Phase transfer cell culture system for self-forming cartilage tissue regeneration

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NANYANG
TECHNOLOGICAL
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**PHASE TRANSFER CELL CULTURE SYSTEM FOR
SELF-FORMING CARTILAGE TISSUE REGENERATION**

SU KAI
SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING

2011

**Phase Transfer Cell Culture System for Self-Forming Cartilage
Tissue Regeneration**

SU Kai

School of Chemical and Biomedical Engineering

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LIST OF ABBREVIATIONS

2D	two dimensional
3D	three dimensional
ACT	autologous cell transplantation
AFM	atomic force microscope
ALP	alkaline phosphatase
ARC	alginate recovered chondrocyte
BMP	bone morphogenetic protein
CCM	chondrocyte culture medium
COL I	collagen type I
COL II	collagen type II
COL X	collagen type X
DIAS	dermis-isolated aggrecan-sensitive
DIC	differential interference contrast
DMEM	Dulbecco's modified eagle's medium
ECM	extracellular matrix
EDC	<i>N</i> -Ethyl- <i>N'</i> -(3-dimethylaminopropyl) carbodiimide
EF	Edge Flourish
ESC	embryonic stem cells
FBS	foetal bovine serum
FESEM	field emission scanning electron microscopy
FITC	fluorescein isothiocyanate
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GAG	glycosaminoglycan

HA	hyaluronan
HEPES	4-(2-hydroxyethyl)-piperazine-1 -ethanesulfonic acid
hMSCs	human mesenchymal stem cells
IACUC	Institutional Animal Care and Use Committees
IGF	insulin-like growth factor
IHC	immunohistochemistry
IPS	induced pluripotent stem cell
MCG	microcavitary hydrogels
MFM	multiple fluorescence microscopy
MMP	metalloproteinase
MSC	mesenchymal stem cell
MSCGM	mesenchymal stem cell growth medium
NEAA	nonessential amino acid
NIAMS	National Institute of Arthritis and Musculoskeletal and Skin Diseases
OA	osteoarthritis
PA	peptide amphiphile
PAG	polyacrylamide gel
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
PEGDA	poly(ethylene glycol) acrylate
PIV	particle image velocimetry
pNIPAm	poly(N-isopropylacrylamide)
PLA	poly lactic acid
PLGA	poly lactic-co-glycolic acid
PTCC	phase transfer cell culture
PVA	poly(vinyl alcohol)

RGD	Arg-Gly-Asp
SMSC	synovium-derived MSC
TEMED	Tetramethylethylenediamine
TGase	transglutaminase
TGF-β	transforming growth factor- β
VEGF	vascular endothelial growth factor
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio] -1,3-benzene disulfonate

SUMMARY

Hydrogels have been widely used as cell-laden vehicles for engineered therapeutic transplantation in regenerative medicine. Taking its advantages of injectability for grafting *in situ* and its superior properties favouring cell residing, more sophisticated applications with a plain gel-encapsulating-cell model are still encountering challenges in long-term tissue developmental efficacy. The major question lies in the space limitation for neo-tissue generation including proliferation of the encapsulated cells *per se* and accommodation of their endogenous extracellular matrix (ECM) generated in the gel bulk. It arises from two material-based backgrounds: i) scaffold degradation fails to match the pace of neo-tissue growth, which is a common drawback for most biomaterials used in tissue engineering; and ii) the modest cell affinity to hydrogel scaffold also hinders cell development, which is due to the intrinsic high hydrophilicity of hydrogel scaffold.

Accordingly, in order to overcome the deficiency of cell affinity and also to improve significantly the living space for the engineered neo-tissues, while still maintaining all the beneficial talents of injectable hydrogels, we developed a novel micro-cavitary hydrogel (MCG) system as well as a novel PTCC strategy to enable *bona fide* neo-tissue self-formation in an artificial graft. By this invention, we hypothesized to upgrade the function of hydrogel scaffold from an assembly of

man-made niches for isolated cells to an integration of micro-incubators that catalyzes self-formation of scaffold-free neo-tissue islets and further expands to achieve full tissue occupation in the grafting area.

The invention of PTCC strategy is elicited from an observation of an ‘edge flourish’ (EF) phenomenon in plain hydrogel culture, which was also witnessed by other researchers: By long-term culture of such constructs *in vitro*, typically using articular chondrocytes seeded in polysaccharide (agarose) hydrogels, in contrast with the conservative tissue development in gel bulk, abundant neo-tissues outgrow the gel edge covering the entire outer surface of the construct and showing *bona fide* cartilaginous phenotype. Given the space limitation and affinity deficiency in gel bulk, this EF effect was shown to be initiated due to spontaneous outgrowth of encapsulated cells (colonies) driven by proliferative expansion and asymmetric pressure selection/orientation, from biomechanical aspect. Through different built mechanical models and computational algorithms, the intensive biomechanical study contributed to further prove that PTCC/EF mechanism is the result from cell oriented outgrowth but not cell migration. Meanwhile, the investigation of cell biomechanology showed there should have a critical distance beneath where PTCC/EF phenomenon can happen.

Based on this PTCC-MCG system, we further proposed to develop and apply an innovative living tissue based scaffolding system (‘Living Scaffold’ or LS) by removing the remaining scaffold material with a gentle dissolution treatment. The

Living Scaffold (LS) is completely free of scaffold material components and enables phenotypically *bona fide* pure neo-tissue formation for artificial graft *per se*. The expansion and fusion of PTCC induced neo-tissue islets within hydrogel bulk constitutes a macroscopic framework of LS that maintains the overall shape and size during removal of hydrogel components. The obtained LS construct can keep encapsulated chondrocytes expressing normal chondrocytic genes, increase the density of cell-secreted ECMs like GAG and total collagen and propel better chondrocytic phenotype as proven by histological staining results when compared to PTCC construct with the scaffold materials inside. Re-seeding cells onto LS construct helped to strengthen its various performances. More significantly, either from macroscopic observation or from microscopic findings, the PTCC-LS system induced cartilaginous implant is comparable to native cartilage.

In summary, on one hand, PTCC-MCG is designed as a physical bioresponsive hydrogel system, contributing to solve the spatial shortage problem of scaffold application in tissue engineering. Therein, more biomechanical tests on plain cell/hydrogel model can be pursued for better understanding PTCC/EF mechanism. On the other hand, successful fabrication of PTCC-LS system, producing a macroscopic porous construct made of pure and living tissue, can perfectly solve the problem of scaffold degradation rate always challenging tissue engineering application. It is expected to employ PTCC-LS system for manipulation of endochondral osteogenesis

and osteogenic angiogenesis. By virtue of the two relative scaffolding systems having been implemented successfully, PTCC strategy opens a novel platform to accommodate cells and neo-tissue for tissue engineering and other therapeutic purposes.

Chapter 1 Introduction

1.1 Cartilage and cartilage lesion

Cartilage is a flexible and opaque connective tissue, found in many places in the body including the joints, the rib cage, the external ear, the tip of the nose, the walls of the windpipe, the voice box (where it provides support and shape) and between intervertebral discs [1-2]. Cartilage defects resulting from trauma damage or chronic illness, impose far-reaching painful and disabling burden to patients, which influences their health and ultimately shorten their life-span. It is known that a lot of people over the age of 65 are suffering from osteoarthritis (OA), and studies reported that only 12.9% of the 55 to 65-year old were free of radiographic OA [3]. Similarly, back pain, which is closely related to degeneration of the intervertebral disc affects up to 35% people in the world [4]. With the growing population of the aged and the obese, cartilage disorders are becoming more and more prevalent nowadays.

1.1.1 Articular cartilage anatomy

Articular cartilage, a firm and elastic structure covering bone endings, can be easily transformed due to pressure and recovers its shape when the force is removed [5]. Microscopically, hyaline articular cartilage mainly contains water, collagen, while proteoglycans together with chondrocytes make up only 1-5% volume of its structure

[6] (**Figure 1.1**). The composition of articular cartilage and a summary of their functions is listed in **Table 1.1**.

Table 1.1 Components of articular cartilage with a summary of their functions [7-9].

Articular Cartilage Components	Morphological Property	Functions
Chondrocytes	Make up about 1-5% of the volume of hyaline cartilage, sparsely spread within the matrix	Maintain the balance of synthesis and catabolism for extracellular matrix (ECM) proteins
Collagen type II	Accounts for 90-95% of total collagen in macrofibrils	Provides the articular cartilage with high tensile strength
Collagen type IX	Cross-linked to the surface of macrofibrils	Provides tensile properties and inter-fibril connections
Collagen type XI	Within or on macrofibrils	Nucleates fibril formation
Proteoglycans	Interweave macrofibrils, and consisted of glycosaminoglycan (GAG) subunits	Offer compressive strength to articular cartilage
Tissue fluid	Makes up about 65-80% of cartilage tissue by water, gases, metabolites and a large amount of cations.	Offers compressive strength and provides nutrition and lubrication for low-friction gliding surface

Figure 1.1 Graphic diagram of articular cartilage's structure.

1.1.2 Current treatments for articular cartilage repair

Due to its aneural, avascular and alymphatic nature as well as immobilization of chondrocytes in dense ECM, cartilage tissue has a limited capacity for self-regeneration after injury from trauma or diseases [8, 10]. Even if cartilage lesions penetrate the vasculature of the subchondral bone and cause local bleeding, the lesions fail to restore ultimately [11-12]. The restoration of structural integrity and function of the newly repaired tissue still remains an issue.

Current approaches, including microfracture, grafting and autologous cell transplantation (ACT), have resulted in pain relief and improvement of joint function to some extent [13]. However, these approaches are still associated with drawbacks such as lack of long-lasting function and graft rejection [14]. A summary of current cartilage replacement therapies and their potential drawbacks is listed in **Table 1.2**.

Table 1.2 An outline of current cartilage replacement therapies and their potential drawbacks [2, 9, 15].

Therapies	Methodology	Drawbacks
Autografting	Grafting a section of cartilage from a less load-bearing site to replace the cartilage defect	Donor site morbidity
Allografting	Cartilage is taken from another individual and then implanted in the damaged site	Possibility of graft rejection
Microfracture	Penetration of subchondral bone to create an opening for migration of cartilage progenitor cells	Unpredictable results that rely on various elements: age of patient, size of the cartilage lesion etc.; high chances for lesion relapse
Autologous Cell Transplantation (ACT)	Isolation of chondrocytes from patient, expansion and culture in the laboratory before implantation	Formation of fibrocartilage instead of hyaline cartilage

On basis of the above mentioned, there is still no approach that can regenerate defective cartilage to its normal function. In recent decades, efforts have been focused on regenerative medicine. A tissue engineering technique was used, by which stimulating factors such as growth factors could be delivered to therapeutic cells such as stem cells or chondrocytes via material-based scaffolds to engineer functional cartilage that would replace the damaged cartilage [16].

1.2 Tissue engineering for cartilage repair

Tissue engineering, a new approach aiming to restore full tissue regeneration and

functionality restoration, has emerged to become a new focus in the search of tissue/organ defects treatment. Categorized to the subject of regenerative medicine, tissue engineering is commonly accepted as an interdisciplinary field which employs both the technologies and tools of engineering and the principles of biological science [17]. Since it first appeared in a publication in 1984 [18], tissue engineering has evolved from a general concept which lacked a clear boundary with biomaterial engineering [19], to a more specific concept with the emphasis on the incorporation of living cells in a biomaterial for therapeutic purposes [20]. The research field has since then been developing fast; a great deal of work has been done on the research of skin, bone, blood vessel, cartilage, pancreas, liver and kidney. Some of the researches have even yielded commercial products that have been approved by Food and Drug Administration (FDA), like tissue-engineered biological dressings such as Dermagraft[®], Apligraf[®] and Cultured Epidermal Autograft (Epicel[®]) [21-24]. Though remaining in its infancy, compared with the developments in other related fields, tissue engineering has great potential to overcome the technical challenges as it faces today.

Tissue engineering generally involves three kinds of strategies, namely, direct implantation of isolated tissue cells, direct *in vivo* application of tissue inducing substances and the use of a cell/scaffold construct with the infrastructure of extraneous natural or synthetic material.

The cell/scaffold approach is gaining more attention as the utilization of a

controlled three dimensional (3D) scaffold where cells are usually encapsulated prior to transplantation can provide several advantages [25]. Unlike the cell growth and proliferation in monolayer culture, the 3D scaffold can encapsulate cells thus provide them with 3D environment for maintaining their morphology to achieve better cell viability. At the first step for the cell/scaffold approach, cells of specific interest are collected from a donor, who could be the patient himself/herself (autologous) or others (allogenic) or even from other species (xenograft). The second step is to purify the collected cells by various mechanical or chemical methods in order to discard ECM and to obtain a cell suspension composed of relatively uniform cell type. This initial cell suspension is used in the third step and expanded for engineering purposes; when the necessary cell number is reached, cells are detached and seeded into/onto a scaffold material. The scaffold, together with seeded cells, is implanted into a patient after optimal *in vitro* cell growth characteristics are observed. The further formation of the tissue *in vivo* is influenced by the structure, strength, biodegradability of the scaffold along with the regenerative potential of patient body [26].

