulphate, two building blocks of ECM, and found that only the gels grafted with gelatin could support cell adhesion [98-100]. Moreover, gelatin-coated gellan hydrogel microcarrier and gelatin-dextran interpenetrating gel network with desirable cell affinity have been recently reported [101, 102], showing excellent potentials of this natural biopolymer in promoting cell affinity.

Other short peptides or ECM proteins such as VAPG (Val-Ala-Pro-Gly, from elastin) [103], YIGSR (Tyr-Ile-Gly-Ser-Arg, from laminin) [104], and laminin [105], have been utilised for similar purposes and obtained interesting results. In summary, these cell-adhesive peptides or proteins / glycoproteins contain specific domain(s) that can be recognised by cellular receptors. Only upon the accomplishment of such recognition, can cell adhesion and following spreading, survival and growth / differentiation be achieved in the artificial microenvironments. This type of strategy aims to establish a cell-material interaction based on specific ligand-receptor bindings. It is of a clear biological mechanism, and has shown satisfactory efficacies at 2D scale so far, therefore has been widely adopted by researchers to introduce cell adhesion onto the bioinert, non-adhesive substrates of common, non-adhesive hydrogels.

1.4.1.2 Specific modifications to 3D matrices

Achievements in 2D modification of hydrogels, especially the approaches dealing with cell-adhesive ECM mimics, have provided clues to 3D cases. After all, the 2D investigations are limited to theoretical research. In practice, therapeutic cells are delivered in 3D vehicles; therefore, their settlement and commitment within 3D hydrogel vehicles are of great importance.

The behaviour of osteoblasts in 3D hydrogel matrices has been investigated for years due to many researchers' interest in musculoskeletal regeneration. Osteoblasts are typical ADC species and thus face the lack-of-anchor challenge in hydrogels. One representative study was from Burdick and Anseth, who seeded osteoblasts onto (2D), and also encapsulated the cells into (3D), RGD-modified PEGDA hydrogels [90]. Their 2D finding was not exceptional from above-mentioned studies that adhesive and spreading osteoblasts were observed on the surface of RGD-modified gels only, and not on unmodified ones. However, their exploration in 3D bulk showed no obvious signs of cell spreading in RGD-modified gels according to the Live/Dead fluorescent staining. No difference in cell viability was indicated between the unmodified and RGD-modified gels; instead, cell loss was found in both groups after 1-2 weeks. The only positive finding was the observation of a 'significant increase in mineralization' in the RGD-modified samples (by macroscopic observation). In another study by Evangelista *et al.*, osteoblasts were encapsulated in RGD-modified alginate hydrogels

[106]. Similarly, cells were hardly found in a spreading morphology in either modified or unmodified matrices, from their fluorescent images in gross view; however, osteoblast viability in RGD-alginate was shown to be higher than in unmodified alginate. It seems that RGD-modification far less effectively facilitates osteoblast spreading in 3D matrices than it does on 2D surfaces.

Incorporation of RGD was applied to promote the survival of progenitor / stem cells that expectedly differentiate into anchorage-dependent lineages within 3D hydrogels. After finding that the viability of encapsulated hMSCs in PEGDA decreased drastically by 90 % in 4 weeks, Nuttelman and co-workers conjugated RGD into the gel bulk, which effectively improved MSC viability by ~ 60% after 1 week as compared with that in the unmodified gel [74, 75]. Interestingly, the less-than-10% living cells in unmodified PEGDA samples could still express osteogenic markers like ALP and osteopontin (OPN) when cultured in conditional medium; however, such an engineered tissue construct is unacceptable – as sufficient cell density must be guaranteed for cell-based therapy, and too many dead cells may pose immunogenic and other concerns *in vivo*. This group proceeded to graft the PEGDA bulk with heparin, a glycosaminoglycan (GAG)-type ECM component with binding domains for several proteins [107, 108]. The authors expected the immobilised heparin to sequester adhesive proteins like fibronectin and further promote cell-material interaction. Results indicated that after 5 weeks, the viability of hMSCs in heparin-functionalised

hydrogels was significantly higher than both unmodified PEGDM and RGD-modified gels; and had no statistical difference with that in heparin + RGD co-functionalised gels. Highest expression of integrin $\alpha 5 \beta 1$ was also presented in heparin-modified group.

This strategy has also benefited the regeneration of other tissue / organs. In a series of works, Mann and West *et al.* conjugated transforming growth factor – β (TGF- β) [109] and several cell degradable / adhesive peptides [110, 111] including RGD to PEG-based hydrogels, and studied the behaviours of vascular smooth muscle cells encapsulated in these matrices. They summarised that: i) the tethering of TGF- $\beta 1$ can increase matrix production; ii) the provision of cell-degradable / adhesive peptides could facilitate cell migration within the 3D matrices; however, both cell proliferation and collagen production in adhesive-peptide-gels were lower than in control gels, albeit the modification of adhesive ligands initially enhanced cell adhesion [110, 111]. Luo and Shoichet demonstrated a creative approach to guide axonal growth in 3D hydrogels [71]. They modified the bulk of agarose hydrogel with photolabile groups, and then used a focused layer to immobilise RGD moieties in selected volumes. Consequently, biochemical channels were patterned to guide cell migration and neurite outgrowth in the 3D hydrogel matrices. Weber *et al.* functionalised the poly(ethylene glycol) methacrylate (PEGDM) hydrogels with cell-recognised peptide sequences – several laminin-derived and one collagen-derived [23, 112]. It was found that certain peptides

could well preserve the viability of the encapsulated islet β -cells, which would fall sharply by $\sim 90\%$ within 10 days in unmodified gels. Enhanced cell-matrix interaction by some of these peptides also increased insulin secretion.

In summary, conjugation of cell-adhesive peptides like RGD is the mainstream to modify 3D hydrogel scaffolds. This approach appears to be feasible to some extent promoting cell survival, and up-regulating cell functions such as cell secretion (of insulin, collagen, etc.), cell migration and tissue calcification. Therefore, it is a feasible and, to some extent, promising method. However, from most studies, one can see that the encapsulated ADCs are scarcely in their favourite spreading morphology even in the presence of RGD. This might be associated with the physically enlacing effects of 3D encapsulation. As previously reviewed in **Sections 1.2** and **1.3.2**, only the spreading morphology is natural for ADCs; otherwise, cell functions and commitments would be compromised. It remains as a substantial challenge to both cell biologists and tissue engineers.

1.4.2 Non-specific modification: with chemical groups and physical hybridisation

The above solutions of setting up cell adhesion onto hydrogel substrates are based on the biological insights of specific ligand-receptor interactions. Into a more physical / chemical way of thinking, researchers have also brought forward some alternative approaches by harnessing of the functional features of certain chemical groups and

physical blends. However, these attempts are generally limited to 2D substrates.

One strategy is to devise functional ionic groups into hydrogel framework. For instance, Tan *et al.* hypothesised that anionic gels at physiological pH would highly swell due to the electrostatic repulsion, and thus are able to trap abundant bioactive proteins that facilitate cell adhesion. They synthesised phosphonic acid-containing hydrogels, namely poly(vinyl phosphonic acid-*co*-acrylamide) or p(VPA-*co*-AM), which were demonstrated to support cell adhesion and proliferation. The authors further speculated that serum proteins were functionally involved during the anionic (i.e. VPA) moieties promoting cell activities, as they had noticed that the presence of VPA apparently elevated protein uptake into the gels [113]. In accordance with this study, Zainuddin *et al.* grafted another phosphate group, namely mono(2-methacryloyloxyethyl) or MMEP, to the hydroxyl groups of pHEMA, turning the surface of pHEMA hydrogel into cell-adhesive followed by cell attachment experiments with human corneal epithelial cells [114]. Incorporation of another anionic and acidic group, sulphonate, has also been reported to enhance endothelial cell adhesion onto the gel surface. Gong's group compared the behaviour of bovine endothelial cells on several negatively-charged hydrogels and found that on the two sulphonate-containing substrates, poly(sodium p-styrene sulfonate) (PNaSS), and poly(2-acrylamido-2-methyl-1-propanesulfonic sodium) (PNaAMPS) were presented with the highest cell density [115]. Similarly, Chaterji and Gemeinhart polymerised

sulphopropyl acrylate potassium-acrylamide (SPAK) into hydrogel networks, on which favourable uptake of serum proteins and elevated adhesion of osteoblasts / preosteoblasts were observed [116].

As compared with chemical modification, to hybridise nano-/micro- particles into the gel bulk appeared more straightforward. One example is from the study by Schiraldi and colleagues. They developed a hybrid hydrogel based on hydrophobic fumed silica and HEMA, which exhibited a remarkable improvement in supporting fibroblast adhesion, with respect to the plain pHEMA gel [117]. Besides, Japanese scientists incorporated inorganic clay into the organic poly(*N*-isopropylacrylamide, pNIPA) hydrogel network, on which the cell adhesion was dramatically enhanced as compared with conventional pNIPA gel [118]. Such exploration might have provided an easier way in terms of processing methodology. Regarding the working mechanism of these blends, the authors of both studies speculated that the addition of silica or clay particles managed to arrest adhesive proteins at the interface, and consequently triggered cell-protein recognition and subsequent cell adhesion. Recently, aiming to elucidate more detailed mechanisms, Wang *et al.* fabricated a simple agarose-silica composite model and investigated the roles of silica additives in improving the gel-cell affinity at the molecular level. Their results confirmed that the hybridised silica aggregates were capable of arresting adhesive proteins from the environment; interestingly, as binding ligands, these proteins activated integrin- $\alpha 5\beta 1$ -specific

pathway for focal adhesion while failing in invoking integrin- β 3-participated signalling cascade. The authors deemed such partial activation could enable modest amount of high-quality cell adhesion and establish a conservative cell affinity on gel matrices [119].

Unspecific modification, such as incorporation of ionic groups or nanoparticles used in 2D cases, has not been widely adopted for enhancing ADC settlement in 3D hydrogels. The reason behind the dearth of such practices is unclear. It can be speculated that modified hydrogel precursors may need to be crosslinked under harsh conditions, which do not allow cell encapsulation; or, the cells can be encapsulated but their adhesion / survival is far below expectation.

1.5 Objective and organisation of this dissertation

The overall objective of this PhD project is to deliver ADCs with injectable hydrogels for therapeutic purposes. The primary challenge to this practice is that ADCs cannot settle well within 3D hydrogels. This is because common hydrogels lack an appropriate interface between their matrices and cells. More specifically, within 3D gel bulk, there is no cell-adhesive anchor to the receptors of ADCs. As a result, cellular FA cannot be activated, and cell survival, spreading and functions are heavily impaired.

Based on this clue and the attempts by pioneer researchers, we realised a potential solution to this problem is to set up ‘adhesive anchors’ within the 3D gel bulk for

ADCs, so that cellular FA can be achieved and a favourable settlement of ADCs can be expected. Notably, the size of these ‘anchors’ is extremely critical: too small ones, such as conjugated ligands at molecular level, would be insufficient to prop up the cellular shape in the fluid surroundings of gel, but too large a size may hamper the injectability of hydrogels in practical use. We supposed the ‘anchors’ to be at micro-scale, serving as cell-adhesive interface in the 3D matrices.

Therefore, we set out to design hydrogel / micro-composite systems in which the fulfilment of FA is highly emphasised. Our methodology is to design and incorporate specific, micro-scale additives into common hydrogel models. These additives are nano-aggregates or microspheres that can expectedly function as ‘adhesive anchors’ to the encapsulated ADCs. Technically, we will utilise nano-aggregates and fabricate microcarriers to be these ‘anchors’, and conduct investigations in the following steps:

- i) By using typical ADC species such as fibroblasts and osteoblasts and ADC-progenitor cells such as MSC, we are to evaluate the FA-promoting effects of these ‘anchors’;
- ii) By encapsulating cell-laden ‘anchors’ into a most common hydrogel, i.e. agarose, we are to evaluate the cell settlement / commitment in 3D hydrogel / micro-composite, and simultaneously elucidate the molecular biological mechanisms behind cell survival / death within 3D gel matrices;
- iii) By projecting an osteogenic model *in vitro* and *in vivo*, we would assess the

engineering perspective of this composite model, and thereby evaluate the efficacies of ADC delivery via this method.

We expect this work to be of potential to overcome the hurdle that ADCs poorly settle down in 3D hydrogels. Meanwhile, we also take advantage of this contradiction between cells and hydrogels for a particular use. Specifically, we will apply our hydrogel / micro-composite model into the area of cell-based tumour immunotherapy. We are to devise a hydrogel / microsphere system in which the activated macrophages are to be transferred, and expect to see the therapeutic immunocytes to exert tumouricidal activities in a period of time but gradually undergo apoptosis due to their confinement within 3D gel matrices.

To summarise, we aim to fabricate and evaluate three different hydrogel / micro-composites for various purposes of cell delivery-based therapy. This dissertation is divided into the following 5 chapters.

Chapter 1, the current chapter, reviews the wide applications of hydrogels in cell delivery-based therapy, the obstacles in transferring ADCs using hydrogels, as well as the corresponding strategies by previous works. The objective, together with outline of this thesis, is presented.

Chapter 2 starts to explore our first hydrogel / micro-composite, namely hydrogel / nano-aggregate composite. The molecular mechanisms of nano-additive in improving cell-affinity of hydrogel substrates at 2D scale are investigated. The 3D settlement of

cells in such composites and the cytotoxicity of the aggregates are critically evaluated.

Chapter 3 reports a hydrogel / microcarrier system for regulating ADC behaviour in an osteogenic model. A novel type of hydrogel microcarriers is fabricated and their capabilities in supporting ADC adhesion / proliferation are evaluated. Cell-laden microcarriers are encapsulated into 3D hydrogels, wherein the settlement as well as commitment of ADCs is compared with conventional pure hydrogels.

Chapter 4 extends the application of hydrogel / micro-composite into the area of cell immunotherapy. We utilise the excellent properties of hydrogels in cell delivery, while taking advantages of their lack of ‘adhesive anchors’ to encapsulated cells. We devise a hydrogel / microsphere composite to targetedly deliver therapeutic immunocytes, confine cell motion and harness cellular products for eradicating tumours. The feasibility of this concept as potential anti-tumour means is evaluated in carcinoma models.

Chapter 5 concludes these studies and suggests realistic future works.

Chapter 2 Hydrogel / nano-aggregate composite: attempts to enhance cell affinity of hydrogels

2.1 Introduction

In Chapter 1, the versatile advantages of hydrogels for biomedical applications and their limitation for ADC delivery have been circumstantiated. Researchers have never stopped tackling the challenge by modifying hydrogels with functional moieties / blends. However, a satisfactory settlement of ADCs in 3D gel matrices, especially a favourable spreading morphology, has yet to be achieved. This is caused by complicated biochemical and biophysical factors in the unique hydrogel microenvironment. Firstly, hydrogels have an inherent, extremely hydrophilic nature, which resists adsorption and unfolding of specific proteins that are necessary for cell adhesion and spreading. Secondly, once encapsulated into hydrogels, cells are immediately immobilised in highly aqueous frameworks and fluid surroundings. Such enlaced cells, regardless of their native morphology, are unable to spread but are constrained to a round shape.

By realising that cells lack but need the ‘anchors’ in hydrogel matrices, we aimed to solve the problem by installing cell-adhesive ‘anchors’ into the gel bulk. With these anchors, ADCs are expectedly able to adhere, activate FA, and establish consequent settlement / commitment to maintain their phenotype. In this chapter, we will

incorporate aggregated Aerosil® fumed silica nanoparticles into hydrogel substrate, and investigate whether such silica additives can serve as cellular anchors. These silica nanoparticles have been widely studied and used in chemical engineering applications, and are well known to form aggregates in various liquid phases [120]. Moreover, they have been reported to improve hydrogel cell-affinity though detailed mechanism remained unclear [117]. Therefore, in this study, we first set out to fabricate a hydrogel / nano-aggregate composite to evaluate whether such nano-aggregates can facilitate cell adhesion on 2D hydrogel surfaces, and elucidate the biological mechanism behind, if any, the positive outcomes. Based on these theoretical findings, we would move on to assess the engineering prospect of such a composite as 3D vehicles for ADC delivery.

2.2 Materials and methods

2.2.1 Silica-hybridised gel: 2D evaluation

2.2.1.1 Hydrogel-silica hybridisation

All chemicals used in these studies were purchased from Sigma-Aldrich (St Louis, MO, USA), and all the oligonucleotides were synthesised by AIT Biotech (Singapore).

Agarose-based hydrogels were prepared from Type VII low-gelling point 2-hydroxyethyl agarose, and the fumed silica nanoparticles, Aerosil® R711, were supplied by Degussa AG (Dusseldorf, Germany). Either the plain agarose hydrogel

(AO gel) or the identical gel hybridised with silica (AS gel) was prepared by transferring 250 μ l of 1% (w/v, in PBS) agarose solution at elevated temperature, with or without incorporation of silica ($\leq 0.1\%$, w/v), into 24-well cell culture plate (made of tissue culture polystyrene, “TCPS”), for which the AS mixture had been pre-treated with sonication and vortex at elevated temperature. The gelation was achieved by cooling the both systems down to the room temperature and curing for 30 min [119].

2.2.1.2 Protein adsorption and cell attachment

The AO and AS hydrogels were first incubated in 50 μ g/ml human fibronectin (FN/PBS) solution or 100 μ g/ml bovine serum albumin (BSA/PBS) solution respectively for 6 hrs on shaker (25 rpm). Being thoroughly rinsed with PBS, the gels were homogenised in 1% sodium dodecyl sulfate (SDS) solution and then subjected to centrifuge (3000 g, 5 min). The total protein in supernatant was quantified using MicroBCATM protein assay (Pierce, Rockford, IL, USA). For *in situ* indication, fluorescein isothiocyanate (FITC)-labelled FN [121] was applied to the both gels and the fluorescent imaging was carried out accordingly [119].

Human dermal fibroblasts (HDFs, Passage 8-15) were purchased from Cambrex (North Brunswick, NJ, USA) and propagated in Dulbecco’s modified eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) with supplements of 10% (v/v) foetal bovine serum (FBS, ‘Gold’, PAA Laboratories, Pasching, Austria), and 100 units/ml penicillin

plus 100 µg/ml streptomycin (“Pen-Strep”, PAA Laboratories). The Pen-Strep (1000 units – 1000µg/ml, or 10 x, in PBS)-sterilised AO and AS gels [117] were pre-equilibrated in DMEM with 50% (v/v) FBS overnight, followed by HDF seeding at a density of 5×10^4 cells per well and controlled with the same cell culture on TCPS. After 24 h, the culture medium together with unattached cells was aspirated off; while the incubation was continued for another 12 h in the renewed medium. The cell viability was tested with Live / Dead fluorescent staining (Calcein-AM / Ethidium bromide, Molecular Probes, Invitrogen), the live (green) and dead (red) cells were observed under fluorescent microscopy after 30-minute incubation (2 µl ‘Dead’ and 0.5 µl ‘Live’ per 1 ml fresh medium) and sufficient PBS rinse [102, 119, 122].

2.2.1.3 Gene expression

Viable cells were harvested after 48 h with the TRIzol® (Invitrogen) reagent from the AS gels and TCPS. RNA was extracted following the TRIzol protocol, and examined / quantified with UV spectrophotometer (A_{260}/A_{280}). Of each sample 0.5 or 1 µg RNA was collected, treated with RNase-free DNase I and converted to cDNA for the subsequent real-time quantitative polymerase chain reaction (PCR) analysis. Relative gene expression values (human integrin $\alpha 5$, αv , $\beta 1$, $\beta 3$) were obtained from iQ™ qPCR system (Bio-Rad, Hercules, CA, USA) and calculated with the comparative $\Delta\Delta C_T$ (threshold cycle) method, with a calculation of $2^{-\Delta C_T}$, where C_T

represents the cycle number when an arbitrarily placed threshold was reached, and $\Delta C_T = (C_{T, \text{target gene}} - C_{T, \beta\text{-actin}})$. All the reverse transcriptional (RT) -PCR reagents used above were purchased from Promega (Madison, MI, USA) except those for real-time system from Bio-Rad. All the primers used are listed in **Table 2.1** [119].

Table 2.1 Primers used for real-time PCR analysis. F and R stand for forward and reverse strands, respectively.

Genes (human)	Primer sequence (both 5'-3') [123, 124]
β -actin	F: GTGGGGCGCCCCAGGCACCA R: CTCCTTAATGTCACGCACGATTTC
Integrin- $\alpha 5$	F: CATTTCGAGTCTGGGCCAA R: TGGAGGCTTGAGCTGAGCTT
Integrin- αv	F: GTTGGGAGATTAGACAGAGGA R: CAAAACAGCCAGTAGCAACAA
Integrin- $\beta 1$	F: TGTTCAGTGCAGAGCCTTCA R: CCTCATACTTCGGATTGACC
Integrin- $\beta 3$	F: GTGACCTGAAGGAGAATCTGC R: TTCTTCGAATCATCTGGCC

2.2.1.4 Immunofluorescent staining

Based on the results from qPCR, the expression of a pair of integrins on AS gels and TCPS at protein level was subsequently evaluated with immunofluorescent staining, by using FITC-labeled anti-integrin $\alpha 5$ and PE-labeled anti-integrin $\beta 1$. Briefly, the paraformaldehyde-preserved (4%, 20 min) cells were first permeabilised with 0.1% Triton X-100 and blocked with 1% BSA, after which the different antibodies were separately applied to indicate integrin $\alpha 5$ (green) and $\beta 1$ (red). Similarly, the HDF focal

contacts on AS gels and TCPS were detected using FAK100 cytoskeleton and focal adhesion kit (Chemicon, Temecula CA, USA), following a similar protocol in which anti-vinculin and FITC-labeled anti-mouse secondary antibody were applied to indicate vinculin (green); the TRITC-conjugated Phalloidin was applied to label F-actin (red); while the cell nuclei were counterstained with DAPI (blue). All the stained samples were imaged with fluorescent microscopy [119].

2.2.2 Hydrogel / nano-aggregate composite: 3D exploration

2.2.2.1 Direct hybridisation

The identical liquefied agarose (in PBS, w/v 2%) supplemented with 0.1 % (w/v) Aerosil® particles were employed to encapsulate fibroblasts. The cell morphology was examined by Live-specific staining as compared with control groups without silica.

2.2.2.2 Functionalised silica aggregate

The raw fumed silica nanoparticles (SiR) were suspended into the 1:1 (v/v) mixture of concentrated H_2SO_4 and 30% (v/v) H_2O_2 for 2 hours, and rinsed in deionised water (DI-water) for at least five times by centrifuge at 15,000 g x 5 min. Then, the particles were resuspended into 10% (v/v) 3-aminopropyltriethoxysilane (APS) for 2 hours at room temperature, centrifuged again and placed into 1% (v/v) glutaraldehyde for another 2 hours [125]. After washing to remove excess glutaraldehyde, these samples

containing aldehyde groups (SiA) were immersed in various protein solutions, namely bovine serum albumin (BSA), calf skin collagen (Col, Type I), or human FN, at 4 °C for 24 hours with occasional shaking, to prepare functionalised SiB, SiC and SiF, respectively.

The immobilisation of proteins onto the silica particles was characterised by Fourier Transform Infrared Spectroscopy (FT-IR). Samples were air-dried in a vacuum oven for overnight and milled with potassium bromide (KBr) and compressed into pellets for IR analysis following the general procedure. Furthermore, the zeta potential of samples in DI-water, PBS and cell culture medium (DMEM with FBS) was detected.

2.2.2.3 Cell-laden silica aggregate

The silica particles for cell culture experiments were sterilised before being functionalised with proteins: SiR and SiA were soaked in 75% ethanol for 48 hours and the subsequent treatments were conducted in cell culture hood. Four types of sample, namely SiR, SiB, SiC and SiF, were pre-equilibrated with cultural medium in cell-non-adhesive 24-well plate for overnight before cell seeding.

Human mesenchymal stem cells (hMSCs, Passage 2) were purchased from Cambrex, and expanded over multiple passages in mesenchymal stem cell growth medium (MSCGM, Cambrex) to prevent unexpected differentiation before induction. One-to-three million hMSCs in 0.5 ml were seeded onto the silica aggregates in each

well. Half of the supernatant was changed the next day for removing the unattached cells and 1 ml of fresh medium was supplemented. At Day 3, 8 and 16, the cell viability was examined with Live-specific staining and determined with WST-1 assay. For the quantitative WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, Roche Diagnostics, Basel, Switzerland) colorimetric assay, 10% (v/v) WST reagent was added into culture medium, followed by a 2-hour incubation and the measurement of absorbance at 450 nm with reference to 620 nm in a microplate reader [102, 119, 122].

For certain groups, cell attachment on the aggregates was observed by field emission scanning electron microscopy (FESEM, JSM-6700F, JEOL Ltd., Tokyo, Japan). Briefly, the samples were preserved in glutaraldehyde (2.5%) at 4 °C for 1 hr, and further fixed with 1% OsO₄ at 4 °C for 2 hrs. After rinse, the samples were dehydrated in gradient ethanol solutions (30, 50, 70, 75, 80, 85, 90, and 95 %, each for 10 min and 100 % for 3 x 30 min) and further dried in vacuum overnight. Platinum was sputtered for SEM contrasting. Besides, the expression of integrins was investigated by RT-PCR and immunofluorescent staining [102, 119, 122].

2.2.2.4 Evaluation of cytotoxicity

To assess the cytotoxicity of such functionalised silica, we applied the aggregates to MSC in two ways. One is to directly add the pre-sonicated silica suspension to the

monolayer cells; the other is to load such suspension to a cell-culture insert with 3 μm pore size, which is commercially available to companion the 24-well plate. The latter method prevents physical touch between the aggregates in inserts and cells on the bottom wells; but during the co-culture process, the randomly de-aggregated / de-assembled silica particles may penetrate through the pore and get in contact with cells. At different time points, the cells were likewise observed with Live-specific staining and determined with WST-1.

In order to more directly visualise the silica intake by the cells, SiC particles were further labelled with tetramethylrhodamine isothiocyanate (TRITC) and co-cultured with hMSCs. Crystal violet solution was used to quench the extracellular fluorescence before the cells were co-stained with Live-specific dye and subjected to fluorescent microscopy, so that only those particles inside the cells are indicated [126].

2.2.3 Statistical analysis

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

2.3 Results and discussion

2.3.1 The 2D hybridised gel: the role of silica

By selection of a well acknowledged attachment-fouling polysaccharide (agarose) hydrogel as the common background for all model substrates, the function of nano-silica hybridization could be highlighted with the possibly induced distinctions in cell adhesion. Practically, an overview of cell attachment suggests an extremely poor HDF adhesion on the plain AO gels - as a result, almost none of the cells remained thereon after the first-time medium change (**Figure 2.1 A**). In contrast, numerable cells remained on the nano-silica-hybridized AS gels with spreading morphology and intact viability (as illuminated by calcein staining in **Figure 2.1 B**), although the cell residing profile cannot compete with the controls on TCPS (**Figure 2.1 C**) [119].

In pursuit of the reason responsible for this cell adhesive variation, as outlined above, environmental protein adsorption was first examined on AO and AS gels, respectively, with functional protein FN and model protein BSA. Quantitative measurements indicate that the amounts of both proteins in AS gels are observably greater (~20.6% for FN and ~70.0% for BSA) than those in AO gels (**Figure 2.1 D**). If deducting the portion by physical entrapment in gel bulk, the difference of protein abundance on each gel surface could be even more significant – as further evidenced by fluorescent imaging with FITC-FN cure (**Figure 2.1 E and F**). The results indicate that all tangible protein binding, instead of the extremely sparse protein entrapment, only occurs in AS

gel and exclusively on the surfaces of the hybridised nano-silica particles or the aggregates thereof where the cell adhesive ligands are provided [119].

Figure 2.1 (A-C) Live/dead assay for fibroblasts grown on plain agarose gel (AO gel, A), agarose gel incorporated with (0.5 mg/ml) fumed silica Aerosil® R711 (AS gel, B); and TCPS (C) at 24 h; (D) adsorbed human fibronectin (FN) and bovine serum albumin (BSA) in 100 µl of AO and AS gels, measured with microBCA assay; (E and F) *in situ* indication of adsorption of FITC-fibronectin in AO (E) and AS (F) gels, with highlight of (F, inset) protein-lying silica isle. Magnification: A-C, E and F: 40 x; F inset 400 x.

It is well understood that cell adhesion to foreign substrates is accomplished via a series of precisely-controlled reactions and events. In general, once the cells are

committed *in vitro*, their transmembrane integrin receptors will be activated and occupied by specific binding ligands that are substantially functional domains located on certain ECM proteins. The virtual recognitions and incorporations between these integrin dimers and their corresponding proteinic ligands, such as the matches of $\alpha 5$ - $\beta 1$ to FN or αv - $\beta 3$ to vitronectin (VN), function to further activate the construction of a vinculin-connected filamentous actin (F-actin) framework – as a specialised cytoskeletal setup in line with the macromorphological action of cell FA. Particularly for ADC species, the maintenance of FA is critical in retaining normal cell morphology and functionality *in vitro*. Accordingly, to study nano-silica mediated ADC focal adhesion, the aspects of examination must include: (1) environmental protein adsorption prior to cell contacts, which determines the presence of adhesive ligands; (2) integrin expression on cell surface, which represents the cell responses via surface receptor turning-over; and (3) vinculin activation and F-actin assembly, which sketches the induced cytoskeletal organisation posing for FA [119].

Therefore, we further analysed the two pairs of integrin dimeric subunits: $\alpha 5$ - $\beta 1$ and αv - $\beta 3$. The mRNA transcription was revealed and quantified with real-time RT-PCR. As shown in **Figure 2.2 A**, mRNAs of the four subunits are all positively detected from the cells on TCPS; while from the cells residing on AS gel, only one pair of the subunits $\alpha 5$ - $\beta 1$ are comparably positive in expression, the other pair αv - $\beta 3$ is totally undetectable. It suggests that among the major signaling pathways for cell adhesion,

which are typically mediated by the recognitions between $\alpha 5$ - $\beta 1$ and FN, or αv - $\beta 3$ and VN (or other $\beta 3$ -involved networks), a comprehensive activation took place in the cells seeded on TCPS; while in the cells attaching to AS gel, merely the $\alpha 5$ - $\beta 1$ -FN channel was connected. Further detection of $\alpha 5$ - $\beta 1$ expression at protein level on both TCPS and AS gel was confirmed by immunofluorescent staining, as illustrated in **Figure 2.2 B** and **C**. Recollecting the general status of cell adhesion, as indicated in **Figure 2.1**,

Figure 2.2 (A) Real-time PCR analysis of integrin subunits $\alpha 5$, αv , $\beta 1$, and $\beta 3$ of HDFs on TCPS and AS gel (*: Below detectable level). (B and C) Immunofluorescent images indicating the expression of integrins $\alpha 5$ (green) and $\beta 1$ (red) on (B) TCPS and (C) AS gel. (magnification: 200 $\times$)

this result explains the excellence of TCPS and also explains the improved but still relatively limited cell affinity of the nano-silica-hybridized AS gels from cellular transcriptional level. Nevertheless, despite these variables involved to the stage of initiation, once the cell adhesion is physically conducted, the cytoskeletal organisation

is equally well posed to favour the focal contacts no matter in the cells on TCPS or AS gel. This equality has been confirmed by immunohistochemical staining. As exhibited in **Figure 2.3**, the traits of focal adhesion are witnessed by the emergence of vinculin (**A** and **B**) and the assembly of F-actin (**C** and **D**) that are equally expressed in cells on both substrates [119].

Figure 2.3 Immunofluorescent staining of HDFs spreading on AS gel (**A**, **C**, and **E**) and TCPS (**B**, **D**, and **F**) at 48 h: (**A** and **B**): FITC-antibody for vinculin (green); (**C** and **D**) TRITC-phalloidin for F-actin (red); (**E** and **F**) survey image by merge of the two panels together with DAPI for nuclei counterstaining (blue). (magnification: 400 ×)

In conclusion, the hybridised nano-silica additives in hydrogel substrates have been found to arrest forerunner proteins from the environmental medium prior to cell contacts. The physical blending of fumed silica nanoparticles, which can be easily processed, has been applied to the commonly used hydrogels and demonstrated encouraging efficacies in cell adhesion. The above investigation has elucidated the exact role of the nano-silica additives for ADC FA against the normally

attachment-fouling hydrogel substrates. Such immobilised proteins are proven capable of activating $\alpha 5$ - $\beta 1$ -specific pathway for cell focal adhesion, but null effect in invoking $\beta 3$ -participated signaling cascade. This partial activation enables modest amount of high-quality cell adhesion on the modified substrate, so that a conservative cell affinity is established in hydrogel scaffolds [119].

2.3.2 The 3D exploration

2.3.2.1 Direct hybridisation

Direct blending of agarose gel, aerosil nanoparticles and cells could ensure the homogeneous distribution of cells and nano-additives in 3D hydrogel bulks. However, the incorporation of silica could hardly change the morphology of cells. There was no obvious sign of cell spreading in silica-added hydrogels, as indicated in **Figure 2.4**.

This result suggests that the direct hybridisation, though minimising the processing complexity, failed to be an effective approach for promoting cell spreading in 3D hydrogels. There might be two reasons for this failure. First, it has been elucidated that, on the surface of AS gels, cell adhesion and subsequent spreading relies on the adsorption of proteins, and there is no directly specific interaction between cells and ‘silica isles’. In 3D circumstances, cells and silica are separated and immobilised by hydrogel frameworks, the chance of occurring ‘protein adsorption to silica → cells contact to proteins → cell spreads’ is much lower – given that the cell affinity achieved

at 2D scale was just conservative instead of promising. Second, due to the unique 3D-enlacing mechanism of hydrogel encapsulation, cells are constrained into a round shape by complicated mechanical forces.