The review below will discuss the main elements involved in cartilage tissue engineering, including cell source, therapeutic growth factor and scaffold. Among these elements, due to its most fundamental role, the continuously advancing scaffolding technique will be accentuated with respect to their applications in tissue engineering. At the moment, cartilage tissue engineering encompassing the three above

mentioned elements in an integrated approach has emerged as a promising technique for cartilage repair [27-28].

1.2.1 Cell source

The ideal therapeutic cells for cartilage tissue engineering should possess the following properties: safety, easy isolation, as well as capability to proliferate and express cartilaginous molecules such as collagen type II (COL II) and aggrecan. In recent years, several cell lineages, including mesenchymal stem cells (MSCs), fibroblasts and chondrocytes, have been employed as therapeutic cells for cartilage regeneration and are discussed below.

1.2.1.1 Chondrocytes

Chondrocytes are the first choice for cartilage tissue engineering due to their existence in native cartilage. Many studies have investigated various chondrocyte sources, including articular [29-30], auricular [31-32], nasoseptal [33-34] and costal cartilage [35-36], for regeneration of cartilage tissue. Chondrocytes obtained from older donors or OA patients also showed their potential in cartilage repair [37-38]

ACT has been utilized clinically to treat cartilage defects in more than 12,000 patients globally since 1987 [39]. Owing to the low cell number in mature cartilage tissue, one of the major challenges in ACT is to obtain sufficient cell numbers before

clinical transplantation can occur. This technique involves the harvest of cartilage tissue from the patient, isolation of chondrocytes from the donor tissue, cell expansion *in vitro*, followed by implantation into the cartilage lesions [29]. One of the major setbacks encountered by ACT is that cultured chondrocytes undergo a dedifferentiation process, namely a change in phenotype and they lose the ability to synthesize cartilaginous ECM after a number of repeated monolayer culture passages [40-41]. Dedifferentiation results in the conversion of the typical spherical shape into a spindle-shaped fibroblastic-like morphology and a shift in the production of ECM from cartilage-specific protein COL II and aggrecan to fibroblast-specific protein collagen type I (COL I) [42], which may substantially weaken the regenerated counterpart's overall quality. Therefore, the maintenance of chondrocytic characteristics is of great significance towards the success of ACT and cartilage tissue engineering.

Several studies reported that dedifferentiated chondrocytes could be recovered to a chondrocytic phenotype in an *in vitro* 3D culture system such as agarose [43], alginate beads [44-45], COL I gel [46-47], COL II gel [48], fibrin glue [49] and so on [50-51]. Also, the delivery of growth factors has been found to contribute to maintain the characteristics of chondrocytes [52-53]. Although the results are encouraging, the redifferentiation effects still demand substantial improvement. The study by Darling and Athanasious demonstrated that the dedifferentiation of passaged chondrocytes was kept even after seeding these cells in alginate beads [54]. Thus, in order to use

chondrocytes as a reliable source of cells for cartilage tissue engineering, a further improved culture system that prevents chondrocyte dedifferentiation should be developed.

1.2.1.2 MSCs

MSCs are multipotent progenitor cells which have the capability to differentiate into a wide range of cell types as occurs in various tissues, including bone, cartilage, muscle, tendon, fat and ligament [55-56]. MSCs are regarded as a superior cell source due to their unique characteristics. Compared to the low number of autologous chondrocytes [54], a large number of autologous MSCs are easy to isolate from numerous tissues, including bone marrow [57], synovial membrane [58], adipose tissue [59], muscle [60], skin [61], bone [62-63] and so on. Furthermore, they can be cultured to expand their numbers in monolayer or scaffolds and their multilineage potential can be maintained during passaging [55]. Lastly and most importantly, MSCs have the capacity to differentiate into chondrocytes when stimulated by appropriate chondrogenic factors [64-67].

There are already various *in vitro* systems developed for chondrogenic differentiation of MSCs. Johnstone *et al.* [65] developed a cell culture system in the form of aggregate culture to facilitate the chondrogenesis of rabbit bone marrow MSCs. Besides, more and more 3D cultures, like agarose- [68], alginate- [69], silk- [70], poly

(ethylene glyco)-based hydrogel [71] etc [72-75], have been explored for cartilage tissue engineering, which provided some positive outcomes. In conjunction with the 3D environment, growth factors have been used and showed their potential in accelerating the production of cartilaginous ECM. It has been reported that the supplement of transforming growth factor- β (TGF- β) [76-78], bone morphogenetic protein (BMP) [79], insulin-like growth factor (IGF) [27, 80] and fibroblast growth factor (FGF) [81] can successfully induce chondrogenesis of MSCs. The effects of growth factors will be discussed later.

A study by Mauck *et al.* demonstrated that bone marrow derived MSCs-laden hydrogels showed inferior mechanical properties to chondrocytes-laden hydrogels after long-term culture [82]. Adipose-derived MSCs exhibited a lower capability to differentiate into chondrocytic cells than other MSCs sources [59, 83-85]. The Sakaguchi group [85] found that synovium-derived MSCs (SMSCs) possessed superiority in chondrogenesis over other MSCs types as characterized by their higher colony-forming efficiency, growth kinetics, and fold increase. As a consequence, more interest is attracted to the use of SMSCs for cartilage engineering.

1.2.1.3 Fibroblasts

Fibroblasts appear as an alternative to MSCs due to the following reasons: 1) a large number of fibroblasts can easily be isolated from skin in a minimally invasive way; 2) fibroblasts can develop toward a chondrocytic phenotype under appropriate cues.

A few researchers have investigated the feasibility of fibroblasts for cartilage repair. Although transplanting fibroblasts/poly lactic acid (PLA) composites directly into cartilage defects failed to form hyaline-like cartilage but fibrous tissue [86], Nicoll *et al.* found that dermal fibroblasts could be differentiated into chondrocytic cells *in vitro* under high density micromass culture with treatment of 40 mM lactate [87]. Likewise, studies demonstrated that dermal fibroblasts could synthesize cartilaginous ECM proteins when cultured on aggrecan after pretreatment with IGF-1 [88] or cultured with demineralized bone powder [89], thus making fibroblasts a potential cell source for regeneration of articular cartilage. In addition, a study by Lee *et al.* suggested that 6 weeks after injection of fibroblasts expressing TGF- β 1 into the cartilage defects, new hyaline cartilage was found as shown by histological analysis [90]. Recently, Deng *et al.* separated dermis-isolated aggrecan-sensitive (DIAS) cells and demonstrated that the cells possessed superiority in chondroinduction potential over unpurified dermis cells [91].

Although fibroblasts have proven to be an optional cell source for cartilage tissue engineering, more work needs to be done to address some latent issues such as the possibility of fibrocartilage formation.

1.2.2 Biological molecules for cartilage tissue engineering

Some biomolecules, no matter in the form of recombinant proteins or by a transgene product via non-viral vectors or viral vectors, have been employed to induce chondrogenesis of mesenchymal progenitor cells [92], to stimulate proliferation of chondrocytes [93-94] and to enhance synthesis of cartilaginous ECM [95]. Among the potential candidates as investigated for cartilage repair, are (1) growth factors of the TGF- β superfamily, including BMPs, IGF-1, and FGFs; (2) transcription factors such as Sox-9; and (3) signal transduction molecules, such as the intracellular proteins SMADs. In this review, mainly the growth factors that belong to the first category are discussed because they are more widely applied for this purpose.

1.2.2.1 Growth factors

TGF- β is a multifunctional peptide growth factor, and comprises at least three known subtypes in human. TGF- β 1 has a vital impact on regulating the inceptive cell-cell interaction triggered by progenitor cells [76], and stimulates chondrocytes to produce new ECM [96]. TGF- β 2 is known to regulate chondrocyte differentiation to hypertrophy via controlling the expression of a special kind of growth hormone [97]. TGF- β 3 exhibits its greater potential in chondrocytic differentiation of MSCs [98]. They also play a role in preventing the formation of blood vessels in cartilage, which

prevents further mineralization and ossification [99]. Palmer et al. transfected bone marrow-derived MSCs with TGF- β 1, and their results showed the formation of cartilaginous tissue and the production of ECM comparable with that of articular cartilage [100]. An increasing number of studies also indicate that MSCs could present chondrocyte specific gene markers and form a cartilaginous matrix by addition of TGF- β 3 during a 14-day to 2-month culture period [101].

BMPs are a family of growth factors with the capability to stimulate the formation of bone and cartilage, and they also belong to the TGF- β superfamily. Several studies have successfully utilized the cDNAs encoding BMPs to induce mesenchymal progenitor cells to differentiate into chondrocytes [79, 102]. Carlberg *et al.* observed that the transfer of BMP-2 cDNA by retroviral vector into C3H10T1/2 cells in micromass culture promoted cartilage formation, and its action was restricted to the infected cell population [103]. Aside from BMP-2, Sekiya *et al.* demonstrated that the supplement of BMP-6 made the pellets about 10-fold heavier and enhanced the production of proteoglycans. In addition, the expression of type II procollagen and collagen type X (COL X) was detectable at 1 week and the levels were augmented at 3 weeks [104]. Nochi *et al.* compared the effects of BMP-2 and BMP-13 expression in chondroprogenitor cells, finding that BMP-13 promoted chondrocytic differentiation to a midway stage, while BMP-2 induced endochondral ossification [105]. Furthermore, the combinational transfer of TGF- β 1 and BMP-2 acted synergistically, leading to

enhancement of chondrocyte redifferentiation and better ECM production [106]. Nevertheless, BMPs have to be used very carefully for cartilage repair as they can also stimulate ossification.

IGF-1, with a molecular structure similar to that of insulin, is a 7.6 kDa polypeptide growth factor which can control glycogen and protein synthesis. Particularly, IGF-1 enhances the expression of proteoglycan and COL II, two major components of chondrocytes in cartilage. Gelse *et al.* evaluated the repair of articular cartilage via cell transplantation as well as transferring IGF-1 cDNA, and found that cells treated with IGF-1 also led to a complete coverage in most cartilage defects with repair tissue [27]. In addition, immunohistochemical staining indicated extensive COL II staining, but weak COL I staining. Another report by Fukumoto *et al.* also described IGF-1-mediated mesenchymal chondrogenesis in periosteal explants from rabbits [66].

FGFs are a group of growth factors playing a role in wound healing and embryonic development [107]. Studies have also shown that FGFs can induce chondrocytic differentiation, and a more detailed research by Hoffmann *et al.* suggested the chondrogenic effect of FGF-3 in this developmental process [108]. In a study conducted by Ellsworth *et al.*, it was revealed that the adenoviral addition of FGF-18 can promote the growth of articular chondrocytes and increase ECM expression [109].

1.2.2.2 Other therapeutic candidates

Sox-9, the major transcriptional factor, is capable of promoting MSCs condensation. Sox-9, together with Sox-5 and Sox-6, is identified to be essential and sufficient for cartilage formation [110-111]. Tsuchiya et al. investigated chondrocytic differentiation of MSCs by overexpression of the Sox-9 gene and their histological staining results showed that both GAGs and COL II were significant but COL X was not stained [112]. In addition, the signal transduction molecules SMADs can regulate chondrogenesis intracellularly by means of gene delivery [113].

1.2.3 Scaffolds

Native cartilage has an extracellular protein matrix which is strengthened by a 3D network of collagen fibrils [114]. A tissue engineered 3D construct can mimic the morphology of the *in vivo* microenvironment. In a 3D construct, most chondrocytes showed a spherical morphology in contrast to the spreaded morphology in monolayer culture. This has been demonstrated to be associated with the production of cartilage-related ECM proteins [40]. Moreover, cells transfected by viral or nonviral vectors show a gradual decrease in transgene expression in monolayer culture, while there is a prolonged transgene expression in 3D hydrogel culture conditions [115-116].

To date, both natural and synthetic polymers as scaffolding vehicles have been studied for cartilage tissue engineering and both of them have their pros and cons [13].

Natural polymers, including agarose, alginate, fibrin, hyaluronic acid, chitosan, collagen and silk fibroin etc, can well interact with cells in order to regulate cellular behavior, while they possess inferior mechanical characteristics, are vulnerable to degradation by host enzymes and there is a potential risk in inducing an immune system response. On the contrary, synthetic polymers such as poly (ethylene glycol/oxide) have superiority in mechanical and degradation properties due to modifications, but are weak for cell/scaffold interactions and can produce toxic byproducts during degradation.

In addition, the material composition of a scaffold is of great importance for cartilage repair, which can generally be categorized as sponges, meshes and hydrogels. Wang and coworkers [70] compared the chondrogenesis of MSCs cultured in 3D porous silk scaffolds with those cultured in silk films. After 3 weeks, the cartilage-specific gene expression at a transcriptional level was not detected or very low in cells cultured in two dimensional films, whereas these genes except for Sox-9 were significantly increased in cells cultured in 3D silk scaffolds. Concerning mesh scaffold, Moroni and co-workers adopted a 3D fiber deposition method to fabricate woven and non-woven fiber networks, in pursuit of a desirable architecture biomechanically similar to native cartilage [117-118]. Williams *et al.* [71] encapsulated MSCs in a photopolymerized hydrogel followed by a 6-week culture in chondrogenic medium with the addition of TGF- β 1. They found that MSCs propagated in the

hydrogels and aggrecan and COL II gene expression were upregulated compared with monolayer MSCs. Among the three types of scaffold on material composition level, hydrogels are promising candidate as scaffold material for cartilage tissue engineering and reviewed in more detail below.