Figure 2.4 Live-specific staining of cells encapsulated in silica-incorporated agarose gels. Magnification: (A) 40 x; (B) 100 x.

2.3.2.2 Functionalised silica aggregates

The above ineffectiveness by direct blending reflects the distinct situation between the theoretical 2D and practical 3D models for cell delivery, which forced us to change our tactics. Since the primary goal of this study is to provide cellular anchors to ADCs, we conceived a plan to install ‘anchored’ cells into hydrogel bulk. Detailedly, our strategy was:

- i) To functionalise the silica nanoparticles with cell-adhesive proteins;
- ii) To seed cells onto these functionalised silica and observe cell adhesion / spreading;
- iii) To encapsulate cell-laden silica aggregates with hydrogels.

As illustrated in **Figure 2.5 A**, we generated hydroxyl groups (-OH) on the silica

particles with concentrated sulphuric acid / hydrogen peroxide and subsequently grafted aldehyde groups (-CHO) via an APS-glutaraldehyde-mediated reaction. This reaction has been widely used [125] and the reaction between APS and glutaraldehyde is illustrated in **Figure 2.5 B**. Next, three different proteins (BSA, collagen and fibronectin) were conjugated via a Schiff-base reaction. The zeta-potential of these products were analysed (**Figure 2.5 C**) and the existence of protein (~ 1650: C=O, amide I; ~ 1545: N-H/C-N, amide II) was witnessed by FT-IR (**Figure 2.5 D**).

Figure 2.5 Functionalisation of silica nanoparticles. (A) Schematic diagram of the reactions to conjugate proteins onto silica; (B) the structure of APS used to generate the intermediate SiA; (C) zeta-potential of various silica products in DI water, PBS and DMEM / FBS; (D) FT-IR spectra of these silica products.

2.3.2.3 Cell adhesion on functionalised silica

Live-specific staining conducted 3 days post seeding suggested that cell adhesion and spreading were only achieved on SiC and SiF, i.e., collagen or fibronectin-functionalised silica aggregates (**Figure 2.6 A**). No living cell remained in SiR and SiB groups. This result is in accordance with our assumptions. Collagen and fibronectin are well-acknowledged cell-adhesive proteins, and were intentionally employed to facilitate cell attachment. BSA is an inert protein and served as control. Notably, the aggregation of cell-laden SiC was more significantly enhanced than other groups at Day 8, which might correlate with established cell-cell communication. The samples in SiC and SiF groups were next examined with SEM. Spreading cell shape as well as the contact between silica aggregates and cell filopodia were clearly displayed.

To further evaluate the cell adhesion on SiC and SiF at a molecular level, we analysed the expression of integrins $\alpha 2$, $\alpha 5$, αv and $\beta 1$ by RT-PCR. As shown in **Figure 2.7 A**, all these integrins were detected at transcriptional level in both groups. It is not surprising that the expression of $\alpha 2$ was higher in SiC than in SiF, because collagen is well known to contain sequence domain that is specifically recognised by $\alpha 2\beta 1$ dimer. Overall, the integrin expression was slightly stronger in SiC, which can be attributed to the fact that collagen has both integrin- and fibronectin- recognised domains. We also chose $\beta 1$ for immunofluorescent staining; and the results confirmed its expression at proteinic level in both SiC (**Figure 2.7 B**) and SiF (**Figure 2.7 C**)

groups.

Taken together, the above results suggest that grafting of collagen or fibronectin could endow the aerosil silica with encouraging cell affinity.

Figure 2.6 Cell adhesion to the functionalised silica aggregates. (A) Live-specific staining of hMSCs in different groups at Day 3 and Day 8; (B) SEM observation of cell attachment. Magnifications are indicated accordingly.

Figure 2.7 Integrin expression of hMSCs in SiC and SiF. Expression of four integrin subunits was analysed at transcriptional level by RT-PCR (A), and integrin $\beta 1$ was probed with PE-labelled anti-CD29 (red) in SiC (B) and SiF (C), with counterstaining of nuclei by DAPI (blue).

2.3.2.4 Cytotoxicity

MSC proliferation on SiC and SiF was quantitatively monitored for 16 days. However, as shown in **Figure 2.8 A**, cell proliferation was not indicated in SiF samples throughout this period. Cell viability in SiC group rose from Day 3 to Day 8 but significantly fell at Day 16. Samples collected from both groups at Day 14 were subjected for Live-specific indication. Cell spreading was found to be not as favourable as at Day 3 (**Figure 2.8 B**).

Figure 2.8 (A) Cell viability of hMSCs on SiC and SiF; (B) Live-specific staining of hMSCs at Day 14.

To discover the reason that might have hindered the cell settlement and growth on these functionalised silica aggregates, we chose SiC samples to test whether they exerted cytotoxicity to hMSCs. Such a concern of toxicity was based on two speculations / considerations. First, cells were shown to adhere to and spread on the aggregates of functionalised silica; however, the aggregates may disassemble into smaller aggregates and further particles that could be taken in by cells [127]. Second, previous studies have demonstrated that a wide variety of nanoparticles induced cell apoptosis via cell endocytosis [127, 128]. Therefore, we designed the two models, as described in **Section 2.2.2.4**, to investigate the potential toxicity of SiC aggregates.

The experimental outcomes confirmed our hypothesis. Cell density in TCPS control group was apparently higher than in the two sample groups in presence of SiC aggregates; cells were sparser in ‘direct’ than in ‘insert’, suggesting that the direct

incubation with silica posed higher toxicity to the cultured cells (**Figure 2.8 A**). Meanwhile, the intake of SiC by cells was probed by fluorescent observation (**Figure 2.8 B**). Determination of cell viability by WST-1 confirmed the inhibition of MSC growth in presence of silica aggregates, as well as the relatively fiercer toxicity of direct incubation (**Figure 2.8 C**).

Figure 2.9 (A) Live-specific staining of hMSCs cultured on TCPS without silica (Control), with SiC placed in the co-culture insert (Insert) and with SiC directly applied to the cell culture medium (Direct); (B) Indication of silica taken into cells (red: TRITC-SiC; green: cell). Crystal violet was employed to quench fluorescence outside cell body before microscopical observation; (C) Cell viability of the three groups. Magnification: (A): 40 x; (B) 400 x.

The detailed biochemical mechanisms for cell death are still unclear. Over the last decade, it has been well known that both physical properties (such as particle size, surface chemistry and crystal structure) [129] and biochemical behaviours of nanoparticles (cell internalisation) [130] lead to their cytotoxicity. In our models, the

cell death should be caused by the cellular uptake of disaggregated SiC or SiF nanoparticles. Our results indicated that human mesenchymal stem cells (hMSCs) settled well on SiC and SiF aggregates within 8 days post-seeding (**Figures 2.6**) but their density became lower at Day 14 post-seeding (**Figures 2.8**); further cytotoxicity study showed that SiC particles ($< 3 \mu\text{m}$, **Figure 2.9**) were taken into cells and induced cell death. The above two findings suggest that cell death is not caused by surface chemistry of nanomaterials, but by the internalisation of SiC disaggregates that are small enough.

2.4 Summary

Our general aim was to prepare a hydrogel / nanoaggregates composite to promote ADC settlement within 3D hydrogel matrices. In light of the 2D investigation on the enhanced cell affinity by silica additives with elicited molecular mechanism, we attempted to produce an identical 3D model by directly suspending cells and nanoparticles into the hydrogel bulk. Nevertheless, it was proved to be ineffective in promoting cell spreading – which reflects the distinct situation between the theoretical 2D and practical 3D models for cell delivery.

By realising that the cells should have been attached to their anchors before hydrogel encapsulation, we functionalised silica particles and selected two types of aggregates with satisfactory cell affinity. Adhesion, spreading and gene expression of hMSCs in

short period were shown to be desirable. These products were expected to function as cellular anchors for supporting ADC settlement within hydrogel vehicles. However, a substantial drawback of such aggregates – the cytotoxicity of disassembled silica at lower scale – was discovered during our monitoring on cell proliferation. Interestingly the identical material, i.e. functionalised silica, appeared as a benign cell carrier at aggregative state but posed cytotoxic concern at lower scale. This would be an important implication to the design and application of nano- / submicro- scale biomaterials. In general, the functionalised silica aggregates were inappropriate to serve as cellular anchors in hydrogels – as initially assumed. We are to seek for new ‘anchors’ in the future study. Also, we gained from this attempt that the correct procedure should be: first loading the cells to the ‘anchors’, and then install the cell-laden micro ‘anchors’ into hydrogel precursors.

Chapter 3 Hydrogel / microcarrier (GC) composite: the control of ADC behaviour

3.1 Introduction

As mentioned in previous chapters, one primary hurdle for general hydrogels for cell delivery is their lack of affinity to ADCs. Our principal strategy to overcome this challenge is to provide specific cell-adhesive ‘anchors’ into hydrogel bulk, in pursuit of a favourable settlement and commitment of ADCs within the artificial 3D matrices. In Chapter 2, we attempted to introduce the aggregates of nano-particles, without or with modifications, as potential ‘anchors’ to support cell adhesion and spreading. These trials, albeit bringing about some conservative amendments in cell affinity of 2D hydrogel substrates, have failed to accomplish a favourable ADC settlement in 3D matrices. Also, the confirmed toxicity of nano-silica could be another substantial caveat. However, the above trials did provide an important implication: we should load the cells to the proposed ‘anchors’ before hydrogel encapsulation, by which both cell settlement and growth can be controlled by modifying the surfaces of ‘anchor’ materials.

Based on this inspiration while sticking to our general ‘anchor-providing’ tactics, in this chapter, we set out to propose a novel model of hydrogel composite, in which another type of ‘anchors’, cell microcarrier, was introduced. As compared with

previously used silica aggregates which were innovatively proposed as cell-laden vehicles, cell microcarriers are of a much more established technique [131]. Microcarriers are microspheres with diameters ranging from 100 microns to a few millimetres, fabricated with a variety of synthetic or natural polymers. With their vast and conveyable cell-loading interfaces, microcarriers offer the dual superiorities in both cell expansion and conveyance, and have emerged as a promising renovation approach for cell adhesion and delivery in the past decade.

Therefore, in this work, we will first practise to design and fabricate the cell microcarrier that supports cell attachment on its spherical surface. Be the microcarriers able to provide anchorage dependence to the conveyed cells, we are to encapsulate the cell-laden microcarriers (MC) into hydrogel to form the gel / microcarrier (GC) system. By projecting engineered osteogenic model with osteoblasts (general ADCs) and MSCs (anchorage-dependent progenitors), we set out to explore whether the GC composite could promote cellular settlement and commitment, and therefore offer a new approach for engineered osteogenesis with injectable hydrogel vehicles. Simultaneously, we would seek to elicit the biochemical mechanisms behind cell survival/death within hydrogel matrices, in pursuit of a better understanding of cell-material interaction and consequently a rational design of materials for regenerative medicine. Our methodology and hypothesis of this study are illustrated in **Figure 3.1** [122].

Figure 3.1 Methodology of fabrication and demonstration of anchorage-dependence upon cellular fates in engineered 3D hydrogel constructs: A) experimental design of two hydrogel models is schematically represented: cells are directly suspended into liquefied hydrogels to form the conventional Gel construct; alternatively, microcarriers loaded with cells are suspended in gel to form the GC construct, offering an interface for cell attachment and spreading; B) theoretical hypothesis is proposed that the distinct cell behaviors in two models are due to the status of integrin receptors [122].

3.2 Materials and methods

3.2.1 Cell culture

Human fetal osteoblasts (hFOB 1.19, American Type Culture Collections ATCC, Manassas, VA, USA; ATCC No. CRL-11372) were cultivated under different conditions specific for their proliferation and differentiation. Routinely under the proliferative condition, the cells were maintained in DMEM/Ham's F12 (1:1) containing 2.5 mM L-glutamine (ATCC), 0.3 mg/ml G418 (PAA Laboratories) and 10% (v/v) FBS ('Gold') at 33.5 °C. When appropriate, cells in monolayer or gel constructs were induced to differentiate under differentiative condition including a

supplemented medium with 10^{-8} M menadione, 100 $\mu\text{g/ml}$ ascorbic acid, and 10^{-8} M 1,25-dihydroxy vitamin D₃, as well as an elevated temperature of 39.5 °C. All these specific conditions and procedures were suggested by ATCC and the inventors of hFOB 1.19, and have been practiced by others previously [102, 122, 132, 133].

Human mesenchymal stem cells (hMSCs, Passage 2) were purchased from Cambrex, and expanded over multiple passages in mesenchymal stem cell growth medium (MSCGM, Cambrex) to prevent unexpected differentiation before induction. When appropriate, cells in gel constructs were cultured in osteogenic medium (OM), comprising DMEM with the supplements of 10% (v/v) FBS (Hyclone, South Logan, UT, USA), 100 units- $\mu\text{g/ml}$ penicillin-streptomycin, 100 nM dexamethasone, 50 μM ascorbic acid 2-phosphate (Invitrogen), and 10 mM β -glycerophosphate [134]. The cultural temperature for hMSCs was kept at 37.5 °C [122].

3.2.2 Fabrication of cell microcarrier

Phytigel[®], one of the commercially available products of gellan gum, was selected to fabricate the gellan microspheres with water-in-oil (W/O) emulsion method. Briefly, gellan was completely dissolved in deionised water (2% w/v) after being heated at 70–80 °C for 15 min. The hot solution was quickly poured into pre-heated 90 °C peanut oil under stirring at ~ 500 rpm for a few minutes. The mixture was finally transferred into large amount of 1% CaCl_2 solution for another five minutes to enhance the gel

strength according to its gelling mechanism [135]. The produced spheres were collected by filtration through a sieve and extensive wash with deionised water before microscopic observation [102].

Size distribution of the beads was analysed with Image J Software (National Institutes of Health, USA) by randomly selecting 121 microspheres from totally 19 visual fields, which had been pre-equilibrated in PBS or cell culture medium for at least 24 hrs. The frequency (%) of diameters was counted and recorded in an increment of 50 μm . To obtain the swelling ratio, the microspheres were first lyophilised for dry weight (W_d) measurement; then being soaked in PBS again for 24 hrs, the wet weight in equilibrium (W_w) was also measured. The swelling ratio is calculated by $(W_w - W_d)/W_d$ [102].

Gelatin was grafted to the raw gellan microspheres. Sodium periodate was used to introduce aldehyde groups onto the gellan microspherical surfaces. The reaction was conducted by immersing the microspheres in sodium periodate solution with shaking and protection from light. For optimisation purpose, various dosing ratios of gellan unit (GU, the repeating tetrasaccharide) to sodium periodate (6:1, 3:1, 1.5:1, and 1:1, mol/mol) against various reaction times (1, 2, 4, 6, 8, and 16 hrs) were applied intercrossingly. The hydrochloride hydroxylamine assay was respectively performed on the products collected from each condition to determine an optimal window for aldehyde production [102].

Then, the oxidised gellan microspheres were immersed in excess amount of gelatin solution (10%, Type B, from bovine skin). The reaction proceeded at 40 °C for 24 hrs with occasional shaking and ended with a reductive treatment with 0.5% NaBH₄, based on which a crosslinking curing was further conducted with 1% solution of *N*-Ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride at 4 °C overnight. The reaction procedure was quantitatively assessed and optimised following the ninhydrin method; while the whole process was also imaged by proteinic staining with Coomassie Brilliant Blue R-250. As the final product, the fabricated gelatin-grafted-gellan microspheres are abbreviated as “TriG” microspheres [102].

3.2.3 TriG Microcarrier culture of ADCs

Two ADC types, HDF and hFOB, were cultured on TriG microspheres, which had been sterilised by soaking and rinsing in a PBS solution of 1000 units/ml penicillin and 1000 µg/ml streptomycin for 24 hrs. The cell seeding process was conducted in 24 or 96-well tissue culture plates that were pre-coated with sterile agarose gels on the bottoms. Taking HDFs as example, 400,000 cells (suspended with 0.5 ml medium) were added onto 35 mg TriG microspheres (dry weight) and cultivated for 24 hrs. Having the unattached cells removed by medium aspiration, the cell-laden TriG microspheres were transferred to new wells and incubated in 1 ml fresh medium that would be renewed every other day [102].

Cell adhesion and growth on TriG microspheres were evaluated with various

methods, together with routinely observation under optical microscope. First, cell proliferation was tested using WST-1 assay, with the samples of cell-laden TriG microspheres being collected on Day 1, 3, 6, 10, and 14; second, cell attachment and morphology were examined with FESEM at Day 7; third, immunofluorescent staining was applied to indicate F-actin and focal contacts in spreading HDFs on Day 7, followed by the samples being subjected to fluorescent microscopy [102].

The microcarrier culture of hFOBs applies an identical cell-seeding protocol as that to HDFs, while the temperature of incubation, as described previously, was set differently at 33.5 °C for proliferation and 39.5 °C for osteogenic differentiation. The loaded cell viability as well as morphology on TriG microspherical surfaces was detected on Day 7 samples using the Live/Dead assay. By cultivation under 39.5 °C for two weeks, the loaded hFOBs were harvested at various time points (Day 3, 7, 10 and 14) and respectively subjected to osteogenic indications, including alkaline phosphatase (ALP) assay and von Kossa staining. Both the ALP and total DNA tests were based on analyses of cell lysates, while the corresponding measurements were conducted using pNPP assay (p-nitrophenyl phosphate liquid substrate, Sigma Diagnostics) and Hoechst assay (Hoechst 33258, Molecular probes, Invitrogen), respectively, following the manufacturers' instructions. DNA quantities were measured, and converted into cell numbers (6.6 pg DNA / cell), by which the ALP outcomes were further normalised against the cell numbers [136]. The von Kossa staining specific to

calcium deposition was directly applied to the hFOB3-laden TriG microcarriers on Day 14 [74, 102, 136].

3.2.4 Settlement of ADC in GC system: investigation in an osteogenic model

Based on our developed TriG microcarrier, we proposed the hydrogel-encapsulating cell-laden microcarrier (GC) system. Both hFOB3 and hMSCs cells attached well onto the TriG microcarriers 1 day post-seeding, and the complex were collected and gently washed against culture medium in a cell strainer (BD Biosciences, San Jose, CA, USA), for the loosely attached cells being removed. Then, liquefied low gelling-point agarose gel (w/v 1.5 %, Type VII, Sigma-Aldrich) was equilibrated at 37.5 °C and employed to suspend and encapsulate the MC. After being cured in silica-rubber molds, the GC constructs (80-100 µl per piece) were obtained and cultured in corresponding medium on a shaker working at 60 rpm. As control, conventional Gel model was prepared by suspending cells into the identical agarose gel at a final density of 8×10^6 /ml in 50-60 µl per construct, and were likewise cultured. The preparation of Gel and GC composites were illustrated in **Figure 3.1 A** [122].

Gel and GC constructs loaded with hFOB3 were cultivated under proliferative condition for 3 days and then the differentiative condition was switched on, with monolayer cells on the tissue culture plates (TP) serving as control. For osteogenic studies of hMSCs, GC constructs were cultivated in OM in comparison with Gel. Cell

viability and apoptosis in the two different models were compared. The osteoblastic viability in the three groups (TP, Gel and GC) was evaluated by Live/Dead and WST-1 methods at the termination of proliferative condition (Day 3) and differentiative condition (Day 7) – some groups were kept under proliferative condition for 7 days for comparison. For MSCs, the cells were likewise stained with Live/Dead dyes at Day 3, 14 and 28, but their proliferation was monitored at Day 0, 7 14, 21 and 28 using a DNA Quantification Kit (Hoechst 33258-based, Sigma-Aldrich). The cell apoptosis was assessed by two methods, namely Annexin-V staining and Caspase-8 assay. The Annexin-V/PI-based Vybrant Apoptosis Kit (Invitrogen) was applied to detect the externalization of phosphatidylserine in apoptotic cells, upon which the cells in early apoptosis showed green fluorescence (FITC-annexin), while those in late apoptosis or already dead showing red (PI). No or little fluorescence could be observed in live cells. The activity of Caspase-8 was analysed with a Caspase-8 Colorimetric Assay Kit (ALEXIS, Lausen, Switzerland), which detects the chromophore pNA (p-nitroaniline) at 405 nm, after the enzyme cleaves its labeled substrate IETD-pNA. Our procedure adhered to the manufacturer's instruction, with the cell number for each test being normalized to 10^6 using the Hoechst 33258-based DNA Quantification Kit [122].

3.2.5 Biochemical analysis for GC system

3.2.5.1 Gene expression: at transcriptional level

Gene expression of integrins and osteogenic markers at transcriptional level were analysed by conventional or real-time quantitative RT-PCR.

First, RNA from cells in monolayer and on the surface of hydrogel substrates were extracted with the TRIzol® (Invitrogen) reagent. RNA from hydrogel constructs / composites was extracted by using the combination of TRIzol® and RNeasy® Mini Plant Kit (QRMP Kit, Qiagen, Düsseldorf, Germany), as specifically reported as bench tips by our group. Briefly, gel constructs were rinsed against PBS, frozen in liquid nitrogen and homogenised in TRIzol®. After phase separation of TRIzol® procedure, the upper aqueous phase was carefully collected (avoid aspirating the mid-phase in white colour) and processed beginning with the 'spin-column' step in the QRMP protocol (step 5). Acquired RNA was examined with UV spectrophotometer (A_{260}/A_{280}) and quantified for further use [122, 137].

Then, RNA from different samples were collected, normalised to 0.5 or 1 µg, treated with RNase-free DNase I and converted to cDNA for the subsequent PCR experiments. For conventional semi-quantitative PCR, the amplification products were analysed in 1.5% agarose gel followed by ethidium bromide (EB) staining. For real-time quantitative PCR, the details have been described in **Section 2.2.1.3**. The primers used in this work are listed in **Table 3.1** [122].

Table 3.1 Primers used in polymerase chain reactions (PCR). F, R, A.T., P.S., and Ref stand for forward strand, reverse strand, annealing temperature, product size and reference(s), respectively [122].

Genes	Primer sequence (both 5'-3')	A.T. (°C)	P.S. (bp)	Ref
For semi-quantitative methods				
Human				
β-actin	F: GTGGGGCGCCCCAGGCACCA R: CTCCTTAATGTCACGCACGATTTC	60	540	[138]
Integrin-α5	F: CATTTCCGAGTCTGGGCCAA R: TGGAGGCTTGAGCTGAGCTT	58	324	[138]
Integrin-αv	F: GTTGGGAGATTAGACAGAGGA R: CAAAACAGCCAGTAGCAACAA	56	288	[138]
Integrin-β1	F: TGTTCA GTGCAGAGCCTTCA R: CCTCATACTTCGGATTGACC	56	452	[138]
Integrin-β3	F: GTGACCTGAAGGAGAATCTGC R: TTCTTCGAATCATCTGGCC	58	544	[138]
For real-time quantitative methods				
Human				
β-actin	F: CCTGGCAGCCAGCACAAT R: GGGCCGGACTCGTCATACT	53	144	[139]
Cbfa-1/Runx2	F: AGTGATTAGGGCGCATTCCT R: GGAGGGCGGCGTGGGTCTCT	50	69	[140]
Alkaline phosphatase	F: GGGAACGAGGTCACCTCCAT R: TGGTCACAATGCCCACAGAT	53	67	[140]
Type I collagen	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCTCT	53	84	[140]
Osteocalcin	F: AGCAAAGGTGCAGCCTTTGT R: GCGCCTGGGTCTCTTCACT	50	63	[140]

3.2.5.2 Gene expression: at proteinic level

Enzyme-linked immunosorbent assay (ELISA). Hydrogel supernatant was collected at various time points for osteocalcin determination by ELISA [136]. Briefly, equal

volume of supernatant was harvested at set time points, and applied to pre-coated specific immunosorbent plates. The procedure was strictly adhering to the manufacturers' (Quidel, San Diego, CA, USA) instructions [122].

Immunofluorescent staining. For the *in vitro* study, cells were either directly applied for immunostaining or prepared via tissue slicing for further treatment and staining. For direct application, the cells in monolayers, on the surface of hydrogels or encapsulated within hydrogels were gently rinsed with phosphate buffer saline (PBS) and fixed in paraformaldehyde (4%, 20 min). Permeabilization with 0.1% Triton-X was specifically applied for the indication of vinculin and F-actin. Afterwards, the samples were blocked in bovine serum albumin (BSA) / PBS solution (2%, 1 hour). Fluorescein-conjugated or unconjugated primary antibodies, and fluorescein-labeled secondary antibody if needed, were applied correspondingly. All samples were applied with DAPI to counter-stain the nucleus [122].

Tissue sections for the indication of collagen type I and osteocalcin were fixed in paraformaldehyde (4%, 30 mins) and blocked with goat serum (1% in PBS) for 1 hour. Corresponding primary antibodies and secondary antibodies were applied for overnight (4 °C) and 1 hour (ambient temperature), respectively. Xylenol orange (2mM in sterilized water) for calcium indication was applied for overnight [141]. A DAPI-containing mounting medium (UltraCruz™, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for both counterstaining and mounting purposes [122].

Western blotting. The activation of focal adhesion kinase (FAK) was assessed by Western blot analysis. Briefly, total proteins extracted from cell lysate in the presence of protease inhibitor cocktail (Roche Diagnostics) were quantified and subjected to SDS-PAGE electrophoresis. Having been verified with Ponceau S staining, the gel was blotted onto nitrocellulose membranes (Bio-Rad) and blocked in 5% non-fat milk-TBST solution for 1 hour. Primary antibodies including anti-focal adhesion kinase phosphorylated at Tyrosine 397 (pFAK Y397) and anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were incubated at 4 °C overnight. Bands were detected by subjecting the membrane to incubation with secondary antibody and chemiluminescence of horseradish peroxidase. The band intensity was analyzed with the software Totallab-100 TM (Nonlinear Dynamics, Durham, NC, USA) to estimate the relative protein expression [122].

All the antibodies and related immunofluorescent dyes used in this chapter are listed in **Table 3.2**.

Table 3.2 Antibodies and related immunofluorescent dyes used throughout the studies. GAPDH, pFAK, PE, FITC, TRITC, and DAPI stand for glyceraldehyde-3-phosphate dehydrogenase, phosphorylated focal adhesion kinase, phycoerythrin, fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate and 4',6-diamidino-2-phenylindole, respectively [122].

Targets	Antibodies or specific dyes	Manufacturers
GAPDH	Primary: Rabbit anti-human GAPDH Secondary: HRP-Goat anti-rabbit IgG	AbCam
Integrin- α 5	PE-mouse anti-human CD49e	BioLegend
Integrin- β 1	FITC-mouse anti-human CD29	
pFAK	Primary: Rabbit anti-human pFAK (Y397) Secondary: HRP-Goat anti-rabbit IgG	AbCam
Vinculin	Primary: Mouse anti-human vinculin Secondary: FITC-goat anti-mouse IgG	Chemicon
F-actin	TRITC-phalloidin	Chemicon
Osteocalcin	Primary: Mouse anti-human Osteocalcin	AbCam
	Secondary: Alexa Fluor 488-goat anti-mouse IgG	Molecular Probes
Type I collagen	Primary: Mouse anti-human Collagen I	Santa Cruz
	Secondary: Alexa Fluor 546-goat anti-mouse IgG or Alexa Fluor 488-goat anti-mouse IgG	Molecular Probes
Calcium	Xylenol orange	Sigma-Aldrich
Nucleus	DAPI	Chemicon

3.2.6 Animal experiments

For the *in vivo* study, all the animal experiments were performed under guidelines approved by the Institutional Animal Care and Use Committees (IACUC), SingHealth, Singapore. Hydrogel constructs with hMSCs were identically prepared and cultured *in vitro* for 10-12 days, before being subcutaneously implanted into the dorsum of nude mice (i-DNA Biotechnology, Singapore). The mice were sacrificed after 30 days post-implantation, after which the specimens were retrieved, fixed in neutral buffered

formalin and embedded in paraffin. The slicing of both *in vitro* and *in vivo* sections (5-10 μm in thickness), as well as the Hematoxylin-Eosin (H&E) staining of *in vivo* sections were conducted at National University Medical Institutes (NUMI), Singapore. Masson's trichrome (Sigma-Aldrich) staining for collagen and Alizarin Red (AppliChem, Darmstadt, Germany) for calcium deposition were performed under on-line available protocols provided by Rosen Lab (Department of Molecular and Cellular Biology, Baylor College of Medicine) and Internet Pathology Laboratory of Medical Education (College of Medicine, Florida State University), respectively. Quantitative analysis of Col and OC expression in representative sections were conducted by using the Olympus automated image analysis system (Olympus, Osaka, Japan) [122, 142, 143].

3.2.7 Statistical analysis

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

3.3 Results and discussion

3.3.1 The TriG cell microcarriers

3.3.1.1 Gellan gel-based microspheres

The aim of this study is to develop a novel model of gellan gel-based microspherical cell carriers for ADC delivery. The raw microspheres were fabricated with a water-in-oil (W/O) emulsion method in which peanut oil, instead of any other organic solvents, was chosen to minimise the potential cytotoxicity. With an average swelling ratio of 17 in PBS, the morphology and dimensions of the equilibrated wet microspheres were respectively examined. The results, as shown in **Figure 3.2**, indicate that a main distribution (frequency of each 50- μm -segment > 10%) [144] of diameters is obtained at 450 – 600 μm (~ 64%), which is comparable to the commercialized or reported microspheres, such as Cytodex-1[®] (147 – 248 μm) [131], Cytoline 2[®] (400 – 2500 μm) [131], as well as the gas foamed PLGA products (500 – 650 μm) [144]. Given the dual gelling mechanisms upon both cationic and temperature (< 40 °C) curing, which is unique to gellan gum, the stability of gellan gel-based microspheres is double ensured under either *in vitro* cultural conditions or human physiological conditions *in vivo* – once constructed, their stability no longer depends on either the valence or the concentration of the ionic environment; and simultaneously, to maintain a mechanical strength comparable to other polysaccharide-based hydrogels, fewer raw materials are demanded with gellan [102].

Figure 3.2 Morphological observation and size analysis of gellan gel-based microspheres. (A) Microspheres prepared by emulsion method with good integrity under optical microscope (scale bar = 500 µm); (B) Main distribution (frequency of each 50-µm-segment > 10%) of diameters of microspheres: ~14%, 450-500 µm; ~18%, 500-550 µm; and ~32%, 550-600 µm [102].

3.3.1.2 Gelatin grafting

The rationale behind the application of gellan gels as the matrix material for microspherical cell carriers is based on their superior mechanical, machinable, and biocompatible properties that may finely facilitate the physical conveyance in practice. However, in order to competently deliver therapeutic cells, the ADC adhesion affinity must be hurdled against the extremely hydrophilic nature of hydrogel substrates that intrinsically and nonspecifically hinders anything binding to plain hydrogels, for which, as a main-stream solution, cell adhesion ligands or machineries are introduced onto the carrier surfaces. In this study, we immobilise gelatin as surface modifying ligands to enable ADC binding to the microspheres. As denatured derivatives of collagen families, gelatin is extensively available and chemically robust; it comprises of abundant RGD

sequences and also receptor sites for RGD-enriched functional proteins, such as fibronectin, which are all well acknowledged cell binding signals or anchors [102].

Based on the raw gellan microspheres, gelatin immobilization experienced three steps of processes by which the fabrication of TriG microspheres was finalized. As demonstrated in **Figure 3.3**, the three steps are, respectively, 1) surface oxidation; 2) Schiff base grafting; and 3) crosslinking curing, in which Step 1 proceeds to oxidize and cleave the two adjacent hydroxyls on the repeating (gellan) saccharide rings (on microspherical surfaces) into a pair of aldehydes [145, 146]; Step 2 functions to conjugate the gelatin amino groups with the newly generated aldehydes on microspherical surfaces via a Schiff base reaction; and Step 3 serves to stabilize and tidy the covalent coating of gelatin via an inter-chain crosslinking mediated by EDC [102].

In Step 1, the effects of periodate concentration and reaction time were optimized to ensure the production of sufficient aldehyde groups and simultaneously minimize the potential damage to the microspherical substrate from the invasive oxidation process. As suggested in **Table 3.3**, the microspheres could be crashed if the oxidation (by NaIO_4) is exerted too long or too intensively. Extracted from the intercrossing trials, the condition with a reaction time of 4 hrs and a GU/ NaIO_4 dosing ratio of 1:1 (mol/mol, by dry weight) was eventually selected, and applied as the optimum that secures the microspheres physically intact while suffices the surface oxidation degree

up to 31% [aldehydes/pairs of (adjacent) hydroxyls, mol/mol $\times 100\%$]. Under this condition, an ideal gelatination rate was yielded as 0.14:1 (gelatin/microsphere by dry weight, in **Figure 3.4**) through the following steps of process [102].

Figure 3.3 Schematic diagrams of fabrication of gelatin-grafted-gellan (TriG) microspheres. Brilliant Blue R staining: (A) raw gellan microsphere; (B) sparse adsorption of gelatin on gellan microsphere without oxidation treatment; (C) intensive and non-homogeneous adhesion of gelatin on oxidized gellan microsphere; (D) water leaching product from (C) without EDC-crosslinking treatment; and (E) final product of TriG microsphere with abundant and homogeneous covalent coating of gelatin made from (C) with EDC crosslinking treatment [102].