1.3 Hydrogels for cartilage tissue engineering

Recently, the rapid development of biomedical materials has greatly expedited the progress of modern biotechnology and tissue engineering. 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 the quality of life for all.

Among the numerous types of biomaterials available as scaffold material for tissue engineering, hydrogels have shown great promise [26, 119-120]. Bodies of organisms are mostly made of hydrogels that vary in diverse forms [121-122]. In

nature, hydrogels are cross-linked hydrated polymeric networks which are insoluble in water. The composition of hydrogels can be natural or synthetic, such as agarose [123] and poly(ethylene glycol) (PEG) [124] respectively. The crosslinking, i.e. gelation, is triggered via different mechanisms (e.g., thermo-, ion- or photo-initiated), and converts precursor solutions into 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 [120]. For application in 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 biomolecules is controlled [26, 120, 125-126]. Moreover, these molecule- or cell-laden hydrogels are often formed by virtue of certain shape and size, such as nano-scale particles [127] and centimeter sheets [128], for meeting different needs in practice such as prompting drug delivery and increasing sufficient cell adherence. 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 [129]. Some of the representative hydrogels are listed in **Table 1.3**.

Table 1.3 Some representative types of hydrogel for biomedical usage [130-133].

Types of hydrogel	Gelling mechanism
<u>Poly (ethylene glycol) (PEG)</u> : Synthetic polymer with several derivatives; very widely used as hydrogel models	Covalent: modified with acrylate and photo-polymerised Example: PhosPEG moulded into mm-cm scale
<u>Poly (vinyl alcohol) (PVA)</u> : Synthetic polymer; good property to form films	Covalent/physical: with gluteraldehyde or by freeze-thaw Example: PVA/ferritin nanofibres
<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
<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

Hydrogels are particularly suitable for cell delivery-based scaffolding therapeutics, due to their unique and versatile advantages [126, 130, 134-135]. First, the liquid precursor of hydrogels containing therapeutic agents/cells is injectable, which makes that they will fit accurately to the defect locally due to the gelatinized nature of the material - as specifically is required for *in situ* therapy. Second, the cell-encapsulating hydrogel can be rapidly fabricated by gelatinizing a cell-suspended macromer solution through various pathways as mentioned in **Table 1.3**, facilitating the transplanted cells to be uniformly distributed in their 3D matrices. Third, the water content and physical properties of hydrogels can be manipulated to precisely mimic their native tissue counterparts. Additionally, the porous structure of hydrogels allows for the

transportation of water and nutrients and the elimination of wastes and metabolites.

In a large number of *in vitro* and *in vivo* studies, hydrogels have been utilized to deliver and accommodate therapeutic cells, aiming for the regeneration of various tissue/organs including cartilage [136]. It is not surprising that hydrogels are the favorable choice as scaffold material for cartilage tissue engineering. On one hand, hyaline cartilage is avascular; once injured, it can not self-regenerate in adults - so cell delivery-based therapeutics are highly demanded. On the other hand, hydrogel bulk is similar to native cartilage in lots of aspects. Moreover, plain non-cell-adhesive hydrogels can maintain the phenotype of encapsulated chondrocytes. Since Elisseeff and Anseth *et al.* employed PEG-based hydrogel to encapsulate chondrocytes [136], their two independent groups have partly focused on the investigation of photopolymerising hydrogels to regenerate cartilage. Bryant and Anseth *et al.* serially explored numerous material factors influencing the encapsulated chondrocytes, such as photoinitiating systems [137], scaffold thickness [138], gel properties [126, 139], gel degradation [140], gel crosslinking density [141-142]. Further, they incorporated tissue-specific molecules and applied spatial patterning to hydrogels, analysing the gene expression and cell metabolism of chondrocytes [143]. Elisseeff's group did studies to the induction of chondrogenesis of MSCs [71], embryonic stem cells (ESCs) [144-145] and ESC-derived MSCs [146] within hydrogel matrices, meanwhile developing novel biodegradable hydrogels for chondrocyte encapsulation [130, 147].

Notably, in their research [148-149], Wang and Elisseeff *et al.* addressed the opinion that the integration of hydrogels with surrounding native tissue was important, and demonstrated that such integration facilitated both immediate functionalities and long-period stability of engineered tissues. At the same time, Tuan and colleagues investigated chondrogenesis and its related signal pathways within a variety of 3D matrices, ranging from agarose or alginate hydrogels [82, 150], fibrous/porous scaffolds [151-153], to vehicle-free micromass [154]. These comprehensive findings at cellular and molecular level have provided biological insight to cell behavior in engineered hydrogels [155-156].

1.3.1 ‘Smart’ hydrogels piloting to the advance of hydrogel

An ideal scaffold not only functions as a mechanical or structural support to facilitate cell proliferation and adjacent ECM secretion while degrading timely to match the subsequent tissue regeneration rate. As one of the most appealing scaffold candidates for tissue engineering, hydrogels possess extraordinary advantages, but yet could not get rid of their drawback that its degradation is difficult to match with the tissue regeneration rate. Furthermore, although the small sized pores inside hydrogel allow free permeation of nutrients and transportation of wastes out of the hydrogel, the pore size is so small that cell migration, proliferation and secretion of ECM are largely limited inside hydrogel, which restricts the tissue regeneration process.

As a consequence, ‘smart’ hydrogels have been designed, inheriting the superiority of hydrogels and intelligently responding to mechanical stimuli, biological signal or environmental change such as spatial variance etc, but interestingly can also direct the behavior of cells [157-159]. Abundant recent research has been covered on bioresponsive hydrogels, which closely mimic natural ECM by responding to enzymes secreted by cells or related cascade enzyme-triggered actions, as summarized in **Table 1.4**.

Table 1.4 Bioresponsive hydrogels (enzyme-specific) for tissue engineering.

Material	Stimulus	Cell type
Oligopeptides	Metalloproteinase	Human fibroblasts
Ac-CGYGRGDSPG [160]	(MMP)	
Polyethylene glycol (PEG) [161]	MMP	Dermal fibroblasts
pNIPAm-Co-AAc [162]	MMP-13	Rat calvarial osteoblasts (RCOs)
Gelatin/gellan [163]	TGase	Fibroblasts NIH T3 cells
Gelatin [164]	mTGase	Retinal tissue
3D fibrin hydrogels modified with $\alpha V\beta 3$ receptor [165]	TGase	Human umbilical cells
CH ₃ (CH ₂) ₁₄ CO-GTAGLIGQRG DS [166]	MMP-2	Dental pulp cells
Nap-FFGEY [167]	(i) kinase (ii) phosphatase	Hela cells
Polyurethane/polycaprolactone/PEG [168]	Elastase	Endothelial cells

Hubbell and co-workers are experts in studying such simulative hydrogels to allow cell migration [160, 169]. They cross-linked PEG-based hydrogels by

oligopeptides (integrin-binding domains (Arg-Gly-Asp-Ser-Pro)), whose sequences were cleaved by fibroblast-secreted matrix metalloproteinases (MMPs) to enable cell infiltration. The *in vivo* evaluation showed the sensitivity of the hydrogels to MMPs, which influenced the cell infiltration efficiency. Following their pioneer work, Raeber *et al.* [161] assessed two 3D PEG-based hydrogel systems that are proteolytically degradable via either a MMP-sensitive or plasmin-sensitive pathway thereby permitting cell migration. The results indicated that only the MMP-sensitive hydrogels induced cell migration. Kim and co-investigators [162] fabricated an injectable pNIPAm-co-AAc hydrogel to mimic ECM and introduced a peptide sequence cross-linked to NIPAm, which could be degraded by MMP-13/collagenase-3. Meanwhile, the poly(AAc) was modified by Arg-Gly-Asp and then the co-polymer was prepared. Rat calvarial osteoblasts were used as model cells to evaluate how the proteolytically degradable hydrogels facilitated cell migration. The results showed that poly(AAc) modified with the Arg-Gly-Asp peptide simulated greater migration and the same effect was observed in MMP-degradable hydrogels.

Natural proteins or their derivatives, such as gelatin that is in the form of denatured collagen, have been widely employed as tissue engineering scaffold and wound dressing. Noticeably gelatin is always cross-linked in order to strengthen its mechanical property [163] usually by transglutaminases (TGases) via propelling to conjugate the γ -carboxamide of Gln and a free amine group [164, 170-172]. By virtue

of TGases performance, Chen *et al.* [164] designed a gelatin-based adhesive. The formed bond catalyzed by TGases was showed as comparably strong and such method supported the improvement of retinal reattachment. Bertoni and co-workers also investigated gelatin hybrid with a natural polysaccharide gellan to increase the proliferation response to the encapsulated fibroblasts [170].

Synthetic and natural hydrogels were also combined for such purpose and an example is the adoption of peptide amphiphiles (PAs) [173]. Jun *et al.* [166] fabricated PAs contained with biological epitopes. The nanofiber network incorporated MMP-cleavable sites and additionally adding type IV collagenase (an MMP) caused its breakdown.

Enzymatic sol-to-gel transitions were studied recently to adapt cell behaviour. Yang *et al.* [167] used naphthyl-pentapeptide, Nap-Phe-Phe-Gly-Glu-Tyr to realize this process. Supramolecular hydrogel could be arranged by using a kinase/phosphatase switch and the supramolecular hydrogel was formed *in vivo*. Cell delivery could be precisely controlled accordingly. Briefly, the function of kinase was to restrain self-assembly by electrostatic repulsion, and the phosphatase was related to hydrogelation. Therefore, such a system is highly dynamic. Since adenosine triphosphate was involved extensively in the reversible process, its level would directly influence the hydrogel status in the presence of the two enzymes.

A phosphoester-PEG hydrogel gelled via photopolymerization was reported

twice by Wang and his group [130, 174] and is another kind of bioresponsive hydrogels without any peptide sequence incorporated. This hydrogel *per se* can undergo hydrolytical degradation and the degradation rate will be increased when alkaline phosphatase (ALP) is present. ALP is a bone-specific enzyme which can be activated by bone-specific protein as secreted by MSCs. Therefore, the degradation rate of phosphoester-PEG encapsulating MSCs can be flexibly adjusted according to the expression level of cell-secreted bone-specific makers.

Although the attempts in creating a bioresponsive hydrogels are quite promising in inducing cell migration or infiltration via biochemical degradation, the degradation of such ‘smart’ hydrogels is still very limited and cell proliferation and secretion of ECM are still greatly limited due to lack of significant space [175]. Innovative methods are still required to address the problems as associated with scaffolds, like the lack of space and the difficulty to match the tissue regeneration rate by degradation.

In response to the problems associated with the scaffold, a ‘scaffold free’ concept has been addressed and extensively explored [176-178]. Cell sheet [128, 179-180], cell pellet culture system [181-182] and cell organoid [177] etc, all belong to the specific termed category of ‘scaffold-free’ constructs. Park and co-workers extracted a chondrocyte/ECM membrane under normal monolayer chondrocyte culture conditions and cultivated it to grow as a scaffold-free engineered cartilage graft [178], which was similar to the known pellet culture for tissue engineering [181, 183-184] or cell sheet

creation for tissue defect repair [185-186]. Waldman and co-workers attempted to obtain a 'scaffold-free' cartilaginous construct formed from calcium polyphosphate substrates [187-189]. An alginate recovery chondrocyte (ARC) method as proposed by Masuda *et al.* [190] and was found to be as not harmful for cells including MSCs [191], has been adopted for creating various 'scaffold-free' cartilage implants [192-194]. Kitahara *et al.* combined ARCs with a HA block to obtain a tissue-engineered osteochondral implant with the cartilage-like free of any scaffold [193]. Han *et al.* re-seeded ARCs into differently shaped molds to create scaffold-free stratified cartilage implants [194]. The common advantage of these products is that they are devoid of foreign scaffold material, which simply and directly bypasses the scaffold-related bottleneck problems. Adversely, the lack of the support from a scaffold makes that these 'scaffold-free' constructs are constrained in their macroscopic size and their ability to withstand mechanical stress.

In smmmry, two challenges raise for the application of hydrogels in cartilage tissue engineering, i.e. the lack of space for cell growth and ECM production and the degradation rate which fails to match the tissue regeneration rate and thus impedes tissue formation. Previously reported bioresponsive hydrogels can reduce their spatial confinement for encapsulated cells to a certain degree, but the problem still exists and breakthrough solutions are still in demand.

1.4 Objective and organisation of this dissertation

The overall objective of this PhD project is to design an innovative scaffolding system to engineer true cartilage tissue for cartilage repair.

The primary challenge for hydrogel application in tissue engineering is that most therapeutic cells cannot reach a desirable growth within 3D hydrogels due to lack of an appropriate interface between their polymeric network and cells. More specifically, within 3D gel bulk, there is no extra space for cells which affects their inclusion in the constraint hydrogel environment. As a result, cellular outgrowth cannot be achieved, and cell survival and function together with tissue formation are heavily impaired.

Accordingly, to overcome both spatial limitations and deficiency of cell adhesive moieties for the neo-tissue formation while still maintaining all the beneficial properties of hydrogels such as injectability, we designed a novel micro-cavitary hydrogel (MCG) system by introducing microcavities inside hydrogel bulk to enable tissue formation. Specially designed microspheres were encapsulated together with cells in the hydrogel and after gelation, dissolved to create microcavities inside the hydrogel bulk to provide significant space for cell proliferation and accommodation of endogenous ECM [195]. The model is an inspiration from an observation of dynamic outgrowth of chondrocytes at the gel-edge of a conventional cell-laden hydrogel system and this phenomenon of active proliferation of cells and ECM secretion at the gel-edge is hence named edge flourish (EF). The developed MCG model can utilize

spontaneous formation of microspherical cavities to introduce multiple gel edges into the hydrogel bulk, thereby promoting and accommodating the outgrowth of the proliferating cells/colonies from the cell-encapsulating gel phase to the micro-cavities - an unconfined phase just like the EF phenomenon. The whole strategy is therefore termed phase transfer cell culture (PTCC). By use of the PTCC strategy, an innovative hydrogel model is presented to generate more space within gel bulk for solving the common hydrogel hurdle as mentioned lastly. Based on this PTCC-MCG model, we further developed a living scaffold (LS) system by removing the remaining materials thus making the whole system scaffold-free. The used dissoluble hydrogels, i.e. alginate, allows its removal by a gentle and harmless solvent such as sodium citrate. As a consequence, PTCC strategy is realized in another way and cells will outgrow the living scaffold and continue to secrete abundant ECM in order to consolidate the construct, so that a pure integral cartilage implant can be obtained.

With the PTCC-MCG system followed by the PTCC-LS system, we believe the problems associated with hydrogel scaffold can be addressed in cartilage tissue engineering application and neo-tissue formation will be obtained.

This dissertation is divided into the following five chapters.

Chapter 1, the current chapter, reviews the cartilage property and its lesion and the wide variety of tissue engineering approaches as applied as well as the needs in cartilage tissue engineering. Hydrogels, the main scaffold material as used in cartilage

tissue engineering and the associated problems are reviewed in detail. The objective, together with the outline of this thesis, is presented.

Chapter 2 starts to explore our first PTCC-MCG, namely hydrogel/cavities composite including chondrocytes, gelatin microspheres and commonly used hydrogels. The construction of the composite system is presented and the cell behaviour in this system is investigated. The mechanism of a micro-additive in improving cell-accommodation of hydrogel substrates at 3D scale is simply investigated.

Chapter 3 presents a more detailed investigation of the biomechanical mechanism of PTCC/EF based on a plain cell/hydrogel model. Agarose hydrogel is utilized and the behaviour of encapsulated chondrocytes outgrowth at the edge of gel is evaluated. From the hydrogel bulk to its surface, cell biomechanical activities are tested and compared layer by layer. Different mechanical models are built to serve for better understanding of the mechanism behind PTCC/EF phenomenon.

Chapter 4 extends the application of the PTCC strategy into the area of a scaffold-free engineered implant. The PTCC-MCG system is used, as it is good for providing significant space for the proliferation of encapsulated cells and the secretion of endogenous ECM by replacing the model hydrogel with easily removable hydrogels, in pursuit of obtaining a living tissue shortly after hydrogel removal. LS construct is designed by dissolving the intrinsic synthetic material part of the hydrogel in order to target the delivery of therapeutic cells, to promote cell proliferation and further to

tissue fusing for scaffold-free construct formation. The feasibility of this concept as potential cartilage defect treatment approach is evaluated in a mouse model.

Chapter 5 concludes these studies and suggests realistic future works.

Chapter 2 Microcavitary Hydrogel-Mediating Phase Transfer Cell Culture for Cartilage Tissue Engineering

2.1 Introduction

In Chapter 1, it is described that the close mimicking of gel-like materials to native tissue has led to extensive research of hydrogels in biomedical engineering in the past decades [17, 196-199]. Noticeably, besides hydrogels there are several scaffold compositions also being used in tissue engineering applications: one of which is the sponge-like scaffold made of poly lactic-co-glycolic acid (PLGA) [200] or collagen [201] etc, while another is the electrospun fibrous scaffold [202-203]. In both the sponge and fibrous scaffolds, cells are seeded after scaffold formation. However, these scaffolds cannot be made as an injectable material. Besides, these scaffolds induce cellular focal adhesion [204-206] which is not appropriate for articular hyaline cartilage regeneration. Generally chondrocytes inside hyaline cartilage maintain their spherical phenotype and keep their robust growth and proliferation rate. The scaffold merely prompting cellular focal adhesion does not aid in maintaining chondrocytic phenotype and further the increased spreading morphology would cause undesired cartilage fibrosis [207-209].

Conventional hydrogel has the advantage of injectability and thus allows in situ tissue development. Hence, much work has been focused on functionalizing hydrogels

to enhance cell transportation and accommodation [210-213], but the breakthrough of hydrogel applications in the field of regenerative medicine is still impeded by limited cell growth and scarce tissue formation [214-215]. These constraints originate from two material-based backgrounds: (i) how to synchronize scaffold degradation with neo-tissue growth, which is a common challenge to most biodegradable scaffolds applied in the current tissue engineering systems [216-222]; and (ii) how to provide cell adhesive moieties due to the intrinsic hydrophilic nature of the hydrogels, which has substantially hindered cellular development [223-225].

Accordingly, to overcome the challenge to engineer neo-tissues while still maintaining the hydrogel own advantages, we first set out to design a novel MCG model to provide significant space for cell growth and accommodation of endogenously secreted ECM of encapsulated cells. The model is inspired from an EF phenomenon as illustrated in **Figure 2.2** and it aims to realize EF inside the hydrogel bulk. The encapsulated cells outgrow the hydrogel bulk into the cavitory phase, thus giving rise to the so-named PTCC strategy.

Comparing our PTCC-MCG system with conventional porous scaffolds like PLGA or collagen sponges or gels where cells could only be seeded after scaffold formation, our system co-encapsulates both cells and (10^2 -microns-scaled) porogens simultaneously during scaffold fabrication, which not only ensures nearly full cell loading efficiency but also maintains injectability of the whole system. With this

invention, we endeavor to transform the traditional role of hydrogel bulk from a mere cell vehicle to an integration of micro-incubators that physically direct and accommodate the growth of neo-tissue inside. In this study, a cartilaginous system was chosen as model system since chondrocytes are one of the non-(typical) anchorage dependent cells, which can maintain viability within the non-cell adhesive hydrogel environment.

2.2 Materials and Methods

2.2.1 Chondrocytes isolation, encapsulation and EF determination

Chondrocytes were isolated from cartilage tissues of porcine articular cartilage. A biopsy was done in a pig and cells obtained in each biopsy were used for one experiment only. Altogether, in this study six repeats were performed using six different pigs. Briefly, cartilage tissue obtained from the joint of 5-month-old pigs was cut into small chips. Chondrocytes were isolated by incubating the cartilage pieces in Dulbecco's Modified Eagle Medium (DMEM) culture medium with 10% (v/v) FBS containing 1 mg/mL collagenase type II at 37°C for 12 hours under gentle stirring. The chondrocytes were then centrifuged and resuspended in chondrocyte culture medium (CCM), which is comprised of DMEM supplemented with 20% (v/v) FBS, 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline, 0.05 mg/mL vitamin C, 100 units/mL penicillin

and 100 mg/mL streptomycin. The cell suspension was then seeded in 75 mL tissue culture flask (Falcon, seeding density 2×10^4 cells/cm²) and incubated in humidified air with 5% CO₂ at 37 °C for routine culture. In this study, passage I cells were used.

To demonstrate the EF phenomenon, chondrocytes were suspended at high density in gel precursor solution (in PBS, agarose 0.02 g/mL, cell density 1×10^7 /mL) at 37 °C and injected into a mold (D = 4 mm, H = 3 mm). After solidification at room temperature, the cell/gel constructs were transferred to the 24-wells culture plate and cultured in the cell culture incubator (5% CO₂, 37 °C) for 3~32 days. The EF phenomenon was observed starting at Day 12 (**Figure 2.2**). This model is named EF-on-Edge model.

2.2.2 MCG construction

2.2.2.1 Preparation of partially surface-crosslinked gelatin microspheres

Gelatin microspheres were fabricated with plain or Rhodamine B (Aldrich) pre-labeled gelatin (sourced from bovine skin, Type B, Sigma) following a double emulsion method. Briefly, 10 mL ethyl acetate was mixed into 30 mL gelatin solution (0.1 g/mL in water) by stirring and the emulsion was further added into 60 mL soybean oil and stirred for 10min. Gelatin microspheres were formed spontaneously in 300 mL pre-cooled (-20 °C) ethanol and rinsed with 1,4-dioxane/acetone in turn. After being air-dried, the microspheres (150~180 μm) were collected via standard sieves (80~100

mesh). A partial surface-crosslinking treatment was conducted on the selected microspheres via suspension in *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide solution (EDC, in 90% ethanol) at 4°C for 12 hours, followed by another suspension in PBS with trace amounts of glutaraldehyde at 4°C for 19 hours. The products were washed in pure ethanol and dried in a vacuum oven.

2.2.2.2 Acellular MCG construction

The partially surface-crosslinked gelatin microspheres were pre-equilibrated in PBS at 4 °C overnight and then suspended in 2 wt% agarose solution (in PBS, solution/microsphere ratio 1:0.2 mL/g) at 37 °C. After injecting into a mold (less than 1 mm thick), the mixture was solidified by cooling down to below 25 °C before a speed-controlled spontaneous dissolution of the encapsulated gelatin microspheres was performed in order to create cavities inside the agarose gel bulk as illustrated in **Figure 2.1**. The rhodamine labeled gelatin (red) was employed to trace the dissolution of spheres and the residual gelatin in the construct was quantified by hydroxyproline test as shown in **Figure 2.2 B**.

Figure 2.1 Schematic demonstration of construction of MCG model.

2.2.3 PTCC in cell-laden MCG system

2.2.3.1 Agarose based PTCC-MCG model

Chondrocytes were co-suspended with partially surface-crosslinked gelatin microspheres in 2 wt% agarose solution as shown in **Figure 2.6**. The final cell density in agarose constructs was around $1 \times 10^7/\text{mL}$. The mixture was injected into a mold and it was solidified by the similar method as mentioned in MCG construction. As control, conventional hydrogel constructs were prepared by encapsulating chondrocytes (cell density $1 \times 10^7/\text{mL}$) without gelatin microspheres in agarose. The constructs were cultured in CCM under shaking (60 rpm) in the cell culture incubator (5% CO_2 , 37 °C) for up to 32 days and the culture medium was changed every 2-3 days. The agarose MCG constructs were utilized to evaluate the overall performance of tissue regeneration.

The self-formation of micro-cavities by delayed dissolution of the co-encapsulated microspheres is demonstrated in **Figure 2.5**. The whole PTCC procedure is recorded

and demonstrated in **Figure 2.7**. In order to trace the fate of hydrogel component in PTCC system quantitatively, the agarose content of lyophilized cell-laden MCG constructs was measured by determining the 3,6-Anhydrogalactose content according to Yaphe and Arsenault [226] in comparison to constructs without cavities. Using pure agarose as standard, the residual agarose content at given time points was calculated according to the following equation: agarose remaining percentage % = W_t/W_0 , where W_0 and W_t denote the relative weight of agarose at day 0 and other given time point respectively. The result is indicated in **Figure 2.10**.

2.2.3.2 Alginate based PTCC-MCG model

The PTCC procedure was also done using alginate-based cell-laden MCG system. Chondrocytes were suspended in 1.2 wt% alginate solution and mixed with surface-crosslinked gelatin spheres to form MCG constructs. The final cell density in alginate constructs was around $1 \times 10^7/\text{mL}$. The alginate MCG constructs were formed by dispensing the alginate-cell suspension drop-wise (40 $\mu\text{L}/\text{drop}$) into a 102 mM calcium chloride solution using pipette. The constructs were cultured in the same conditions as the agarose constructs. Cells embedded in the alginate could be re-isolated without any harm using the ARC method [190], and subsequently used for analysis of the gene expression of aggregated cells grown in the unconfined phase as well as scattered cells grown in the hydrogel phase.

2.2.4 RNA extraction

2.2.4.1 RNA extraction from EF-on-Edge model

As described in section 2.2.1, the constructs of EF-on-Edge model were prepared. The EF layer was separated from gel bulk by punching out the surface part from the whole construct. The top and bottom surfaces of the solid gel bulk were also carefully cut off as parts of the EF layer. The EF layer and gel bulk were thus distinguished and separated for subsequent RNA extraction using a combination of TRIzol® (Invitrogen) and RNeasy® Mini Plant Kit (Qiagen, Düsseldorf, Germany), as specifically reported by our group [213].