From Step 2 through Step 3, a surface gelatination was carried out on gellan microspheres via an instant Schiff base conjugation followed by a gradual EDC curing. As illustrated in **Figure 3.3 B** and **C**, non-aldehyde primed microspherical surface only anchors the physically adsorbed gelatin, which is rather sparse and extremely unstable; while in contrast, the oxidatively primed surface attracts tremendous amount of gelatin

attachment. However, due to the rapid nature of Schiff base reaction in Step 2, when

Table 3.3 Effects of periodate dosage and exposure time on oxidation degree of gellan microspheres [102].

GU ^a : IO ₄ (mol/mol)	Oxidation degree (%) ^b						
	1 hr	2 hrs	3 hrs	4 hrs	6 hrs	8 hrs	16 hrs
1 : 6	62 *	**	--	--	--	--	--
1 : 3	30	58	78 *	--	--	--	--
1 : 2	5	16	29	37	69 *	--	--
1 : 1	7	12	20	31	47	74 *	**

a. GU: gellan unit, the repeating tetrasaccharide;

b. Oxidation degree (%): generated aldehydes / pairs of adjacent hydroxyls (mol/mol) × 100%;

*: ~ 50% of microspheres collapse;

**: ~100% of microspheres collapse.

gelatin molecules randomly approach to the aldehyde-primed microspherical surfaces, the fast chemical immobilisation of the front-runners easily launch an extensive entrapment among the rest, therefore large portion of the entire attachment is actually attributed to effects of physical imbedding instead of covalent bonding, which is also vulnerable to water leaching (as demonstrated in **Figure 3.3 D**) thus is unstable. This kinetically induced heterogeneity of grafting would negatively influence the quality and consistency of the surface gelatination. This transitional flaw was surmounted via an EDC curing in Step 3. The water leaching procedure synchronizes with the EDC-mediated crosslinking reaction, among which the crosslinking procedure is even slower, so that the physically entrapped gelatin could be first leached (dissolved) off

and then covalently linked back to the immobilised front-runners via EDC. The overall procedure is equal to gradually converting the majority of gelatin attachment from a

Figure 3.4 Variation of gelatination rate verse oxidation time on gellan microspherical substrate. The gelatination reaction proceeded at 40 °C for 24 hrs in 10% gelatin solution, in which the employed gellan microspheres had been oxidatively treated with a GU/periodate ratio of 1:1 (mol/mol) [102].

physical imbedding to covalent immobilisation, by which the gelatination is enhanced in quantity and reinforced in stability, besides, the stacking of gelatin chains also gets chance to be reorganised orderly on the microspherical surface. The treatment and evolvement in Step 3 ultimately fulfilled the abundance, stability, and homogeneity for the surface gelatination. The finalised TriG microspheres are imaged in **Figure 3.3 E**. No statistical difference in size distribution was found before and after oxidation; and the aldehyde leftover on the finalised microsphere was below detectable level [102].

3.3.2 Microcarrier culture of HDFs

Two major categories of vehicles have been developed to convey living cells for

tissue repair with injectable perspective. They are respectively cell-laden carriers and cell-encapsulating scaffolds, among which the microcarriers are more favourably designed for ADC delivery, since the survival of most ADCs, including fibroblasts, osteoblasts and endothelial cells, requires an activation of focal adhesion against appropriate substrates. In this study, a typical ADC model, HDFs, was selected and subjected to TriG-based microcarrier culture with a clinical perspective for wound healing and skin regeneration [102].

Figure 3.5 Proliferation of human dermal fibroblasts (HDFs) on TriG microspheres. The cell proliferation was assessed using the WST-1 colorimetric assay [102].

The HDF proliferation on TriG microspheres was kinetically monitored in parallel with microscopic imaging and quantitative assessments (WST-1 assay). Chronologically, the cells demonstrated a rapidly growing tendency with favourable

morphology shortly after the transplantation; a confluence was subsequently achieved within ten days by propagating up to 310% of the starting cell population; thereafter the cell proliferation was slightly down-regulated to a lower plateau level where the propagation rate is floating around 260%; while the ECM production was dramatically expanded to fuse the inter-microspherical gaps. The whole procedure is demonstrated in **Figure 3.6**. Despite the moderate down-regulation of cell proliferation upon over confluence, the cell growth-induced microspherical conglutination (as highlighted in **Figure 3.6 D and G**), which had also been seen from other microcarrier cultural models, is believed to be beneficial for the constitution of neo-tissues with better continuity and integrity [102].

The HDF focal adhesion on TriG substrate was successfully manifested with immunofluorescent staining, specifically, the major focal contacting indicators, including the oriented F-actins and activated vinculins, were both positively labeled with fluorescent dyes and respectively displayed in red (**Figure 3.6 H**) and green (**I**). Incorporating with the bluish counterstaining for the nuclei (**J**), a survey image is generated and demonstrated in **Figure 3.6 K and L** [102].

It is well acknowledged that cell focal adhesion is initiated via a series of specific interactions - recognitions and incorporations occurring between the ECM-based adhesion ligands (like RGD sequence) and the corresponding transmembrane receptors (integrins) on cell surfaces. Positive signals are conducted associating with the

activation of intracellular proteins, typically vinculin, by the order of which the

Figure 3.6 (A-D) Optical microscopic morphology of HDFs cultivated on TriG microcarriers at Day 3 (A), 7 (B) and 14 (C), and cell growth-induced inter-microspherical conglutination (D); (E-G) FESEM observation of HDFs cultured on TriG microcarriers at Day 7, with highlight of cell conglutination (G); (H-L) immunofluorescent staining of HDFs on the TriG microcarriers: (H) TRITC-phalloidin for F-actin (red); (I) FITC-antibody for vinculin (green); (J) DAPI for nuclei counterstaining (blue); and (K and L) survey image by merge of (H), (I) and (J). Magnification: (A-C, H-K): 40 x; (D): 100 x; (E) 220 x; (F): 430 x; (G): 330 x [102].

downstream events are triggered: the assembly of actin monomers results in a filamentous network (F-actin) that enables and promotes the orientation of cytoskeleton and consequently spreads out the whole cell body. For the ADC species, a focal adhesion-related, spread morphology directly favours the retaining of their normal phenotype and functionality, otherwise pathways to trans-differentiation or apoptosis may easily be activated. To summarise, upon the cell-substrate interaction, the existence of specific ligands is crucial for the induction of focal adhesion, while the organisation of F-actin and the activation of vinculin are both critical markers to evidence it. In this study, the HDF focal adhesion on TriG microspheres was facilitated with the covalent coating of RGD-enriched gelatin and manifested by the imaging indications of vinculin and F-actins [102].

3.3.3 Microcarrier culture of hFOB: proliferation and differentiation

Given the successful trials with HDFs, another functional ADC species, hFOBs, was also seeded onto TriG microspheres as a novel injectable osteogenic model undergoing microcarrier culture. The proliferation stage lasted for 7 days under 33.5 °C and ended at over 90% confluence, when the viable cell population and morphology, together with the ECM-based inter-microspherical conglutination, were recorded by microscopic imaging and further screened with Live/Dead assay. As demonstrated in **Figure 3.7 A and B**, both the cell density and viability are desirable. The cultural

conditions were altered for differentiation perspective by elevating the cultivation temperature to 39.5 °C and also lasting for 14 days. Positive ALP production was first detected at Day 3, followed by significant up-regulation till Day 10, and the increase was stopped at Day 14 (**Figure 3.7 C**). As one of the early bone markers, the emergence of ALP may suggest an evoking of osteogenesis that proceeds prior to the initiation of bony mineralization. Accordingly, significant mineralization was not detected until Day 14. The imaging under von Kossa staining is demonstrated in **Figure 3.7 D**. These both sets of data indicate that the commitment of hFOB3 loaded on TriG (GgG) microspheres are in line with an early osteogenic differentiation [102].

Figure 3.7 Proliferation and osteogenesis of human fetal osteoblasts (hFOB 1.19) on TriG microspheres. Under proliferative conditions: (A) Optical microscopic observation with highlight of cell population and the cell growth-induced inter-microspherical conglutination, and (B) cell viability indication with fluorescent Live/Dead assay (scale bar = 500 μm). Under differentiation conditions: (C) Alkaline phosphatase (ALP) production vary in from Day 3 through Day 14, and (D) von Kossa indication of osteogenic mineralization at Day 14 (Calcium deposition highlighted by red arrows and circles, scale bar = 200 μm) [102].

3.3.4 GC model: osteoblastic viability and apoptosis

In comparison with monolayer hFOBs that were almost viable at both Day 3 and Day 7 (**Figure 3.8 A & B**), the osteoblasts encapsulated in conventional Gel appeared to die in mass as early as Day 3 (**Figure 3.8 C**). Continuing cell death was observed at Day 7 under both proliferative (**Figure 3.8 D**) and differentiative condition (**Figure 3.8**

E). On the contrary, almost all cells in GC remained viable till Day 7 (**Figure 3.8 F & G**) and maintained a spreading shape (**Figure 3.8 inset**); both the cell viability and morphology were comparable to those in monolayer (TP) [122].

Figure 3.8 Analysis of osteoblastic viability and apoptosis. A-G) Cells in monolayer (TP, A and B), Gel (C-E) and GC (F, G and inset) were applied with Live/Dead staining at the terminal of Day 3 and 7. Gel samples at Day 7 under both proliferative conditions (PC, D) and differentiative conditions (DC, E) were provided, and cellular morphology in GC was highlighted (inset); H) cell proliferation in the three models was examined with WST-1 colorimetric assay; I-K) Annexin/V-PI staining for apoptosis was conducted to TP (I), Gel (J) and GC (K); (L) Caspase-8 activity in the three groups at Day 3 and 7. Magnification: 40 × (A-I and K) and 100 × (J and inset) [122].

Cell amounts in different groups were further quantified by WST-1 colorimetric assay, showing the density in both TP and GC groups kept increasing – faster under PC or lower under DC, whereas that of Gel decreased by more than 50% till Day 7 (**Figure 3.8 H**). As shown both qualitatively and quantitatively by the above, cell growth is enhanced in GC but retarded in Gel [122].

To determine whether Caspase-8-mediated apoptosis is responsible for the cell death in Gel, we conducted fluorescent staining to investigate whether the cells went through apoptosis, and enzyme activity assays to see whether the apoptosis was mediated by Caspase-8. Both apoptotic (positive for Annexin-V, green) and dead (positive for PI, red) cells were observed to be much less in TP and GC (**Figure 3.8 I & K**) than in Gel (**Figure 3.8 J**). In addition, the activity of Caspase-8 in Gel was significantly higher than in two other groups (**Figure 3.8 L**). Thus, Caspase-8 plays a role in initiating cell apoptosis in the conventional 3D Gel constructs [122].

3.3.5 GC model: integrin expressions, focal adhesion and cytoskeleton

Following the above, the expression of cellular integrin receptors was examined due to their essential functions of mediating cell-matrix interaction. There was no significant difference among the different groups at either Day 3 or 7 in terms of integrin expression at transcriptional level, as suggested by RT-PCR results (**Figure 3.9 A**); all the three integrin types were highly expressed. Similarly, the expressions of both integrin $\alpha 5$ (**Figure 3.9 B, D & F**) and $\beta 1$ (**Figure 3.9 C, E & G**) at protein level were confirmed in all groups including the Gel, albeit in which the cells were constrained in shape and diminished in viability. The above results suggest that integrin expression in Gel is as high as in TP and GC, although such conventional hydrogel has been well acknowledged to be lack of integrin ligands. Thus, cell death was not caused

by the inhibition of integrin expression [122].

Figure 3.9 Analysis of integrin expression and focal adhesion from hFOB3 cells in the three groups of TP, Gel and GC. A) RT-PCR for transcriptional levels of integrins ($\alpha 5$, $\beta 1$ and αv); B-G) immunofluorescent staining to detect the expression of integrins $\alpha 5$ (red) and $\beta 1$ (green) in TP (B and C), Gel (D and E) and GC (F and G), with typical zones magnified (insets); H) western blotting to study the phosphorylation of focal adhesion kinase (pFAK); I-N) immunofluorescent staining for vinculin (green) and F-actin (red) in TP (I and J), Gel (K and L) and GC (M and N). Nuclei are counterstained in blue with DAPI (B-G, and I-N). Magnification: $40 \times$ (D-G normal), $100 \times$ (B and C normal, D-G insets), $200 \times$ (B and C insets, K-N), and $400 \times$ (I) [122].

As the intracellular events downstream to integrin, FA and cytoskeleton reorganisation were further investigated. The activation of FAK was investigated by western blotting; significantly more intensive bands were present in TP (100%) and GC (81%) groups than that in Gel (27%, **Figure 3.9 H**). Consistently, the expression of

vinculin protein (universal focal adhesion marker) was clearly observed in TP and GC (**Figure 3.9 I & M**) but not in Gel (**Figure 3.9 K**). Both studies reflect that the activation of FA in GC is as precise as that in TP, but declined in Gel [122].

Assembly of actin cytoskeleton was subsequently examined with fluorescent-labeled Phalloidin. Normally, a filamentous network of F-actin is formed in spreading osteoblasts in monolayer, like the situation in TP (**Figure 3.9 J**). Again, only contracted and unorganized F-actin could be seen in Gel (**Figure 3.9 L**), indicating the unsuccessful reorganisation of cytoskeleton therein, with the consequence that cells failed to spread. In contrast, the GC model seemed to effectively maintain the stretching morphology of the immobilised cells, with distinguishable F-actin filaments (**Figure 3.9 N**) [122].

3.3.6 GC model: insight into biochemical mechanisms

It remains a substantial problem in the practice of bone tissue engineering that the efforts to conduct osteogenesis in injectable hydrogels could be easily defeated by the mass depletion of cells, due to the lack of a proper interface between hydrogel matrices and ADC species. To overcome this obstacle, we developed a hydrogel-microcarrier composite to deliver osteo-progenitor cells while maintaining their viability and functionality. Comprehensive studies with osteoblast cell line (hFOB) and potential therapeutic cells (hMSCs) have proved that, the establishment of anchorage

dependence is prerequisite to facilitate cell survival and differentiation in such artificial microenvironments for these two cell types [122].

The findings that osteoblasts and MSCs under osteogenic induction survived poorly in Gel model are in agreement with some previous studies related to the death of ADCs in general hydrogels. Researchers from Anseth's group have demonstrated that, when immobilised in normal PEGDA (polyethylene glycol diacrylate) gels, the viability of hMSCs decreased from almost 100% initially to less than 10% at 4 weeks [74], and that of murine pancreatic β -cells fell $\sim 80\%$ at Day 10 post-encapsulation [23]. Similarly, the primary bovine osteo-progenitor cells were observed by others to lose some 20% population in agarose gels after one week [73]. Accordingly, researchers have attempted to conjugate bioactive substances into the gel bulk and achieved some improvement in the cell viability [23, 75]. However, the cells were still unable to spread out in the matrices, and the mechanisms behind cell death – which we further proved to be apoptosis – remained unclear. To outline the intracellular events that lead to apoptosis, we started from investigating the expression of integrins, which play essential roles in mediating cell-material interaction. It is well acknowledged that general hydrogels *per se* hardly provide any specific ligands for integrin binding; hence, there could be two possibilities: (1) the expression of integrins was strongly inhibited due to the lack of ligands, so the cells went apoptosis in the same way as they did on the non-adhesive two-dimensional substrates [64]; (2) the extent of integrin expression

was not largely altered though they were unligated – but just these unligated integrins triggered apoptosis via recruitment of Caspase-8 [147]. It was suggested by our experimental data that the first possibility might not be primary, as there was no significant variation in integrin expression among the TP, Gel and GC groups. Subsequently, outcomes of highest Caspase-8 and poor focal adhesion in Gel appeared to be consistent with the observed mass apoptosis and thus verified the second hypothesis. The situations in GC and TP were similar: since the cells established attachment to their favourable interfaces namely microcarriers or commercial TCPS (tissue culture polystyrene), their requirement of anchorage dependence was fulfilled. As a result, FA – which could only be triggered by integrin ligation in physiological condition – was activated and the cells survived [122].

Notably, both cell types exhibited a genuinely spreading morphology in the proposed GC model, according to gross observation and F-actin staining, which indicate a successful reorganisation of cytoskeleton. Outspread of ADCs, is not only the natural phenotype of such cells, but also, more importantly, a prerequisite to normal survival of ADCs. Several previous studies have confirmed that cell spreading is required to avoid cell apoptosis. A study by Colotta group has demonstrated that endothelial cells could not be rescued from apoptosis if the cells were not permitted to spread – even when the integrin-binding RGD peptides had been provided [59]. Chen *et al.* also suggested that round ADCs were prone to apoptosis while the spreading

ones survived and proliferated [46]. As we assumed, in comparison with the numerous cell-adhesive small molecules that could be hybridized into gel framework, the microcarriers were more appropriate as cellular anchors. They not only provided cell-adhesive sites with biochemical considerations, but also offered a spherical interface between cells and the 3D aqueous matrices. Consequently, cells could overcome the 3D enlacement and fully spread out into their natural morphology. Moreover, technically, these microcarriers were initially designed in hundred-micron size with both cell cultivation and injection considerations, which precisely avoided being in too small diameters to afford the attachment of cells in 10-to-100 of micrometers, or too large a size that hampers the injectability of assembled hydrogels in practical use [122].