2.2.4.2 RNA extraction from agarose-based PTCC-MCG model

PTCC strategy was launched in agarose based cell-laden MCG constructs. To extract RNA, the whole construct was homogenized and RNA from specimens was extracted using the combination of TRIzol® (Invitrogen) and RNeasy® Mini Plant Kit (Qiagen, Düsseldorf, Germany) [213]. The acquired RNA samples (from cell-laden constructs) were treated with RNase-free DNase I and converted to cDNA for subsequent PCR experiments.

2.2.4.3 RNA extraction from alginate-based PTCC-MCG model

PTCC strategy was also tested in alginate based cell-laden MCG constructs. Before RNA extraction, ARC method [190] was used to distinguish the cell islets induced by PTCC procedure from scattered cells/colonies remaining in the gel-bulk. When obvious cell aggregations could be observed using optical microscope, alginate based MCG constructs were collected and dissolved in a buffer containing 55 mM sodium citrate, 0.15 M sodium chloride at pH 6.8, for 10 minutes at 4 °C to remove alginate in order to release scattered or aggregated cells. The resulting suspension of chondrocytes (mixture individual and aggregated cells) was filtered by a cell strainer (40 µm Nylon; BD Falcon, Bedford, MA). The aggregated chondrocytes on the strainer and the scattered cells in the filtrate were collected respectively. These two populations of cells were centrifuged separately and then the cell pellets were washed before resuspending them in PBS. Accordingly the two distinct forms of chondrocytes were separated from each other and RNA was extracted by TRIzol® (Invitrogen).

2.2.5 Real Time RT-PCR

For real-time quantitative PCR, the relative gene expression values were obtained from iQ™ qPCR system (Bio-Rad, Hercules, CA, USA) and calculated with the comparative threshold cycle method (named as 'ΔC_T method' by the manufacturer), as normalized to the housekeeping gene. All the RT-PCR reagents used above were purchased from Promega (Madison, MI, USA). All the primers used are listed in Table

1, and all the oligonucleotides were synthesized by AIT Biotech (Singapore).

Table 2.1 PCR primers used in this study.

Genes	Primer sequence (both 5' – 3') [a]
GAPDH[227]	F: ACCCCTTCATTGACCTCCAC R: ATACTCAGCACCAGCATCGC
HPRT1	F: GGACTTGAATCATGTTTGTG R: CAGATGTTTCCAACTCAAC
RPL4	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC
TBP1	F: AACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG
Collagen II[227]	F: GCTATGGAGATGACAACCTGGCTC; R: CACTTACCGGTGTGTTTCGTGCAG
Aggrecan[227]	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC
RhoA	F: AGCTGGGCAGGAAGATTATG R: TGTGCTCATCATTCCGAAGA
Integrin β 1	F: TGCCAAATCATGTGGAGAATGTAT R: GTCTGTGGCTCCCCTGATCTTA
Sox9	F: GCTGGCGGATCAGTACCC R: CGCGGCTGGTACTTGTA
COMP	F: GGCACATTCCACGTGAACA R: GGTTTGCCTGCCAGTATGTC

[a] F and R stand for 'forward' and 'reverse' sequence, respectively.

2.2.6 Biochemical assays

'Live/Dead' dye staining (Molecular probes, Invitrogen Singapore) was used to

determine the viability of chondrocytes. The loaded viable cells were indicated with green fluorescent calcein. Biochemical assays were performed on the lyophilized cell-laden MCG constructs. The specimens were digested with 1 mL papain solution (0.3 mg/mL, mixed with 0.1 mM disodium ethylenediaminetetra acetic acid in 0.2 mM dithiothreitol) for 24 hours. By centrifugation, the supernatant containing digested cell lysate was collected for measurement of cell numbers [228] (via DNA quantification using Hoechst 33258 dye), collagen content [229] (via hydroxyproline quantification), and glycosaminoglycan (GAG) content [230] (with dimethylmethylene blue).

2.2.7 Histology

For histological evaluation, paraformaldehyde (4%)-preserved sample were cut to micron-sized slices by paraffin-sectioning [231]. The obtained sections were subsequently deparaffinized and respectively stained with H&E, Safranin O and Masson's trichrome dyes. Collagen II primary antibody (2 µg/mL in PBS, MAB8887, Chemicon) and Anti-IgG (5 µg/mL in PBS, InvitrogenAlexaFluor, 488) were utilized for collagen II staining, Collagen I primary antibody (2 µg/mL, mouse monoclonal IgG, Santa Cruz Biotechnology) and Anti-IgG (5 µg/mL, InvitrogenAlexaFluor, 543) were utilized for collagen I staining and NCAM antibody with fluorescein conjugates (sc-7326 FITC, Santa Cruz Biotechnology) was applied for NCAM staining.

2.2.8 Scanning electron microscopy (SEM)

Field emission SEM (FESEM, JSM-6700F, JEOL Ltd., Tokyo, Japan) was used to examine the microstructure of MCG constructs. Briefly, the glutaraldehyde (2.5%)-preserved MCG constructs were treated with 1% OsO₄ at 4 °C for 2 hours, then dehydrated in gradient ethanol solutions and further dried in vacuum overnight. Platinum was sputtered for SEM contrast.

2.2.9 Animal experimentation

All the animal experiments were performed under guidelines approved by the Institutional Animal Care and Use Committees (IACUC), SingHealth, Singapore. Specimens with chondrocytes (agarose model) were implanted subcutaneously in the dorsum of athymic nude mice after 7~10 days culture *in vitro*. Briefly, the nude mice were anesthetized by Ketamine (40 mg/Kg) and Diazepam (5 mg/Kg) mixture and the back was disinfected with 70% ethanol and iodine. Then 2 incisions were through the full thickness of the skin of the back of each animal. Subsequently the specimens were implanted subcutaneously through each of the incisions. After suturing the animals were left for 3 or 6 weeks and then euthanized by cervical dislocation after undergoing anesthesia. The embedded constructs were harvested from the subcutaneous tissue for analysis. In total, 12 animals were used, each with 2 specimens consisting of PTCC samples and Control (gel without cavities) samples inserted. For each time point, 6

animals would be euthanized and 12 specimens in 2 groups could be obtained.

2.2.10 Statistical Analysis

Biochemical data from cell-laden hydrogels constructs with cavities were compared to that obtained from cell-laden hydrogel constructs without cavities with equal incubation times using student's t-test, with 3 specimens in each group. The significant level was set as $p < 0.05$. Results are reported as mean $\pm$ standard deviation.

2.3 Results

2.3.1 EF phenomenon

As presented in **Figure 2.2**, cellular outgrowth is visible after 12 days of culture in a simple cell-laden agarose hydrogel construct *in vitro* and after further culturing till day 40, an abundant neo-tissue outgrowth is generated at the gel edge, forming a continuous scaffold-free phase that covers the entire outer surface of the construct. This phenomenon is recognized and defined as EF. The Safranin-O staining used in this typical EF-on-Edge model revealed high cell proliferation and ECM production such as GAG in this outgrowth layer (**Figure 2.2**).

Figure 2.2 Histological illustration of EF phenomenon in EF-on-Edge model. The panels from left to right respectively indicate, in plain agarose hydrogel, cell seeding on Day 3, cell outgrowth on Day 12, and neo-tissue formation around the gel (EF effect) on Day 40. In these histological indications, positive glycosaminoglycan (GAG) production is demonstrated with red Safranin-O stain.

2.3.2 Evaluation of the EF layer from EF-on-Edge model

The EF phenomenon was evaluated by phenotypic evidences based on mRNA and protein level expressions. As depicted in **Figure 2.3 A**, there is higher expression of RhoA, cellular connective proteins such as COMP and Collagen Type II, transcription factor Sox 9 and Aggrecan in the cell colonies at the surface than in the gel bulk at almost all time points investigated. In particular, highest expression of RhoA, Collagen II, Sox 9 and Aggrecan were recorded on day 18 for both surface and bulk cell colonies.

The secretion of the ECM was assessed by GAG and total collagen production as shown in **Figure 2.3 B**. There was an increasing production of both GAG and total collagen at the gel surface and gel bulk from day 10 to day 32. The production of GAG was slightly higher in the gel bulk than the gel surface, while total collagen production

was much higher in gel surface than gel bulk on day 32.

Figure 2.3 (A) Analysis of the expression of relevant cartilaginous markers at transcriptional level from porcine chondrocytes in agarose gel-surface and gel-bulk by quantitative real-time PCR (n = 3). The values represent the levels of expression relative to that of the internal control gene (GAPDH). Cells at surface (n = 3, gray); Cells in bulk (n = 3, white). The values represent the means $\pm$ standard deviations. The selected time points of analysis are respectively 3, 10, 18 and 32 days. * Differences between different constructs at the same culture time are not significant ($p > 0.05$) and ** differences between different constructs at the same culture time are significant ($p < 0.05$). (B) Graphical representations of the secretion of ECM in agarose gel-surface and gel-bulk. From left to right, the panel illustrates respectively the secretion of GAG and total collagen, as a function of culture time at 10, 18 and 32 days. The values have been normalized by the dry weight of the gel surface and gel bulk, and represent the means

± standard deviations. * Differences between different constructs at the same culture time are not significant ($p > 0.05$) and ** differences between different constructs at the same culture time are significant ($p < 0.05$).

2.3.3 Construction of acellular MCG

The MCG model was created in order to render the EF effect from the outer edge of the gel construct to the inner surface by creating cavities within the gel. The establishment of the MCG model was clearly indicated by the pre-labeled Rhodamin B fluorescence gelatin microsphere degradation as monitored both quantitatively (**Figure 2.4**) and qualitatively (**Figure 2.5**). The delayed dissolution of water soluble gelatin microspheres was achieved by the pre-crosslinked treatment of only the surface layer of the gelatin microsphere and the dissolved gelatin molecules were subsequently released through the highly permeable gel network.

Figure 2.4 Kinetics of cavity creation quantified by amount of gelatin release percentage over 6 days.

Figure 2.5 Achievement of cell-laden MCG construct: the row panels from the top to the bottom are respectively a schematic illustration and fluorescent indication of cavity creation in cell-laden hydrogel bulk. The selected time points of detection are respectively 30 minutes, 2 hours, and 2 days.

2.3.4 Construction of PTCC-MCG model

Figure 2.5-2.7 demonstrates the PTCC strategy and procedure in the MCG model. It proceeds in four steps: (i) Therapeutic cells were co-suspended and then co-encapsulated in agarose hydrogel together with gelatin microspheres that were fabricated with a diameter of 150-180 μm and lightly crosslinked in spherical surface layer as indicated in **Figure 2.6**; (ii) The hydrogel constructs co-encapsulating cells and microspheres were then incubated in a chondrocyte growth medium for a prolonged

period. During the first two days, while the cells were settling down in the gel bulk, the co-encapsulated gelatin microspheres gradually dissolved to create similar sized cavities and the MCG model was established. The whole procedure was recorded and assessed by monitoring the disappearance of pre-labeled red Rhodamine B fluorescence in gelatin microspheres both quantitatively and qualitatively as shown in **Figure 2.4** and **Figure 2.5** respectively. At the same time, the cells remained viable within the hydrogel as shown by the live/Dead staining after 2 days (**Figure 2.5**).

Figure 2.6 Schematic demonstration and optical microscopic illustration of fabrication and formation of ‘cell-and-sphere co-encapsulating gel’ construct, as the early step of MCG construction.

While reaching the third step, as the culturing continued, the EF phenomenon occurred at the gel-cavity interface at about 15 days; following by trans-phase sprouting and outgrowth of cell colonies, which gradually developed into scaffold-free neo-tissue islets and eventually filled up the cavities with densely populated cells and

endogenous ECMs that both showed *bona fide* morphologies as those in their native (tissue) counterparts. This process is shown schematically and histologically in the first and second rows of **Figure 2.7** using H&E and Masson's Trichrome staining which stained the islets blue. In the third row, the results of the Safranin-O staining are presented; and in the bottom row, multiple fluorescence microscopy (MFM) is shown, which indicated the high viability of the cells throughout the neo-tissue islet generation and the increase in total collagen production (Collagen type I was also tested and the results were negative) from the cells in the MCG construct. (iv) During further culturing, these scaffold-free islets acted as neo-tissue cores and demonstrated strong tendency to expansion and fusion with each other and also with isolated cells or colonies remaining in gel bulk. This resulted in a piece of scaffold-free 3D macroscopic tissue.

Figure 2.7 Demonstration of PTCC strategy and procedure in MCG model. The row panels from the top to the bottom are respectively schematic illustration (top row) and histological indication (second through bottom row) of PTCC procedure. In the second row, hematoxylin and eosin (H&E) stain is applied to survey the histological evolution, except for that in the ‘filling-up’ image in which Masson’s Trichrome stain is applied to reveal positive collagen production in blue; in the third row, Safranin-O stain is applied to reveal positive GAG production in red; and in the bottom row, multiple fluorescence microscopy (MFM) is applied, in which fluorescent DAPI (4',6-diamidino-2-phenylindole) stain is applied to position the cell nuclei in purple, simultaneously green fluorescent calcein stain is applied to image the ‘proliferation’ and ‘outgrowth’ processes, while immunohistochemical (IHC) stain conjugating with green fluorescent chromophore is applied to image the ‘sprouting’, ‘filling-up’ and ‘fusing’ processes with type II collagen as the primary antibody.

2.3.5 Gene expression and ECM production in PTCC-MCG model

Comparison between the PTCC-MCG model and a plain hydrogel model was made based on the mRNA and protein expression. As shown in **Figure 2.8**, the expression of RhoA, Sox 9, Aggrecan and Collagen Type II is higher in cells encapsulated in the PTCC-MCG model compared to the control group. At the protein level, GAG and total collagen production was also higher in the PTCC-MCG model.

Figure 2.8 Practicable illustration of the superiority of micro-cavitary hydrogel (MCG) employing phase transfer cell culture (PTCC) strategy ('PTCC' samples), in contrast with plain agarose hydrogel without alteration ('Control' samples) *in vitro* and *in vivo*. **(A)**. Analysis of the expression of relevant cartilaginous markers at transcriptional level from porcine chondrocytes in PTCC samples and Control samples, adopting agarose hydrogels, by quantitative real-time PCR (n = 3). The values represent the levels of expression relative to that of two internal control genes (HPRT and RPL4). Cells in PTCC samples (n = 3, gray); Cells in Control samples (n = 3, white). The values represent the means $\pm$ standard deviations. The selected time points of analysis are respectively 3, 10, 18 and 32 days. **(B)**. Investigation of the secretion of extracellular matrix (ECM) in PTCC samples and Control samples. The row panels from the top to the bottom respectively illustrate the secretion of GAG and total collagen, as a function of culture time at 3, 7, 12, 18, 24 and 32 days. * Differences between different constructs at the same culture time are not significant ($p > 0.05$) and ** differences between different constructs at the same culture time are significant ($p < 0.05$).

Further examination was done in an alginate hydrogel based PTCC-MCG model from which, by dissolving the alginate construct with sodium citrate, the neo-tissue islets in the cavities and the scattered cells remaining in gel phase were separately harvested. Their gene expression profiles are shown in **Figure 2.9**. These results again confirmed that the proliferation profile and maintenance of the chondrocytic phenotype is superior in the EF layer.

Figure 2.9 Analysis of the expression of relevant cartilaginous markers at transcriptional level from porcine chondrocytes in PTCC samples, adapting alginate hydrogels for distinguishing between aggregated cell islets and individual cells in bulk, by quantitative real-time PCR ($n = 3$). The values represent the levels of expression relative to that of two internal control genes (HPRT and RPL4). Mixed cells at the initial stage ($n = 3$, black); Aggregated Cell islets ($n = 3$, gray); Cells in gel-bulk ($n = 3$, white). The values represent the means $\pm$ standard deviations. The selected time points of analysis are respectively at 4 (initial stage), 16, 24 and 32 days. * Differences between different constructs at the same culture time are not significant ($p > 0.05$) and ** differences between different constructs at the same culture time are significant ($p < 0.05$).

2.3.6 Fate of agarose in PTCC-MCG system

With regards to the dissolution of the hydrogel scaffolding material, the results in **Figure 2.3 D** show the kinetics of agarose degradation till 32 days, in addition to the percentage of agarose as remaining. The clearance of the polysaccharides exhibited a linear molecular release profile.

Figure 2.10 Graph of kinetics of agarose degradation in PTCC and Control sample quantified with relative agarose remaining percentage till 32 days. The curves are marked as cyan and magenta color respectively for PTCC and Control samples.

2.3.7 *In vivo* evaluation of PTCC-MCG model

The subcutaneously implanted MCG constructs in nude mice were harvested and evaluated after 3 and 6 weeks of implantation. Immunostaining indicated that there is a higher secretion of both GAG and Collagen type II throughout the MCG construct, which gives rise to a continuous phase of ECM among the cells (**Figure 2.11**). This is compared to the plain hydrogel control. Collagen Type I was also tested and the results were in both constructs negative.

Figure 2.11 Histological evaluation of PTCC samples cultured *in vivo* for 6 weeks in contrast with Control samples and native cartilage (left, center and right column respectively), both PTCC and Control samples adopting agarose hydrogel. In the first row, Safranin-O stain is applied to reveal positive GAG production in red; in the second row, immunohistochemical (IHC) stain conjugating with green fluorescent chromophore is applied to image the samples with primary antibody of type II collagen.

2.4 Discussion

2.4.1 EF phenomenon

After a long term culture of cell-laden hydrogel constructs *in vitro*, in contrast with the modest cell growth appearing in gel bulk, there was an abundant neo-tissue outgrowth along the gel edge, showing microscopic flourish of both cell proliferation and ECM production. This phenomenon has been observed in various individual systems that are all made of hybridizations between non-(typical) anchorage dependent cells and non-cell-adhesive hydrogels. This setting implies the preconditions for the occurrence

of the EF phenomenon: (i) the 3D cell-laden microenvironment in hydrogel bulk lacks focal adhesive functionality for cell attachment; and (ii) being entrapped and confined in the framework-lattice of a hydrogel scaffold where there is a lack of focal adhesive settlement, the cells are still capable of maintaining high viability and proliferative tendency. Among these two situations, phenomenon (i) is characteristic for non-cell-adhesive hydrogels including photo-crosslinked polyethylene glycol (PEG) based gels and neutrally or negatively charged thermo-cure polysaccharide gels such as agarose, gellan gum, and alginate; phenomenon (ii) generally is a characteristic of non-(typical) anchorage dependent cells such as chondrocytes and numerous cell types among endodermal origin. As a consequence, then non-anchorage depended cells are encapsulated in a hydrogel system, cell colonies located deep in the gel bulk will experience limited growth due to the physical constraint by the scaffold framework, while those located adjacent to the edge of the gel construct will continuously expand toward the gel surface and ultimately sprout out to form the EF layer on the interface as indicated in **Figure 2.2**.

To further investigate the properties of the cells in the EF layer, chondrocytic markers and ECM protein expressions in such as cell/hydrogel system were tested. The observed greater expression of RhoA at surface layer reflects a higher proliferative profile of the local cell population that ultimately constitutes the EF. In addition, the maintenance of chondrocytic phenotype was found to be better in the cells residing in

surface layer based on superior gene expression and proteinic yields of the cartilage markers including chondrogenic transcription factor (Sox9), cartilaginous collagen (type II) and proteoglycan (aggrecan/GAG).

2.4.2 Set up of PTCC-MCG system

The results and performances in the EF-on-Edge modeling system did inspire the design of the PTCC system to generate multiple interfaces within the gel bulk via the MCG model, which renders the EF effects from the outer edge of the gel construct to the inner surface of the cavities inside the gel. As a consequence, the EF effect can also occur inside the hydrogel construct, translating it to a practical methodology for engineering applications. It also converts the role of hydrogel from a non-cell-adhesive substrate to a productive nursery for cells to grow (**Figure 2.6**). It is important that the ratio of the weight of microspheres (porogens) to the weight of hydrogel does not exceed 0.3: 1 as too many microcavities within the hydrogel would compromise the mechanical properties of the construct and result in difficulties during handling. Also, the size of the microcavities should be within a diameter of 100-500 microns. Gel constructs with cavities smaller than 100 μm cannot be sufficiently distinguished from common gel systems since they are also porous in nature. Since the EF layer is typically 6-8 cell layers' thick, which is approximately 200-250 μm , the cavity size

should not be larger than twice of the EF layer thickness (500 μm) because such large cavities cannot be fully filled up by outgrowing cell colonies.

The results in **Figure 2.5-2.7** have proven the feasibility of the PTCC-MCG system. The two days' delayed dissolution of water soluble gelatin microspheres was obtained by the pre-crosslinking treatment in the surface layer of the microspheres. The dissolved gelatin molecules were released through the well permeable gel network which created micro-sized cavities within the hydrogel construct. The outgrowth from the edges created within the hydrogel will later develop into scaffold-free neo-tissue islets when all the cavities are filled with densely populated cells and their endogenous ECMs. Further culturing of the MCG construct revealed the strong tendency of expansion from the islets, which led to fusion of these islets with each other or with scattered cells, giving rise to a maximally scaffold-free 3D macroscopic tissue that showed *bona fide* morphologies similar to native cartilage (**Figure 2.7**). The chondrocytic markers used in the EF evaluation were tested on the cells in our PTCC-MCG system and it was confirmed that this system is indeed superior over the plain hydrogel model. Phenotypic markers are preserved and also there are neo-tissue islets formation as indicated in comparison between islets and scattered cells (**Figure 2.8-2.9**). All these results demonstrate the feasibility of our system to fabrication, maintain and even enhance the viability and phenotype of the used chondrocytes.

It has also to be noticed that the gelatin microspheres as used in this PTCC

strategy are in the range of a few hundreds of microns, and therefore the injectability of our PTCC-MCG system is maintained. It was found that injecting the PTCC construct is the same as injecting a plain hydrogel during routine experimental operation. The clearance of the 3D network of the agarose scaffolding material, due to the physical crosslinking in this gel, will gradually disentangle under the growing pressure from the expanding neo-tissue islets and ends with a removal via linear molecular release. This, at a certain level, addresses the requirement for degradation of the scaffold as used in the field of tissue engineering and regenerative medicine and eventually leaves a nearly scaffold-free neo-tissue.

2.4.3 Mechanisms of PTCC in MCG construct

As the design of PTCC-MCG system is an adoption of EF phenomenon, mechanistic studies on the initiation of EF phenomena such as medium diffusion, gelatin presence and interfacing mechanics were carried out with the EF-on-Edge.

The variable of medium diffusion in hydrogel is always a vital factor for all types of cell culture. It is excluded from being a dominant reason for initiation of EF effect, though it remains a substantial factor responsible for the development of EF outgrowth because of the consistent outcomes yielded from both the EF-on-Edge model and PTCC-MCG system. In the EF-on-Edge model, EF arises at direct exposure to ambient medium; while in PTCC-MCG system, PTCC proceeds inside the gel body which is

barely exposed to the penetrated medium in cavity. The comparable results (**Figure 2.2 and Figure 2.7**) in these contrasting situations prove that medium diffusion has no significant role in the initiation of the EF effect and PTCC phenomenon. In fact, a consensus exists long among hydrogel users: as long as the construct is made small enough the generally well permeable hydrogels do not hinder molecular leveled transportation therein [232]. This is further confirmed by an additional experiment. When the serum concentration is varied in the formulation of the culture medium, both the EF-on-Edge and the PTCC in MCG can still occur, but only somewhat sooner or slower.

The potential contribution of soluble gelatin to the initiation of PTCC in MCG is regarded as negligible. This is based on two facts: first, in the EF-on-Edge model, the EF phenomenon occurs without any involvement of gelatin (**Figure 2.2**); second, in the PTCC-MCG system, the dissolution and clearance of the gelatin component is accomplished within 2~7 days (**Figure 2.4**), which is much earlier than the initiation of the trans-phase cell growth on day 15 (**Figure 2.7**). Furthermore, the morphological evaluation of the outgrowing cell colonies showed that the cells did not spread. Although chondrocytes are non-typical anchorage dependent cells, they still show a preference for focal adhesion formation if the conditions allow. If the gelatin had played a critical role in the initiation of the trans-phase growth of cells residing adjacent to gel-cavity interface, it would have induced focal adhesion formation in

these cell populations since it is a potent cell adhesive protein [233-234]. However, experimental outcomes did not indicate any commitment of focal adhesion in chondrocytes which could cause spreading cellular morphology (as shown in **Figure 2.2** and **Figure 2.7**). Putting all these evidences together, gelatin microspheres only played a role as porogen for the establishment of the MCG construct. Any conclusion of cell growth by gelatin is minimal.

The nature of the EF phenomenon is attributed to a biomechanical response between the hydrogel scaffold and the cell colonies inside. An asymmetric compression exerted by hydrogel frame orientates the growing direction of the cell colonies located at the surface of hydrogel towards the outside. When scattered cells are seeded in the lattice of the hydrogel framework, the polymeric backbone of the gel frame is flexible enough to allow cells to proliferate into colonies. The growth continues until the colony reaches a certain critical size that is confined by maximal flexibility of the employed materials. Along with the expansion of cell colonies, the resisting pressure by gel framework increases accordingly. If the cell colonies are encapsulated deep in the gel bulk, they will receive symmetric compressions from the surroundings. When the pressure increases and manages to balance the expanding force of cell division, further cell proliferation is mechanically prohibited. On the other hand, cell colonies at the surface layer of a hydrogel experience an asymmetric compression from the surrounding hydrogel as the local compressive modulus sharply decreases with the

reducing material density from the bulk side toward the interface. The cell colonies residing there will receive lower pressure from the interface side and it is not sufficient to counteract the expanding force of cell division. Driven by this outward mechano-orientation, the outgrowth of cell colonies from the surface layer of hydrogel is then initiated. Therefore, cell colonies may appear in spherical or spindle shape depending on their local mechano-homogeneity as shown in **Figure 2.12**. In agarose gel based working conditions as are particularly used in the current model study, a ‘critical thickness’ of the surface layer for EF occurrence is observed, i.e. a depth varying between one to two hundred microns under the interface. Below this depth is the gel ‘bulk’, while it is free of EF-on-Edge. This finding suggests that in the PTCC-MCG system, by manipulating the inter-cavitary distance below the ‘critical thickness’, a completely ‘bulk-free’ construct can be produced in which all-pervasive EF effects will enable a full-scale histogenesis followed by comprehensive neo-tissue occupation (proven both *in vitro* and *in vivo* as indicated in **Figure 2.5-2.7** and **Figure 2.10-2.11**).