3.3.7 GC model: osteoblastic functionalities

Two typical osteogenic markers [133, 148], namely ALP and OC, of hFOB cells were first evaluated at protein levels. In contrast to ALP (Day 3: 0.44 ± 0.19 ; Day 7: 0.37 ± 0.15) and OC (Day 3: 20.0 ± 0.15 ; Day 7: 19.0 ± 0.88) levels in Gel group, the activity of ALP in GC was more than doubled (Day 3: 1.11 ± 0.11 ; Day 7: 1.34 ± 0.31), starting from Day 3 (**Figure 3.10 A**); and OC secreted in GC was elevated more than 50% (Day 3: 36.16 ± 1.43 ; Day 7: 38.22 ± 0.56 ; **Figure 3.10 B**). Accordant results were obtained from assessment of transcriptional levels of ALP and Col I, which

unsurprisingly showed low and descending values in Gel but significant ascending values in GC from Day 3 to Day 7, after the application of differentiative condition at the end of Day 3; the relative values of ALP and Col I expression in GC reached 9.17 and 5.05 folds of those in Gel at Day 7 (**Figure 3.10 C & D**), respectively. In summary, the proposed GC model is superior in providing hFOBs of high viability, favourable morphology and normal functionalities, to the common Gel model where the cells are tethered in spheroidal shape and on their way to apoptosis [122].

Figure 3.10 Analysis of the expression of osteogenic markers from hFOBs in the three groups of TP, Gel and GC. A) alkaline phosphatase (ALP) activity; B) osteocalcin (OC) production; C and D) real-time quantitative RT-PCR for the gene expression of ALP and type I collagen (Col I) [122].

3.3.8 Engineering explorations of GC: (1) biochemical study

Based on the establishment of material model and the mechanism investigations of cell survival/apoptosis with osteoblasts, we aimed to evaluate the engineering potential of GC system with the cell source of therapeutic potential – hMSCs. Similar to the situations in osteoblasts, the viability of hMSCs cultured in OM was sufficiently high on MC (one day before being encapsulated, **Figure 3.11 A**) and in GC till Day 28 (**Figure 3.11 B, D & F**), but obviously decreased in Gel from Day 3 to 28 (**Figure 3.11 C, E & G**) [122].

Quantitative measurement on cell proliferation revealed that the cell numbers at Day 28 rose in GC to 219% but dropped in Gel to 28% of those in Day 0 (**Figure 3.11 H**). Moreover, the desirable spreading morphology of osteogenic lineage and successful reorganisation of F-actin cytoskeleton were only achieved in GC (**Figure 3.11 I**) but not Gel (**Figure 3.11 J**). In terms of apoptosis, cells that are positive for Annexin-V and/or PI were scatteredly found in GC (**Figure 3.11 K**) but largely presented in Gel (**Figure 3.11 L**). The activity of Caspase-8 was found to be higher in Gel than in GC throughout the experiments, with most distinct ratio of 222% at Day 21 (**Figure 3.11 M**) [122].

Figure 3.11 Analysis of hMSCs viability and apoptosis. A) Cells-laden microcarriers were observed under optical microscope and by Live/Dead staining; B-G) Cells in GC (B, D and F) and Gel (C, E and G) were applied with Live/Dead staining at the terminal of Day 3, 14 and 28; H) Cell proliferation in GC and Gel was examined with DNA quantitative kit till Day 28; I-J) Cell shapes in GC (I) and Gel (J) was indicated by probing F-actin cytoskeleton (red); K-L) Annexin/V-PI staining for apoptosis was conducted to GC (K) and Gel (L); (M) Caspase-8 activity in GC and Gel till Day 28. Magnification: 40 $\times$ (A-G), 100 $\times$ (K-L) and 200 $\times$ (I-J) [122].

The production of ALP and OC from the hydrogel constructs was determined at Day 7, 14, 21 and 28 post-induction. The activity of ALP, which is an early-to-mid marker of osteogenesis, sharply increased from Day 7 to 14 (0.19 ± 0.05) and maintained the relatively high level till Day 28 (0.14 ± 0.05) in GC, but remained lower values in Gel (peak at 0.07 ± 0.04 at Day 14, **Figure 3.12 A**). More distinct variance was shown in the secretion of OC, which was the typical late marker of osteogenesis. In Gel, it was scarcely detectable till Day 14 and presented at very low levels at Day 21

(0.11 ± 0.01) and 28 (0.23 ± 0.02), which were merely 2.6% and 4.1% of the values at same periods in GC (**Figure 3.12 B**) [122].

Figure 3.12 Analysis of the expression of osteogenic markers at protein level from hMSCs in GC and Gel till Day 28. A) ALP activity; B) OC production [122].

Real-time PCR further indicated that though slight difference was shown in the expression of the earliest marker Runx2/cbfa-1 (**Figure 3.13 A**) [148-150], the mRNA levels of ALP, Col I and OC in GC were respectively 2.84, 6.19 and 4.48 folds of those in Gel at Day 28 (**Figure 3.13 B-D**). All these biochemical measurements indicate that the MSCs under osteogenic induction survived, proliferate and exerted osteogenic commitment in GC, but underwent apoptosis and failed to show signs of mature differentiation in Gel [122].

Figure 3.13 Analysis of the expression of osteogenic markers at transcriptional level from hMSCs in GC and Gel till Day 28. A) Runx2/cbfa-1; B) ALP; C) Col I; D) OC [122].

3.3.9 Engineering explorations of GC: (2) *in vitro* and *in vivo* histology

For *in vitro* histology, results from Von Kossa staining revealed that mineralization was established in GC matrix during Day 14 and 28, but not well achieved in Gel throughout the experiments (**Figure 3.14 A & B**). Similarly, large amounts of both Col I and OC were deposited in GC matrix during this period (**Figure 3.14 C & E**). Neither marker was detected in Gel groups (**Figure 3.14 D & F**), coinciding with the gene expression and ELISA results described above [122].

Figure 3.14 Histological evaluation of *in vitro* hMSC-laden GC (A, C and E) and Gel (B, D, and F) samples collected at Day 14 and 28. A and B) Von Kossa staining for calcium deposition; C and D) immunofluorescent staining for Col I (red); E and F) immunofluorescent staining for OC (green). Nuclei were counterstained in blue. Magnification: 100 × (A-F) [122].

For *in vivo* histology, the existence of cells in both GC and Gel after 30-day implantation was confirmed by H&E staining (**Figure 3.15 A & B**). Collagen fibrils indicated in blue by Masson's trichrome staining were shown to be enriched and well organised in GC (**Figure 3.15 C**) but not in Gel (**Figure 3.15 D**). Unlike *in vitro* result, calcium deposition was detected by Alizarin Red not only in GC (**Figure 3.15 E**), but also in Gel (**Figure 3.15 F**) to some extents. Unsurprisingly, significantly higher expressions of OC and Col I were indicated in GC than in Gel by IF (**Figure 3.15 G-J**), as further verified by quantitative analysis: OC and Col I expressions in GC were respectively 6.1 and 8.2 folds of those in Gel (**Figure 3.15 K**). Calcium distribution was further explored with the co-staining of Col I in different colours, and it could be observed that the calcium mostly existed together with Col I matrix in GC (**Figure 3.15 L**) but simply clumped with nucleus in Gel – no evidence of being associated with Col I (**Figure 3.15 M**). Overall, the formation of osteogenic matrices was better

achieved in GC than in Gel [122].

Figure 3.15 Histological evaluation of in vivo hMSC-laden GC (A, C, E, G, I and K) and Gel (B, D, F, H, J, and L) samples collected at 30 days post-implantation. A and B) H&E staining for gross view; C and D) Masson's trichrome staining for collagens (blue); E and F) Alizarin red staining for calcium deposition (red-purple); G and H) immunofluorescent staining for OC (green); I and J) immunofluorescent staining for Col I (red); K) Quantitative analysis of OC and Col from representative in vivo samples; L and M) co-staining for calcium (red) and Col I (green). Nuclei were counterstained in blue. Magnification: 200 × (A-J, and L-M) [122].

In the case of osteogenesis, bone marrow-derived MSC differentiate into osteoblasts via osteochondral ossification, which can be characterised at specific stages with specific markers [148-150]. The earliest osteogenic marker is the transcriptional factor Runx2/Cbfa1 that drives the pluripotent mesenchymal cells to preosteoblasts by initiating the expression of many bone-specific genes. The maturation of preosteoblasts

is accompanied with elevation of ALP activity and Col I production, and accomplished with the evidence that OC – the most typical marker for late-stage osteogenesis – is steadily secreted. During this period, calcium deposition and matrix mineralization occur in response to complicated actions by ALP, OC and some other proteins, installing the formed apatite crystals to collagen fibrils. Accordingly, we evaluated these factors to investigate cell functionalities or osteogenic extents in hydrogel constructs. For hFOB3 as well-committed model cells, we showed that their activities as bone cells were optimised in GC; both mRNA levels of two mid-stage markers (ALP and Col I) and protein levels of ALP and the late-stage marker (OC) were significantly higher than those in Gel after induction. Interestingly, the transcriptional levels of the two genes were even higher in GC than in TP, which tallied with previous findings that microcarrier-based system could enhance osteogenic phenotype in comparison with monolayer [131]. For hMSCs as undifferentiated and pluripotent cells, we demonstrated that a successful osteogenesis was achieved in GC. Differentiation appeared to be also still initiated in Gel since a comparable expression of the earliest marker Runx2/cbfa1 was observed; however, it was then suspended before more mature preosteoblasts were borne, according to the extremely poor expressions of those mid-to-late markers. In contrast, cells in GC were proved to express markers for all stages towards osteogenesis, and particularly, a sharp increase of OC amounts from

Day 14 to 21 post-induction indicated that the maturation process started within this period [122].

Histological evaluations *in vitro* and *in vivo* have further evidenced the potential application of GC in bone engineering. The *in vitro* mineralization and the formation of Col I and OC (as mid- and late- stage marker respectively) in GC sections were clearly observed at Day 28. Calcium deposition was probed to spread all over the GC matrices, unlike in Gel only a handful of dots were positive. Results from *in vivo* tissue sections were overall in consistent with those *in vitro*, in which the framework of collagen fibrils were distinctly better organised and higher expression of Col I and OC were found in GC than in Gel, as visualised by Masson's and IF stainings, suggesting a more favorable formation of extracellular matrices in GC implants. A minor difference between *in vitro* and *in vivo* results could be noted, that certain level of calcium deposition was detected from *in vivo* Gel samples. We attributed it to the complicated *in vivo* environment, where the effects of blood or humoral fluids could not be neglected. However, when simultaneously staining the calcium and collagen deposition, we found that the situations in GC and Gel were entirely different: mineralization in GC took place in collagen network, in the same manner of native tissue [148, 151]; while the calcium deposition in Gel was randomly distributed and surrounding the cells. However, cells in native bone tissue do not directly touch the hydroxyapatite. We speculate from this observation that the mineralization in GC was contributed in a

more endogenous way – substantially similar to that in native tissue, in comparison to the random and uncontrollable calcium adsorption in Gel [122].

3.4 Summary

In summary, our study has provided a solution for the settlement and commitment of ADCs in hydrogels, and evaluated it in the osteogenic model with attention of both scientific and engineering aspects. In terms of basic study with biochemical insights, we have addressed the importance of anchorage-dependence to the delivered ADCs in 3D environment, and elicited that the lack of integrin ligation to “cellular anchors” – rather than inhibition of integrin expression – is responsible for cell apoptosis in conventional 3D hydrogel (Gel). Absence of such ligation brought about mass cell death in osteoblasts (committed ADCs) and failure of osteogenic commitment of MSCs (anchorage-dependent progenitors). In terms of engineering explorations, we have demonstrated a GC system that facilitated both survival and differentiation of osteo-progenitor cells, while maintaining their favourable spread morphology in hydrogel matrices. In addition, the whole system is a general material model that preserves all the advantageous features of hydrogels as cell vehicles / scaffolds, and can be tailor-made and expediently prepared with commonly used G (gel) and C (microcarrier) without any additional step of chemical graft / modification; both G and C types can be customised according to the researchers’ convenience. In the future, we

expect to see GC or similar models being expanded to various applications of regenerative medicine, wherever the mass transfer of therapeutic ADCs are in great demand [122].

Chapter 4 Hydrogel / microsphere composite: Injectable immunoconstruct for *in situ* tumour therapy

4.1 Introduction

Studies reported in the previous two chapters aimed to improve the settlement and commitment of ADCs in hydrogel matrices. For tissue engineering and related cell-based therapeutics, conveyed cells are building blocks of regenerated tissue, and are expected to maintain high viability, retain normal phenotype as well as survive for a long period of time. The GC composite would be a potential solution for ADC delivery for such purposes. Alternatively, we realised that in some other applications, cells are delivered to target sites for exerting specific functionalities, e.g. secreting cytokines or growth factors; however, these introduced cells, especially exogenous ones, are not expected to permanently live in the host tissue. In this chapter, we will take advantage of the unfavourable settlement of cells in 3D hydrogel matrices, to design an injectable hydrogel composite for localised cell therapy.

Through the past decades of crusading against cancer, it has been increasingly realised that the lack of appropriate immune modulation over tumour cells substantially accounts for its medical intractability [152]. The establishment and progression of tumour occurs within a favourable microenvironment, wherein the neoplasm is able to prevent adaptive immune responses, impair antigen presentation, as well as promote

angiogenesis. The tumour cells dynamically influence the levels of cytokines and other components in their niche, in order to withstand and prevent host immune response. Eventually, the tumour tissue is nurtured into a relatively isolated autonomy surviving from the host immune attack [152-154]. During the recent years, cell-based immunotherapy has emerged as a promising treatment modality for cancer, as it can help to modulate effective immune reaction against tumour [155, 156]. Specifically, T cells, natural killer (NK) cells and dendritic cells (DC) have been modified / activated and delivered – mostly without material vehicles – to incite effective immune responses in a number of basic and clinical trials. These practices, albeit need significant improvement in terms of both efficacy and safety, are believed to hold a huge promise of future success [155]. Towards a better governing of the actions and fate of the introduced therapeutic cells, cell-based immunotherapy faces at least two technical challenges. First, the administrated cells need to target and settle at the aberrant site. Undesired localisation – either unsuccessful delivery or poor settlement – of therapeutic cells would not only impair the remedial efficacies, but, more severely, give rise to potential safety concerns arising out of off-target cells spreading and proliferating all over the body, provoking acute immune response or forming ‘new’ tumours. Second, host immune response should be avoided during the process of cell transfer, so that the delivered cells are not harmed by prompt immune rejection. We set out to engineer a proper vehicle to deliver the therapeutic immunocytes to the aberrant

site, retain there for a sufficient period of time, confine cell motion and harness cellular products / signals with the aim of re-programming local immune homeostasis and eradicating tumours. Based on the above conception, an *in vitro* hydrogel / microsphere system was developed to encapsulate the immunocytes into microspheres which were then suspended in liquified hydrogel and solidified *in situ*, with which necessary cytokines could be released for a period of time and the immunocytes would eventually undergo apoptosis provided the absence of “adhesive anchors”. The dual-layer encapsulation of immunocytes would also minimise the risk of leakage of the immunocytes and their debris after death [157].

4.2 Materials and methods

4.2.1 Cell culture

Murine macrophage Raw 264.7 (ATCC Number TIB-71), human breast carcinoma MCF-7 (ATCC Number HTB22), human hepatocellular carcinoma HepG2 (ATCC Number HB-8065), human lung carcinoma H1299 (ATCC Number CRL-5803), and human cervical carcinoma HeLa (ATCC Number CCL-2) were maintained in DMEM that contains 2.5 mM L-glutamine, 100 units- μ g/ml penicillin-streptomycin and 10% (v/v) FBS (‘Gold’). For regular sub-culture or various experimental purposes, all cells were detached with trypsin except Raw 264.7 $M\Phi$ with cell scraper [157].