Figure 2.12 Optical microscopic illustration of cell interaction in EF-on-Edge model. The column panels from left to right respectively indicate, in plain agarose hydrogel, cells forming spherical colonies, and the subsequent different fate of the colonies located in either the favouring interface (in the upper row) or the obstructive bulk (in the lower row).

Based on the promising results of the PTCC-MCG construct compared to normal hydrogel scaffold, it is essential to intensively study the mechanism of PTCC. Because PTCC strategy was regarded with the same principle as EF phenomenon, it is reliable and practicable to use the EF-on-Edge model in order to conduct an in-depth investigation of PTCC mechanism.

2.5 Conclusion

In conclusion, inspired by the coincidentally observed EF phenomenon during routine work with cell-laden hydrogels, an innovative 3D cell cultural strategy PTCC is designed and practiced within a jointly invented MCG system, mediated by an interfacing mechano-responsive nature of the processing, and also translational trials are accomplished both *in vitro* and *in vivo*. By this invention, new possibilities are created to engineer histogenesis in a hydrogel based scaffolding system for non-anchorage dependent cells.

Chapter 3 Probing cellular mechanobiology of EF phenomenon in three-dimensional culture with agarose matrix

3.1 Introduction

In Chapter 2, PTCC strategy has already been successfully realized and its promising positive effects *in vitro* and *in vivo* (as seen in **Figure 2.5-2.7** and **Figure 2.10-2.11**) have also been shown. We have also attempted to understand the mechanism of the EF phenomenon from where the PTCC strategy was derived. EF can be regarded as similar to PTCC as detailed in Chapter 2. Hence the investigation of the mechanism of EF can simplify the exploration of the much more complicate phenomenon of PTCC and the resulted explanation will hold for both EF and PTCC. In Chapter 2, the results was provided of the gene expression and ECM secretion from gel edge and gel bulk, which revealed that cells grew better at the edge of the gel than those confined in the gel bulk, although optical microphotographs showed a different cell colony shape in different location of the hydrogels.

In this chapter, we will report an in-depth investigation of the EF mechanism using biomechanical analysis, in pursuit of proving the driven force for the PTCC/EF phenomenon. The EF phenomenon will be studied quantitatively and systematically. Several models will be incorporated to analyze the EF phenomenon of chondrocytes under the confinement of agarose hydrogel to evaluate the mechanobiological behavior

of chondrocytes embedded in the 3D agarose hydrogel. For consistency with our previous study as detailed in Chapter 2, agarose was used as the hydrogel material.

3.2 Materials and methods

3.2.1 Chondrocytes isolation and culture

Chondrocytes isolation and culture for use in this study were the same as described in Chapter 2 (see Section 2.2.1, Page 32).

3.2.2 Preparation of agarose hydrogels

To create the EF model, chondrocytes were suspended at a certain density in gel precursor solution (in PBS, agarose 0.02 g/mL, cell density 1×10^6 cells/mL) at 37 °C and injected into various molds for different subsequent biomechanical measurements. After 5 minutes, the cell/gel construct solidified at room temperature and can be incubated in the cell culture incubator (5% CO₂, 37 °C) for a predetermined number of days.

3.2.3 Characterization of the mechanical properties of agarose hydrogel

Atomic force microscopy (AFM, Asylum Research, model MFP-3D) was used to measure the micro scale linear elastic moduli with water immersion capability of a flat, homogeneous, agarose substratum with a thickness of 700 μm under swollen condition.

A contact mode with a spring constant of 34.59 nN/m was applied via the silicon nitride cantilevers (Sharpened Microlevers, Crest Technologies). Both the velocity of the cantilever and indentation depth were well controlled during the test. In addition, the unloaded cantilevers were calibrated before the test by measuring the thermally induced motion for gels under water immersion. **Figure 3.1** shows the schematic details of AFM indentation and the *force-indentation* relation in microscopic mechanical characterization, from the following *indentation-deflection* relation can be deduced [235]

$$z - z_0 = d - d_0 + \sqrt{\frac{k(d - d_0)}{(2/\pi)[E/(1-\nu^2)] \tan(\alpha)}} \quad \text{Equation 3.1}$$

where $k = \frac{3E_{\text{SiN}} I}{L^3}$ is the stiffness of the silicon nitride cantilever.

This equation is used to determine the linear elasticity of substratum (E) by fitting the *indentation-deflection* curves from the AFM measurement. For a small deflection of the cantilever, the Hertzian contact can be assumed and then the relation between the applied force F and indentation on an elastic medium is

$$F = \frac{2}{\pi} \frac{E \delta^2}{(1-\nu^2)} \tan \alpha \quad \text{Equation 3.2}$$

where δ is the indentation depth, E is elastic modulus, ν is Poisson's ratio of polymer, and α is the half-cone angle of cantilever tip.

Figure 3.1 A schematic representation of AFM indentation and force-deflection relation

3.2.4 Microscopy and measurement of the normal distribution of chondrocytes

The microscopy assay is based on a laser scanning confocal microscope (Pascal 5, Carl Zeiss, Germany), which is integrated with an online CO₂ incubator (Carl Zeiss, Germany). The illumination source is an argon ion laser with a maximum power of 1 mW and excitation wavelength of 488 nm. A 10× objective (NA: 0.3) was used in this study.

Figure 3.2 Schematic of the method used for the characterization of chondrocyte distribution along z direction in agarose hydrogel.

The encapsulated chondrocytes (1×10^6 cells/mL) in agarose hydrogel (2% w/v in PBS) were cultured for 19 days at 37 °C and 5% CO₂ atmosphere. The medium was refreshed every day. In order to monitor the normal distribution of chondrocytes, the sample was transferred every alternating day to an online CO₂ incubator attached on the microscope stage at 37 °C with 5% CO₂ atmosphere. A stack of phase contrast images ($n = 7, i = 100 \mu\text{m}$) of a selected region of the sample was made up to 19 days of incubation as illustrated in **Fig. 3.2**. The bottom focal plane is 100 μm , which is closest to the rigid coverslip and the top focal plane is 700 μm which is close to the surface of hydrogel. At each time point, 9 stacks of images from at least three identical samples were captured from 3 distinguished regions. The cell counting was done by using MetaMorph software (Molecular Devices, Inc. version 7.1.7.0. Sunnyvale, California, United States).

3.2.5 3D multiple-particle tracking

To quantify the local 3D deformation of agarose hydrogel induced by the encapsulated chondrocytes, confocal images of the 0.2 μm fluorescent latex microbeads (fluorescein isothiocyanate (FITC)-labeled, Invitrogen)) embedded in the hydrogel were captured using a laser scanning confocal microscope (Pascal 5, Carl Zeiss, Germany) equipped with an argon laser with an excitation wavelength of 488 nm and maximum power of 25 mW as illumination source, and a 50x objective (NA. 0.5) lens. Microbeads that were embedded just above the target chondrocytes were first identified using the computer-controlled microscope stage. A series of 150 images with the an identical XY position were then captured with a resolution of 2048 by 2048 pixels over the range of 30 μm along z axis with an interval of 0.2 μm as the calibration set. The in-focus microbead image ($z = 0$) is a spot with a sharp and narrow intensity profile across the diameter of the image as shown in **Figure 3.3**. When the microscope stage is moved away from the in-focus plane, the pattern of the defocus microbead image deforms according to the level of z displacement. At first, the width of the microbead image increases with the peak of the intensity profile decreases, and then a series of ring intensity patterns are formed as the distance of the object from the in-focus plane increases. This assay relies on the z position related intensity pattern of a microbead to determine the position of the bead as caused by the movement of a chondrocyte. A

series of images of microbeads were analyzed using the MetaMorph software to extract the grey level across the diameter of the image of the microbead as intensity profile as shown in **Figure 3.3**. A set of images of a microbead was taken with the total span of 20 μm and an interval of 1 μm during a certain time and then the relative movement of the microbeads in the z direction can be obtained by comparing the individual intensity profiles with the set of calibration profiles using a square error minimization method. This method allows the determination of the current relative displacement of the bead in the z direction by identifying the minimum value of error among the 150 intensity profiles from the calibration set. The tangential displacements of microbeads in agarose hydrogel in x and y axes were obtained by monitoring the intensity-weighted center of mass of the in-focus microbead images using the track-object algorithm provided by MetaMorph.

Figure 3.3 Assay to quantify the local 3D deformation of agarose hydrogel caused by chondrocytes. The calibration images set taken from the microbead of interest in the

hydrogel. Indicated z positions are the relative position of computer-controlled micro stage to the in-focus focal plane. The measurement of z displacement is achieved by analyzing the pattern of microbeads grey image.

3.2.6 Preparation of polyacrylamide-agarose hydrogel

Chondrocytes extracted from cartilage tissues of porcine articular cartilage were suspended at high density in gel solution (in PBS, agarose 0.02 g/mL, cell density 1×10^6 /ml) at 37°C and injected into aminosaline functionalized coverslips ($\phi = 30$ mm) which readily cross-link with agarose hydrogel. In brief, coverslips were first cleaned in Piranha (7:3 v/v H₂SO₄: H₂O₂) at 80°C for 2 hours and washed in distilled water. After drying under a stream of N₂ gas for 15 minutes, the coverslips were immersed in 95% water containing 1% 3-aminopropyltriethoxysilane for 10 minutes and washed three times with acetone, alcohol, and distilled water. The functionalized coverslips were used immediately after drying under 120 °C for 45 minutes [236].

To determine the surface tension of the cell-laden agarose hydrogel as caused by ‘edge flourish’ phenomenon, a thin layer of polyacrylamide gel (PAG) embedded with 0.2 μ m fluorescent latex microbeads (fluorescein isothiocyanate (FITC)-labeled, Invitrogen) was added to the top of the solidified agarose hydrogel to serve as the markers for the measurement of surface tension as shown in **Figure 3.4**. The PAG was prepared by adding monomer acrylamide (40% w/v, Sigma) 8% v/v and cross-linker N,N’-Methylene bis-acrylamide (BIS, 2% w/v, Sigma) 0.1% v/v in distilled water. Fluorescent microbeads at a 1/1000 volume concentration were added to the solution to

serve as the markers to quantify the gel surface deformation. The polymerization of PAG was initiated and the speed was controlled by adding 1/1000 of N,N,N',N'-Tetramethylethylenediamine (TEMED, Sigma) and 1/100 of ammonium persulfate (10% w/v, Sigma) in volume concentration. The total duration for full polymerization has to be 30 minutes to allow the microbeads to be fully precipitated down to the surface of agarose hydrogel located underneath the PAG sheet [237].

Figure 3.4 Schematic illustration of polyacrylamide-agarose hydrogel for the characterization of surface tension caused by 'edge flourish' phenomenon.

To prepare a thin sheet of PAG, a 12 μL of mixture was dropped onto the solidified agarose hydrogel and immediately overlaid with a piece of inert plastic sheet throughout the polymerization process. This is to control the degree of spreading and thickness of PAG sheet on the surface of cell-laden agarose hydrogel. The actual thickness of the swollen gel was measured by focusing the agarose hydrogel surface up to the PAG surface with the microscope. The thickness of gel under swollen condition is estimated to be 6.5 μm . For all experiments, the thickness of PAG is around 6.5 μm

and the thickness of the cell-laden agarose hydrogel is around 500 μm .

3.2.7 Live cell microscopy assay for surface tension characterization

Live cell microscopy was performed using an inverted light microscope (Olympus IX71) equipped with a LUCPlanFLN 40x /0.60 Ph2 objective (Olympus) and a motorized stage (BioPoint 2, Ludl Electronic Products). PAG overlaid cell-laden agarose hydrogel on functionalized coverslip was mounted onto a microscope perfusion chamber where the desirable environmental parameters were controlled. Throughout the experiment, the temperature of the chamber was maintained at 37°C using a Tempcontrol 37-2 Digital and Heating Unit (Leica). 5% Carbon dioxide and the humidity of the microscope stage were controlled by a humidifier system (CTI-Controller 3700, Leica). In order to assess the displacement of microbeads caused by the EF phenomenon, photomicrographs were taken of the cells both with phase-contrast optics to visualize the cell morphology and location as well as with fluorescein illumination at 490 nm to excite the fluorescent microbeads with the emission at 515 nm at different time points.

A program known as particle image velocimetry (PIV) was coded in MATLAB to quantify the displacement of microbeads caused by EF phenomenon. PIV calculates the average movement of many nearby particles inside a small window directly from a *pair* of digital images taken at two contiguous moments [238].