4.2.2 Materials system

Cell-laden microspheres (MS) were fabricated via an emulsion solvent method. Briefly, 5×10^7 Raw 264.7 M Φ cells were suspended in DMEM / PEG (2 ml / 30 μ l) solution, and then mixed with 2 ml liquefied agarose (w/v 2.5% in physiological saline, low gelling-point, Type VII, Sigma Aldrich). This mixture was vigorously shaken and quickly poured into 20 ml peanut oil, followed by a 3-minute stir at 38 °C and a 10-minute stir at 26 °C, both at 375 rpm on a magnetic stirrer. The mixture was collected and centrifuged for 3-5 times against cultural medium, till the oil phase was completely removed from above the sedimentary beads. These beads, actually M Φ -laden microspheres (MS), were harvested and cultured in 6-well culture plate. At expected time points, they were collected and submerged in serum-free medium supplemented with 1 μ g / ml lipopolysaccharide (LPS) for activation, which took place on a shaker at 60 rpm for 6 hours. After being rinsed in cultural medium to remove excess LPS, the MS were suspended into agarose/DMEM and each 120 μ l of the mixture was injected through the cut-tips onto the silicon rubber sheet, where they solidified into constructs in minutes. Such cell-laden gel / microsphere constructs (GS) were cultivated for future use. Agarose solution and peanut oil used above were sterilised and pre-equilibrated (37.5 ± 1 °C) when appropriate. The experimental work flow is illustrated in **Figure 4.1** [157].

Figure 4.1 Immunocyte-laden GS system for localised tumour therapy. (A) Schematic diagram of the GS system set-up; (B) Gross view of fabricated MS, with Live-specific fluorescent staining indicating cell viability; (C) Illustration of LPS exchange in MS: FITC-labelled LPS specifically binds to encapsulated MΦ or freely diffuses from/to the hydrogel bulk; (D) Gross view of GS construct with Live-specific indication of viable cells [157].

4.2.3 Gene expression

RNA extraction and real-time PCR were described in previous chapters. The primers used in this study are listed below in **Table 4.1**.

For PCR-array techniques, total RNA was likewise extracted from hydrogel constructs and then converted into cDNA but with an array-companionate first-strand synthesis kit (SA Biosciences, Frederick, MD, USA). The product was diluted as required from 20 to 106 μl, and applied to the mouse cytokine RT² Profiler PCR Arrays (PAMM-021, SA Biosciences) strictly adhering to the recommended

procedures. After PCR reaction performed in the iQTM qPCR system, the gene expression profiles from Array were processed with the SA Biosciences online data analysis system [157].

Table 4.1 Primers used in PCR. Recommended annealing temperature is 55 °C. The primers for β -actin and TNF- α (gene name: *tnf*) are following Ref [158] and [135], respectively; others are designed using OLIGOTM 6 software (Molecular Biology Insights, Inc, Cascade, CO, USA) [157].

Genes	Primer sequence (both 5'-3')
β -actin	Forward: TGTCCACCTTCCAGCAGATGT Reverse: AGCTCAGTAACAGTCCGCCTAGA
Tnf	Forward: CCACGCTCTTCTGTCTACTG Reverse: GCTACGGGCTTGTCCTC
Ifnb1	Forward: GCTGCGTTCCTGCTGTGCT Reverse: CGCCCTGTAGGTGAGGTTGAT
Csf1	Forward: GCTGCTGCTGGTCTGTCTCC Reverse: CACAGGCCTTGTCTGTCTCC
Il10	Forward: TTTCTTTCAAACAAAGGACCAGC Reverse: GGTCTTCAGCTTCTCACCCAGG

The amounts of TNF- α were quantitatively determined by ELISA. Under corresponding cultural / stimulating conditions, equal volume of supernatant was harvested at set time points, and applied to pre-coated specific immunosorbent plates. The performance was strictly adhering to the manufacturers' (eBioscience, San Diego, CA, USA) instructions [157].

4.2.4 Statistical analysis

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

4.3 Results and discussion

4.3.1 Dual-layer hydrogel composite

The system utilises a dual-layer hydrogel / microsphere composite to deliver and accommodate therapeutic cells. We chose agarose to prepare both gel and microspheres, and murine M Φ Raw 264.7 cell line as a model of immunocytes. Once activated, they start to secrete desired cytokines / chemokines, which not only directly attack the carcinoma cells but also expectedly recruit host immunocytes into the vicinity via dynamic crosstalk – this characteristic is hardly replaceable by other options. As illustrated in **Figure 4.1 A**, the immunoconstruct was fabricated via four simple steps: (1) Fabrication of MS: M Φ were homogeneously suspended into liquefied agarose, and the mixture was added dropwise into oil to produce spheres (average diameter: ~ 100 μ m) via the emulsion solvent method; (2) Activation of M Φ : after equilibration in culture medium for 1-2 days, the MS were incubated in lipopolysaccharide (LPS) solution for 6 hours to challenge the M Φ into an active state; (3) Clearance of excess LPS: the MS upon stimulation were transferred to and rinsed in fresh medium, for

removing excess LPS; (4) Construction of GS composite: another layer of gel was applied to suspend and encapsulate the MS: this provides a number of advantages. First, this mixture is injectable and solidifies to form the immunoconstruct *in situ*. This dual-layer design provides for injectability and localisation – the two necessities of *in situ* therapy, and helps in retaining cell motion. The inner microspheres are of micro scale, and the outer patch of hydrogel is in liquid form during transfer – the mixture becomes easily injectable. Secondly, the outer layer of liquefied hydrogel guides the MS to the target site, and solidifies locally to immobilise the whole constructs long-term under physiological conditions. Thirdly, agarose and this genre of hydrogels have been well-acknowledged to be bio-inert and effective in immuno-isolating the delivered cells from host rejection. Lastly, the double-layer design additionally prevents cellular leakage or other unwanted motion from the system. Encapsulated cells were indicated by the ‘Live’ specific staining to be homogeneously distributed and highly viable in MS (**Figure 4.1 B**) and GS (**Figure 4.1 D**) [157].

Right from the age of Coley’s toxins, LPS was well-established as being efficacious in activating macrophages and some other immunocytes [159, 160]. We employed LPS as an agent to challenge $M\Phi$ in our model. We took advantage of the permeability of hydrogel to remove excessive LPS at Step 3, as excess LPS can have potential side effects. Since LPS interacts with $M\Phi$ in the specific manner of ligand-receptor binding, it was assumed that unbound LPS could freely diffuse into and out from the hydrogel

matrices. Indeed, we demonstrated that the FITC-conjugated LPS could quickly enter (in seconds) and completely exit the pure, cell-free, agarose spheres (**Figure 4.1 C**). Meanwhile, in the M Φ -laden MS with identical agarose bulk, the LPS immediately bound to the cells; however, after washing, only the-bound LPS was found in the spheres. It supports our hypothesis that unbound and excessive LPS molecules would not remain in the construct. Bound LPS remains to the cells and would not be released in an eruptive manner. This washing step effectively lessens the amount of LPS to the required safer level. Furthermore, we compared the viability of encapsulated M Φ in both MS and GS construct, showing that the LPS challenge did not significantly affect the survival of Raw cells [157].

4.3.2 Activation of the GS system

To investigate the activation of M Φ in GS construct, which is a prerequisite in our proposed therapeutics, we examined the gene expression of M Φ by screening a total of 86 genes with PCR Array technique. Cytokines including TNF superfamily, interferons and interleukins were tested from three groups namely M Φ in monolayer as control ('Ctrl'), in GS construct before ('S0') and 6 hours after ('S1') activation. The results, illustrated in the scatter plots of **Figure 4.2 A** and presented in detail in **Table 4.2**, indicated that the cells in 'S1' were effectively activated, with the expression of 14 genes (TNF, colony stimulating factor or CSF, etc) being turned on or more than 10

folds, and that of 10 genes (including some TNF superfamily members and interleukins) being reasonably elevated (> 2 folds). For the 'S0' group, the levels of most of the cytokine genes were not substantially affected, whereas a couple of cytokines like TNF and IL-10 were mildly up-regulated (~ 3 folds). We further selected four genes, namely TNF- α [161], IFN- β 1 [162], CSF-1 [163], and IL-10 [164], which were previously reported to have tumouricidal activity, for quantitative real-time PCR analysis. Here, another group 'S2', which stands for M Φ in GS construct incubated in fresh medium for another 24 hours after the 6-hour activation and washing step, was supplemented. The outcomes, as shown in **Figure 4.2 B**, were consistent with the PCR array result, confirming that all the four cytokines were drastically up-regulated in 'S1' after activation. Three of the genes with the exception of IL-10 were markedly down-regulated in 'S2', suggesting that cytokine expression eventually turned off 24 hours after the removal of LPS (plus washing). Given that the produced cytokines are sufficient to destroy the malignant cells, this 'turn-off' effect might be a benign factor to the therapeutic practice, by which the overall content of secretions could be controlled and the reaction time be shortened [157].

Table 4.2 PCR Array analysis of $M\Phi$ gene expression in monolayer (ctrl) and in GS constructs before (S0) and after (S1) 6-hour challenge [157].

GenBank	Symbol	Description	S0 vs. ctrl		S1 vs. ctrl	
			Trends	Folds	Trends	Folds
1. Tumor necrosis factor (TNF) superfamily (SF)						
NM_013693	Tnf	TNF	↑	4.33	↑	13.81
NM_009452	Tnfsf4	TNF (ligand) SF, member 4	N.D.	--	N.D.	--
NM_010177	FasI	TNF SF, member 6 (Fas ligand)	N.D.	--	N.D.	--
NM_009403	Tnfsf8	TNF SF, member 8	=	0.54	↓	0.46
NM_009404	Tnfsf9	TNF SF, member 9	=	1.16	↑	2.08
NM_009425	Tnfsf10	TNF SF, member 10	↑	2.52	↑	5.42
NM_011613	Tnfsf11	TNF SF, member 11	N.D.	--	N.D.	--
NM_008764	Tnfrsf11b	TNF receptor SF, member 11b	N.D.	--	N.D.	--
NM_011614	Tnfsf12	TNF SF, member 12	=	1.05	=	0.9
NM_023517	Tnfsf13	TNF SF, member 13	↓	0.35	↓	0.25
NM_033622	Tnfsf13b	TNF SF, member 13b	=	0.51	↓	0.25
NM_019418	Tnfsf14	TNF SF, member 14	↓	0.18	↑	3.31
NM_177371	Tnfsf15	TNF SF, member 15	=	0.78	↑	44.57
NM_183391	Tnfsf18	TNF SF, member 18	=	0.69	=	0.61
NM_011616	Cd40lg	CD40 ligand	N.D.	--	N.D.	--
NM_011617	Cd70	CD70 antigen	=	1.77	=	1.15
NM_010735	Lta	Lymphotoxin A	=	1.9	↑	3.43
NM_008518	Ltb	Lymphotoxin B	=	1.43	↑	2.36
2. Interferons (IFN) and interleukins (ILs)						
NM_010503	Ifna2	IFN alpha 2	N.D.	--	N.D.	--
NM_010504	Ifna4	IFN alpha 4	↑	8.91	↑	3.78
NM_010510	Ifnb1	IFN beta 1, fibroblast	↑	2.34	↑	65.71
NM_008337	Ifng	IFN gamma	N.D.	--	N.D.	--
NM_010554	Il1a	IL 1 alpha	=	1.56	ON	--
NM_008361	Il1b	Interleukin 1 beta	=	1.98	ON	--
NM_019451	Il1f5	IL 1 family, member 5	N.D.	--	N.D.	--
NM_019450	Il1f6	IL 1 family, member 6	N.D.	--	ON	--
NM_027163	Il1f8	IL 1 family, member 8	N.D.	--	N.D.	--
NM_153511	Il1f9	IL 1 family, member 9	N.D.	--	ON	--

NM_153077	Il1f10	IL 1 family, member 10	N.D.	--	N.D.	--
NM_031167	Il1rn	IL 1 receptor antagonist	↑	2.71	↑	12.71
NM_008366	Il2	IL 2	=	1.62	↑	2.05
NM_010556	Il3	IL 3	N.D.	--	N.D.	--
NM_021283	Il4	IL 4	N.D.	--	N.D.	--
NM_008371	Il7	IL 7	N.D.	--	N.D.	--
NM_008373	Il9	IL 9	N.D.	--	N.D.	--
NM_001005506	Il14	IL 14 Taxilin alpha	=	1.05	=	0.69
NM_010548	Il10	IL 10	↑	3.42	↑	177.05
NM_008350	Il11	IL 11	↑	2.29	=	1.25
NM_008352	Il12b	IL 12B	N.D.	--	N.D.	--
NM_008355	Il13	IL 13	N.D.	--	N.D.	--
NM_008357	Il15	IL 15	↑	2.26	↑	4.14
NM_010551	Il16	IL 16	=	0.80	=	0.60
NM_019508	Il17b	IL 17B	↑	2.40	=	0.97
NM_145834	Il17c	IL 17C	=	1.28	=	0.51
NM_145856	Il17f	IL 17F	↑	5.12	=	1.83
NM_008360	Il18	IL 18	=	1.70	↑	3.01
NM_001009940	Il19	IL 19	↑	3.67	↑	16.54
NM_021380	Il20	IL 20	N.D.	--	N.D.	--
NM_021782	Il21	IL 21	N.D.	--	N.D.	--
NM_053095	Il24	IL 24	N.D.	--	N.D.	--
NM_080729	Il25	IL 25	N.D.	--	N.D.	--
NM_145636	Il27	IL 27	↑	2.14	↑	28.60

3. Bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs)

NM_009755	Bmp1	BMP 1	N.D.	--	N.D.	--
NM_009756	Bmp10	BMP 10	N.D.	--	N.D.	--
NM_007553	Bmp2	BMP 2	↑	2.49	N.D.	--
NM_173404	Bmp3	BMP 3	N.D.	--	N.D.	--
NM_007554	Bmp4	BMP 4	↑	4.19	↓	0.46
NM_007555	Bmp5	BMP 5	↑	3.88	N.D.	--
NM_007556	Bmp6	BMP 6	=	1.62	=	1.25
NM_007557	Bmp7	BMP 7	N.D.	--	N.D.	--
NM_007559	Bmp8b	BMP 8b	N.D.	--	N.D.	--
NM_008107	Gdf1	GDF 1	=	1.17	↓	0.29
NM_145741	Gdf10	GDF 10	=	1.62	=	1.25
NM_010272	Gdf11	GDF 11	=	1.21	=	0.77
NM_011819	Gdf15	GDF 15	↑	2.80	↑	2.20

NM_019506	Gdf2	GDF 2	N.D.	--	N.D.	--
NM_008108	Gdf3	GDF 3	N.D.	--	N.D.	--
NM_008109	Gdf5	GDF 5	N.D.	--	N.D.	--
NM_010834	Mstn	Myostatin	N.D.	--	N.D.	--
NM_008110	Gdf9	GDF 9	=	1.32	=	1.29
NM_010564	Inha	Inhibin alpha	N.D.	--	N.D.	--
NM_008380	Inhba	Inhibin beta-A	N.D.	--	N.D.	--

4. Other genes

NM_007778	Csf1	Colony stimulating factor 1	↑	2.12	↑	30.02
NM_009969	Csf2	Colony stimulating factor 2	=	1.62	ON	--
NM_007795	Ctf1	Cardiotrophin 1	=	0.98	OFF	--
NM_198858	Ctf2	Cardiotrophin 2	N.D.	--	N.D.	--
NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)	N.D.	--	N.D.	--
XM_284344	Fbrs	Fibrosin	=	1.48	=	0.9
NM_008002	Fgf10	Fibroblast growth factor 10	N.D.	--	N.D.	--
NM_013520	Flt3l	FMS-like tyrosine kinase 3 ligand	=	1.25	↓	0.48
NM_010094	Lefty1	Left right determination factor 1	=	0.82	=	0.62
NM_008501	Lif	Leukemia inhibitory factor	↑	8.79	ON	--
NM_010798	Mif	Macrophage migration inhibitory factor	=	1.79	=	1.03
NM_170727	Scgb3a1	Secretoglobulin, family 3A, member 1	=	0.50	↓	0.30
NM_007926	Scye1	Small inducible cytokine subfamily E, member 1	=	1.87	=	0.79

(A)

(Figure 4.2 continued in next page)

Figure 4.2 Examination of the activation of GS system. (A) Scattering plot from PCR Array technique to indicate the variation of gene expression profiles in GS samples (S0 and S1), as compared to monolayer control (Ctrl) without activation; (B) Real-time PCR analysis of four cytokines in GS samples (S0, S1 and S2) as compared to monolayer control without activation; (C) Determination of TNF- α secretion from GS construct [157].

Following the gene analysis of M Φ at transcriptional level, we singled out TNF- α as the major tumouricidal component, because TNF- α is well acknowledged as a major effector molecule regulating *in vitro* tumour killing by immunocytes [165]. We determined its secretion by ELISA (enzyme-linked immunosorbent assay) at proteinic level. The activation of M Φ was more apparently displayed in **Figure 4.2 C**, with a sharp increase (~ 30 folds) in TNF- α production after the 6-hour challenge followed by a marked decrease (by $\sim 80\%$) after washing and incubation for another day. The secretion of TNF- α was maintained at a lower level throughout the remaining observation period of 5 days. This ELISA result is consistent with PCR data, and the overall trend of TNF- α secretion is in agreement with previous studies [166], suggesting that the release of this cytokine from GS constructs could be controlled – being triggered only via biochemical activation (at desired time) and turned down in days. More importantly, after removal of LPS, the turn-down was spontaneously achieved without any genetic or chemical manipulation. The high dose of TNF- α released in a short period of time could directly favour tumour regression *in situ*, and the marked lowering of TNF- α could minimise potential inflammatory side-effects. Such an activation and control of cytokine release can hardly be achieved in pure cytokine-administration based therapeutics without delivery of living cells. To summarise, both gene expression and TNF secretion tests suggest that the M Φ encapsulated and immobilised in 3D hydrogel matrices can be adequately challenged

into an activated state to vibrantly release a series of cytokines and other active tumouricidal molecules. Unlike the use of a single agent in conventional cytokine therapy that has frequently faced resistance, the tumouricidal cocktail from the living cells in GS contains various components that target several pathways and may cooperatively augment the efficacy of one single cytokine, so as to achieve maximum anti-tumour effect as well as attract local immune response [157].

4.3.3 Efficacy of GS system on carcinoma growth

The efficacy of this system to inhibit the growth of carcinoma cells was methodologically evaluated in two steps. As designed, the immunoconstruct exerts its tumouricidal effects by secreting cytokines instead of direct immunocyte-tumour contact; therefore, we first assessed the impact of secretion from the activated MS on tumour cells. As shown in **Figure 4.3 A**, we collected the supernatant after LPS challenge (Sup A) and that after wash and incubation in refreshed medium for another 24 hours (Sup B). Fresh culture medium (as control) and the two supernatants were applied in parallel experiments to the prepared adherent human breast carcinoma MCF-7 cells. After 24, 48 and 72 hours, MCF-7 viability and apoptosis were examined with 'Live' and Annexin-V/PI staining respectively. Both supernatants apparently inhibited the proliferation of viable MCF-7 while triggering their apoptosis (**Figure 4.3 B**). It is not unreasonable that Sup A exhibited a stronger effect than Sup B did, as the

former was supposed to contain more TNF- α and other cytokines and excessive LPS. Further quantitative test of cell viability by WST-1 assay confirmed that, after 72 hours of incubation, both supernatants significantly decreased the MCF-7 viability to 33 % (Sup A) and 42 % (Sup B) of control (**Figure 4.3 C**). From these results, it was particularly interesting to note that Sup B could effectively curb the growth of carcinoma cells, suggesting that the washing step in pursuit of higher safety would not compromise the efficacies of activated MS [157].

Next, we utilised a co-culture system that models *in vivo* situation, in which the prepared GS constructs were placed in an insert and the carcinoma cells were cultivated on the bottom of the culture wells (**Figure 4.3 D**). Such commercially available co-culture system enables the two compartments to share a common medium environment, but isolates the GS constructs from physically touching the bottom layer of target cells. It can be envisaged that, in potential clinical therapy, the constructs would be implanted into and thus definitely touch the tumour tissue; however, we preferred avoiding their contact during *in vitro* assessment as we required a strict monitoring of how the GS-released cocktails worked. We intentionally chose three categories of cells: MCF-7 and human hepatocellular carcinoma HepG2 cell line, as representatives of TNF- α -sensitive cells; human cervical carcinoma HeLa and human lung carcinoma H1299 cell lines, which are not sensitive to TNF- α ; and hMSCs and HDFs that are not carcinoma but normal cells. Suppression of cell growth was clearly

indicated in the first category: the mean viability of MCF-7 was 69.8 % (48 hours) and 38.9 % (96 hours) and of HepG2 was 81.6 % (48 hours) and 53.5 % (96 hours) of their corresponding controls (**Figure 4.3 E and F**). However, no significant decrease in cell viability was found in the second category (**Figure 4.3 G and H**), correlating with the lower sensitivity of these cells to $M\Phi$ -produced cytokines. It is not surprising that the two normal cell types were not induced to apoptosis after stimulation (**Figure 4.3 I and J**), as they may lack abundant receptors to the TNF-superfamily cytokines. In addition to the quantitative measurement, live-specific and apoptotic staining further confirmed the apoptosis and decreased density of the MCF-7 cells co-cultured with GS (**Figure 4.3 K**). Similarly, HepG2 cells were morphologically observed to turn round and wrinkled after treatment, which is the typical behaviour of apoptotic cells (**Figure 4.3 L**). On the contrary, and in accordance with WST-1 data, no apparent change was discovered between the treated and control groups of HDF (**Figure 4.3 M**) [157].

(Figure 4.3 – continued in next page)

Figure 4.3 Examination of the effects of GS system on carcinoma growth. (A) Schematic illustration of supernatant experiments: two types of supernatant from activated MS were applied to MCF-7 carcinoma cells; (B) Live-specific staining (green) and Annexin-V/PI staining (red mixed with yellow-green) and (C) quantitative cell viability of tested cells; (D) Schematic illustration of co-culture experiments: activated GS constructs were co-cultured with target cells; (E-J) Quantitative cell viability of MCF-7, HepG2, H1229, HeLa, hMSC and HDF, respectively; (K) Live-specific staining (green) and Annexin-V/PI staining (red mixed with yellow / green) of MCF-7 cells; (L and M) Morphological observation of HepG2 and HDF cells, respectively, without (upper) and with (lower) co-culture with GS [157].

4.3.4 Long-term fates of therapeutic cells

The long-term fate of the engineered immunoconstructs is of high concern in successful cancer therapy. M Φ s are expected to never proliferate in an uncontrolled manner, or escape from the gel encapsulation. Therefore, we set out to explore the survival and settlement of M Φ in GS system. Since no previous work has investigated the biology of immunocytes residing in three-dimensional (3D) artificial matrices, we first compared anchorage-dependent, anchorage-independent and gel-encapsulated cultures of M Φ . Results from viability tests and cell counting (**Figure 4.4 A**), coincidentally, suggested that M Φ proliferated only in an anchorage-dependent manner, reaching confluence within three days on TCPS. However, in the anchorage-independent condition, cells tended to agglomerate when cultured on the non-adhesive gel, while maintaining their viability. This is distinct from some typical ADC types like osteoblasts, which would quickly go through apoptosis once failing to spread [64]. The M Φ behaved in a mid way: they did not proliferate or undergo apoptosis. Similarly, gel-encapsulated cells, which technically can not be isolated and counted, did not proliferate but remained viable as indicated by the viability tests. Hence, hydrogel manages to suspend the macrophages homogenously in its 3D matrices while prohibiting their expansion. To study the long-term containment of M Φ after delivery, the use of dual-layer GS system was compared with the application of hydrogel alone to suspend and convey the cells, i.e., the immunocytes were directly

suspended in hydrogel without undergoing the microencapsulation step. The latter was capable of encapsulating cells and forming constructs as well, however after only a few days of culture, increasing numbers of cells were found to leak out of the gel, attached onto the bottom of the culture well (**Figure 4.4 B**). Such a situation would have disastrous consequences *in vivo*: the undesired release of therapeutic cells may bring forth unpredictable outcomes, probably including acute and global immune response, blockage of blood vessels and/or, ironically, transformation into a new tumour. In contrast, no cell leakage was found in the GS constructs after a culture period as long as 3 months, with the entire gel constructs remaining intact. Therefore, the dual-layer design could offer a convincing restraint to the movement of the delivered cells and confine them to the target area only. Further, long-term survival of gel-encapsulated $M\Phi$ was monitored till 12 weeks (or 3 months) by Live/Dead staining (**Figure 4.4 C**) and 8 weeks (or 2 months) by WST-1 determination (**Figure 4.4 D**). Cell viability was determined to reduce by half after 4 weeks and most $M\Phi$ failed to survive at the termination of observations in either MS or GS. The overall outcome, that the encapsulated $M\Phi$ gradually lost viability, could actually be a beneficial result with respect to the long-term fate of $M\Phi$ *in situ*. On the one hand, we expect to re-establish immunosurveillance by the delivery of living therapeutic immunocytes; on the other hand, we do not expect these introduced cells (even if they are autologous) to permanently live in the constructs after they complete their mission. We anticipate cell

therapy to be accomplished in a controlled manner: delivering cells with a right vehicle, limiting them in the right place, harnessing their therapeutic activity over a right period of time, and ideally, silencing them in a right way afterwards. In the case of M Φ -laden GS constructs, the cells die in a few months in a relatively spontaneous manner, without any supplements of chemical reagents or radiation. Unsurprisingly, the 3D gel encapsulation *per se* could influence the commitment of these immunocytes and lead them to death. This is because, as examined above, the M Φ proliferate only in an anchorage-dependent manner, that is, they require attachment to a favourable substrate with specific ligands (like some protein domains). The 3D hydrogel networks possess a highly hydrophilic and bio-inert microenvironment in which the suspended cells find nothing to attach. Consequently, cells can hardly expand or freely move, due to a combination of biochemical signals and spatial constraint. In a relatively short period, they manage to be stimulated and function to produce cytokines, which has been experimentally confirmed above (**Figure 4.2**), for the therapeutic purposes; through a longer period of time, they gradually lose their viability [157].

Figure 4.4 Long-term viability monitoring of GS constructs. (A) Investigations on the anchorage-dependent growth of MΦ; (B) Observations of cell leakage from single layer hydrogel or dual layer GS; (C) Live/Dead staining of MΦ in MS and GS constructs through 12 weeks; (D) The trends of MΦ viability in MS and GS through 8 weeks [157].

4.4 Summary

Taken together, we have designed an immunocyte delivery platform and demonstrated its anti-tumour efficacies *in vitro* for potential localised tumour immunotherapy. Compared with other engineered cells used for therapeutic purposes, immunocytes, which are essentially part of the native immune system, can be expediently activated by incubation with established reagents/cytokines, avoiding genetic modification or other treatments that could cause safety risks as well as cell

damage. Once activated, they start to secrete cytokines / chemokines, which not only directly attack the carcinoma cells but will expectedly recruit host immunocytes into the vicinity via dynamic crosstalk. Technically, the dual-layer hydrogel composite preserves all the advantageous features of hydrogel (injectability, selected permeability, etc.), and is capable of achieving a genuine localisation and physical immobilisation of therapeutic cells. Moreover, this is a general material model; in practical operation, the hydrogel type can be customised according to the surgeons' convenience and individual tumour's condition. In general, the immunoconstruct presents effective elimination of carcinoma cells, as well as high safety – no gene transfer, cell leakage or unwanted proliferation. We recommend exploring this system for various applications in tumour immunotherapy. For instance, autologous immunocytes can be isolated from the patient, cultured / expanded *in vitro*, activated *ex vivo* and delivered back to the host with such immunoconstructs, by which the treatment can be initiated at a desired location and implemented in a controlled manner [157].

Chapter 5 Conclusions and future work

5.1 Conclusions

In these studies, three hydrogel / micro-composite systems were proposed and their potentials for therapeutic cell delivery were evaluated. The exploration of the first composite, namely hydrogel / nanoaggregates, offered interesting insights into the ADC-hydrogel interactions but was limited to 2D scale due to the concern of material toxicity. The second platform, i.e. the GC composite wherein the ADC-laden microcarriers were encapsulated in hydrogel, showed competence as ADC-delivery vehicle and tissue-regeneration scaffold. Additionally, the third hydrogel / microsphere system – with macrophages doubly encapsulated inside microspheres and hydrogel – provided a feasible means for *in situ* tumour therapy, by conveying immunocytes and forming immunoconstructs into aberrant neoplasm tissue.

The most essential finding from the above investigations is that the settlement and commitment of ADCs in 3D hydrogel matrices are highly regulated by their status of being ‘anchored’. Specifically, the ligation of integrin receptors and activation of FA are required for ADCs to maintain their phenotype and normal functionalities. Therefore, for different application backgrounds, researchers can manipulate to provide or vacate such ‘adhesive anchors’ within hydrogel matrices in order to control the settlement of delivered cells. This conclusion is drawn from both mechanism

investigation and engineering exploration presented in this dissertation.

Firstly, the biochemical mechanism behind ADC survival / death within 3D hydrogel matrices was elicited. For ADCs encapsulated in highly hydrated 3D network of hydrogels, the specific binding between cellular integrins and extracellular ligands is required for their survival. The fulfilment of such ligation – instead of the expression of integrins *per se* – is the key for avoidance of apoptosis in ADC population. Unligated integrins may recruit Caspase-8 that triggers integrin-mediated cell death, and provision of microscopic ‘anchors’ could effectively avoid apoptosis and promote cell growth within 3D gel networks.

Secondly, a potential solution for ADC delivery with hydrogel vehicles was proposed and examined in both *in vitro* and *in vivo* osteogenic models. Our devised GC composite were shown to be a promising cell-delivery vehicle as well as tissue engineering scaffold, in which the proliferation and differentiation of osteoblasts and osteo-progenitor cells (hMSCs) were favourably supported. Notably, the injectable microscopic ‘anchors’ not only provided platforms for cellular FA but also overcame the enlacement by gel frameworks and therefore allowed cells to fully spread out into their natural morphology. This is in accordance with the above biological elucidation. Compared with other reported approaches such as grafting RGD or FN into hydrogel bulks, our GC model would be a more promising ADC-delivery vehicle as well as tissue engineering scaffold.

Thirdly and reversely, the absence of ‘adhesive anchors’ could be utilised to constrain cell growth for certain cell-delivery purposes. We innovatively demonstrated that a GS system free of ‘adhesive anchors’ was suitable for delivery of immunocytes. Due to the advantageous features of hydrogel composite, activated macrophages could be conveyed to the right place and secreted tumouricidal cytokines for an expected period of time; nevertheless, introduced cells were unable to proliferate / migrate, or survive for too long due to the unfavourable, enlacing hydrogel microenvironment. Therefore, the fate of delivered cells could be controlled in a relatively natural manner – without any chemical / genetic manipulation. This approach, originating from fundamental knowledge and our findings of cell-material interaction, would emerge as a new strategy for cell delivery especially in which the potential side-effects of cell transfer are of extremely high concern.

5.2 Future outlook

Towards the advancement of regenerative medicine and cell-therapeutic biotechnology, continued efforts are expected on expanding the application fields of hydrogel / micro-composite system for cell delivery. Particularly, future study can be directed to several critical aspects including: i) providing more sophisticated information on cell biology in 3D environment, ii) utilising more different cell types for conveyance, iii) combining auxiliary strategies such as the co-delivery of proteins /

genes; and iv) comprehensively studying the degradation properties of these hydrogel materials – in the presence and the absence of living cells.

For more deeply scientific insights, substantial work remains to interpret cell biology in 3D hydrogel matrices. Besides the integrin-FA pathway that functions essentially in mediating cell survival / death, other receptors for cell-material interaction and cell-cell communication have been witnessed active in regulating cell behaviours as well [62]. Beyond the biochemical mechanisms, biophysical and particularly mechanical factors are equally important in determining cell fate [167, 168]. Therefore, interdisciplinary approaches ranging from genetic microarrays, physical modelling, electrochemical sensing, mechanical testing and computational simulation are all helpful.

For enlarging the application fields of the materials platform, i.e., the remarkable hydrogel / micro-composite systems, multiple cell types for various therapeutic purposes can be employed. Aside from osteoblasts and MSCs, a wide variety of ADC species have been used for regeneration of different tissue / organs – such as myoblasts for muscle regeneration, fibroblasts for skin transplantation, and endothelial cells for vascular engineering. Moreover, for the progenitor cells, e.g. MSCs and ESCs, their commitment in the identical material model would vary under different inductive circumstances, and thus the differentiation of stem cells into different lineages in hydrogel composites need to be thoroughly investigated.

Also, regenerative functions of hydrogel / micro-composite system may be enhanced with the assistance of growth factor release and / or gene transfection. For example, bone morphogenic proteins (BMPs), vascular endothelial growth factors (VEGFs) or neurotrophins could be combinedly used to potentially accelerate osteogenesis, angiogenesis or neurogenesis respectively. Likewise, plasmids encoding these proteins (growth factors) could be incorporated via viral or non-viral vectors into the gel composite for enhancing purposes. Recently, RNA interference (RNAi) with unique gene-silencing efficacies has been shown to hold promise in regenerative medicine as well as cancer therapy [169], and could provide an alternative tool for controlling cell differentiation in the likes of GC composite.

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PUBLICATION LIST

1. Review papers:

1.1 **CM Wang**, RR Varshney, DA Wang. ‘Therapeutic cell delivery and fate control in hydrogels and hydrogel hybrids’, by invitation & submitted;

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2. Research articles:

2.1 **CM Wang**, GN Adrianus, N Sheng, SK Toh, YH Gong, DA Wang. ‘In vitro performance of an injectable hydrogel / microsphere based immunocyte delivery system for localised anti-tumour activity’. *Biomaterials*, 2009, 30: 6986-95;

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3. Conference proceedings

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