A linear stress-strain relationship was implemented to describe the PAG mechanical behavior in the computational analysis on performing to quantify the surface stresses generated by the EF phenomenon using SEM analysis. The mechanical response of PAG to the external forces was computed using the following stress-strain relationship:

$$\varepsilon = [D]^{-1}\sigma$$

Where ε is elastic strain vector, $[D]^{-1}$ is the flexibility or compliance matrix of the material, and σ is the stress vector.

3.2.8 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 Agarose hydrogel elasticity and structure

AFM mechanical characterizations were carried out to determine the elastic modulus of agarose hydrogel. The elastic modulus served as the boundary condition in the FEM analysis of stresses from the microbead displacements caused by the chondrocytic EF phenomenon. AFM measurements on a flat, homogeneous agarose substratum under

swollen conditions are shown in **Figure 3.5** where a full cycle of a loading and unloading process is presented by the indentation-deflection ($z - z_o$ vs. $d - d_o$) curve.

Figure 3.5 Experimental measure of AFM indentation curve for agarose substratum using contact mode. The red and blue curves represent the deflection of AFM cantilever during loading and unloading process respectively.

Due to the high content of water, agarose hydrogel is almost incompressible and thus its Poisson's ratio is close to 0.5. The initial deflection and height of cantilever, d_o and z_o can be identified directly from the experimental curves. Therefore, the slope of any two points on the curve can be used to calculate the value of E from **Equation 3.1**. However, the choice of points is too arbitrary in order to yield an

accurate estimate. Therefore, an optimized approach is employed here. By iteratively specifying E and using **Equation 3.1** to calculate z at every d from the measured data, the average difference (L_2 norm) between $z^{experimental}$ and $z^{calculated}$ along the loading force curve can be minimized when an optimal elastic modulus is found. This approach ensures that every single point along the experimental loading force curve is taken into consideration in the calculation of the E value. In **Figure 3.6**, an optimal Young's modulus corresponding with the minimum value of error along the curve can be found for each of these tests to minimize the differences between **Equation 3.1** and experiments. The minimum average difference (L_2 norm) between $z^{experimental}$ and $z^{calculated}$ among five individual measurements taken at different locations on an agarose substratum was found around $E = 36.13 \pm 4.05$ kPa.

Figure 3.6 Errors (L_2 norm value) between the calculated and experimental measures of AFM indentation curves for agarose substratum using contact mode.

3.3.2 Chondrocyte normal distribution

The biophysical analysis of chondrocyte activity in agarose hydrogel was first analyzed by characterizing the distribution of chondrocytes over a long period of time. Phase contrast images of chondrocytes from different focal planes were captured using laser as the light source which has a narrower depth of view than the conventional light source and allows to distinguishing the individual focal plane at an interval of 100 μm . The number of cells was counted at a certain focal plane layer over an area of 0.849 mm^2 at a distance of 100 μm from bottom, until 700 μm to the surface of the hydrogel.

The density of cells increased constantly over time with an approximate growth rate of 2.3×10^4 cells per day as shown in **Figure 3.7 A**. However, the distribution of growth (**Figure 3.7 B**) varied among the normal position of focal plane even though hydrogel is supposed to be through and through homogeneous. For the focal plane closest to the bottom (focal plane 100 μm , **Figure 3.7 B**, deep blue), the number of cells decreased over time due to the adhesion of chondrocytes to the surface of the coverslip. Cells attached to the coverslip did not exhibit the phenotype of chondrocytes; therefore none of these cells were counted in a focal plane of 100 μm even though a decrease in cell spreading can be observed over time. The cell number from focal plane 200 μm to 500 μm remained consistent over a long period of time, even though a slight deviation in cell number can be observed; still it was insignificant compared with the

overall cell growth. This indicates that the cell proliferation in these focal planes was relatively inactive compared with the overall growth of cell density in agarose hydrogel, we defined this regions as the bulk region. The region between the surface till 100 μm down is defined as the edge of the hydrogel. Initially, no or a limited number of cells were counted before day 5 of incubation at the focal plane of 700 μm , which is just underneath the surface of hydrogel. Thereafter, the cell number was then increased significantly over time and this focal plane has the highest growth rate of cell number throughout the hydrogel. Focal plane 600 μm had the maximum number of cell count over all focal planes after day 7. In addition, the number of cell count increased linearly over time in this focal plane.

Figure 3.7 The growth and distribution of chondrocytes in 3D agarose hydrogel. **(A).** The overall growth of chondrocyte was quantified by the cell density (per volume) as a function of time. The growth rate is $45.895 \text{ cells} \times \text{mm}^{-3}/\text{day}$. **(B).** Quantification of cell distribution among different focal planes as a function of time.

The spatiotemporal evolution of the cell population in normal xy direction revealed a significant increase of cell number on the edge compared with the bulk region of the hydrogel. This observation indicates that the growth patterns varied across the normal position in the hydrogel. The inhomogeneous trend of cell distribution however can be caused by the migration of cells or directed cell growth. Therefore, further characterization of the cell mechanical response is needed to justify the mechanism of such a trend.

3.3.3 Measurement of local 3D hydrogel deformation by 3D multiple-particle tracking

Matrix deformation probing has been done to understand the mechanism of cellular activities such as adhesion, migration, contractility, and matrix remodeling. The matrix deformation is probed by direct and indirect approaches, where the direct approaches are to monitor the 3D projection of the matrix movement by using phase contrast microscopy (e.g. differential interference contrast, DIC), or fluorescent microscopy. The indirect approaches utilize the embedded microbeads as tracers, for tracking the movement of matrix by using confocal microscopy [239-242]. 3D single-particle tracking schemes are widely used in the study of single molecule application such as the intracellular myosin movement along the actin filament. These 3D single-particle tracking schemes probe the displacement by analyzing the size and the diffraction

patterns of the out-of-focus microbeads.

In this assay, we developed an assay to probe the 3D trajectories of the multiple microbeads embedded near to the encapsulated chondrocytes. This assay allows us to quantify and understand the 3D movement of the chondrocytes in real time. The deformation of agarose gel caused by the encapsulated chondrocytes can be observed and measured via the displacement of fluorescent microbeads. The 3D trajectories of microbeads over time were then used to represent the activities of chondrocytes in a spatiotemporal approach. We also took advantage of our assay which allows us to observe the matrix deformation and the time-dependent cell morphology changes in real time and to investigate the mechanism or the migration mode involved in matrix deformation. This is the optimal assay that allows us to investigate the 3D cellular mechano-transduction which is more closely related to the *in vivo* condition.

We first collected a set of microbead images with a specific pattern of grey level in accordance to the distance between the in-focus plane ($z = 0$) and the position of the computer-controlled micro stage. The total set of 150 images spanned across $\pm 15 \mu\text{m}$ with $0.5 \mu\text{m}$ interval in z direction. This set of images was defined as calibration set and the respective grey level patterns were used as the references for the measurement of relative z displacement. The precision of our assay in the z direction was found to be $0.589 \mu\text{m}$. Spontaneous displacement of microbeads in agarose hydrogel without the presence of chondrocytes was monitored and the 3D trajectories of the microbeads

were measured using the assay. The passive displacement of microbeads in 2% w/v agarose hydrogel was restricted to minimally 1 μm in all three directions up to 48 hours. This indicates that the newly developed 3D particle tracking assay allowed us to measure the local 3D deformation of agarose hydrogel caused by the activities of encapsulated chondrocytes in real time up to submicrometer precision. In addition, the cell morphological changes can be monitored simultaneously in order to study the mode of migration.

3.3.4 Patterns of 3D hydrogel deformation caused by encapsulated chondrocytes

A cell migrating through a 3D agarose hydrogel matrix deforms it locally in all three directions. To monitor matrix deformation, 0.2 μm diameter polystyrene microbeads were embedded before the cells were encapsulated in the matrix. The 3D movements of the microbeads in the solidified matrix were tracked using a single-particle tracking method based on confocal microscopy to monitor the local 3D deformations of the matrix near to the cell. The time-dependent coordinates x , y , and z of each microbead in the hydrogel was monitored in real time after the initial calibration along the z axis for each individual microbead. The displacement in x and y directions was obtained by tracking the intensity-weighted center of mass of the in-focus microbead images using the track-object algorithm provided by MetaMorph. The relative positions of the microbeads in z direction were obtained by analyzing the diffraction pattern of

microbeads with a 0.589 μm resolution.

Using the developed 3D particle tracking assay, we monitored the time-dependent coordinates x, y, and z of each bead in the matrix in real time. The 3D trajectories of microbeads revealed the local deformation of hydrogel caused by the movement of chondrocytes were insignificant compared with the overall movement of cells, as shown in **Figure 3.8**.

Figure 3.8 Local 3D deformation of agarose matrix around chondrocytes. **(A)**. Phase contrast image of chondrocytes overlaid with the fluorescent microbeads. Microbead B and C located 3.02 μm and 4.91 μm from centre respectively, microbead D located 7.92 μm away from the edge of chondrocytes. The arrows represent the direction of tangential displacements of each microbeads. Scale bar, 20 μm . **(B-D)**. 3D trajectories of microbeads located at three different locations with respect to chondrocytes.

The microbeads located just above chondrocytes showed significant displacement in three axes compared with the microbeads at some distance away from cells as well as the negative controls. The microbead trajectories just above the chondrocytes revealed the presence of outward stresses from chondrocytes towards the hydrogel surface. The local deformation of hydrogel on the surface of a chondrocyte showed overall positive displacement up to 20 μm towards the surface within 48 hours tracking duration. Furthermore, the tangential displacements and locations of microbeads also showed strong evidence for outward stresses generated by chondrocytes beneath the microbeads.

The high resolution time lapse imaging of chondrocytes in agarose hydrogel allowed the characterization of the mode of cell motility that caused local deformation of hydrogel. The morphology of the chondrocytes remained spherical throughout the 48 hours observation as shown in **Figure 3.9**. This indicated that chondrocytes did not migrate via the mesenchymal mode which establishes cell polarity and proteolytic degradation of the local matrix [243]. This observation is supported by the lack of ECM proteins that work as adhesion sites for the chondrocytes to generate traction force for cell polarity formation. Studies have shown that the lack of ECM proteins in

3D hydrogel may convert the mesenchymal motility mode to amoeboid motility mode where the cells remain rounded and migrate in the 3D hydrogel by contractility-dependent squeezing through the pre-existent pores in the matrix [244]. Although some spikes were formed around the boundary of chondrocytes, yet no significant pseudopods were observed in 48 hours duration. These findings suggest that the local deformation of hydrogel around chondrocytes is not originating from the migration of chondrocytes. Furthermore, pure agarose hydrogel was found to be insufficient in supporting cell migration in the absence of ECM proteins as anchorage sites in any concentration. Given the upwards deformation of hydrogel around the chondrocytes and the rounded morphology of chondrocytes, it is plausible to conclude that the encapsulated chondrocytes exhibited oriented outgrowth towards the surface of the hydrogel and causing the upwards stresses on the surface of cellular clusters.

Figure 3.9 The morphology of the chondrocytes encapsulated in agarose hydrogel. Scale bar, 10 μm .

3.3.5 Displacement field of agarose hydrogel surface under ‘edge flourish’ phenomenon

The details of interaction of encapsulated chondrocytes in agarose hydrogel with PAG surface is shown in **Figure 3.4** where the bulk deformation of PAG occurred by the protrusion of chondrocytes via the ‘edge flourish’ phenomenon. The embedded fluorescent latex microbeads located just above the agarose hydrogel surface are used as markers to track the PAG deformation caused by chondrocytes. The measurement of displacement field of the microbeads is obtained from a pair of images taken from different time points. The quantitative numbers of the displacement field are obtained by the particle image velocimetry (PIV), where a cross-correlation function is introduced to derive the local motion of beads statistically.

A representative chondrocyte cluster located approximately 7 μm underneath the layer of fluorescent microbeads was chosen and captured as phase contrast image as shown in **Figure 3.10 A**. Prior to quantify the beads’ displacement field using PIV, the systematic drift error that caused by the misalignment from the microscope stage movement was corrected by overlaying the pair of fluorescent images taken from different time points. Pseudo-colors were applied to the fluorescent microbeads which appear *green* captured at an earlier time point, and *red* for later time point. When two images were overlaid, microbeads with zero displacement throughout the observation

are shown as *yellow* dots whereas a pair of nearby red and green dots indicates that the embedded bead is displaced by a distance between the two dots as shown in Fig. 10B. For reader's reference, the cell boundary was also marked by the white dash lines traced from **Figure 3.10 A**.

Figure 3.10 Tangential deformation of PAG surface caused by ‘edge flourish’ phenomenon on the XY plane. **(A)**. A phase-contrast image of chondrocytes cluster, encapsulated in the 2% w/v agarose hydrogel covered with a thin layer of PAG embedded with a layer of fluorescent microbeads close to agarose surface. **(B)**. Overlaid fluorescent images of the $0.2\mu\text{m}$ microbeads embedded at the bottom of PAG. The fluorescent image is taken at the same field of view as in A. **(C)**. Bead

displacement (pixel) vector field computed from two fluorescent images taken from two different time points ($t = 44$ hours). The missing and strayed vectors are interpolated and shown in red arrows. **(D)**. Tangential (x - y) displacement field U_{xy} induced by ‘edge flourish’ phenomenon from PIV calculations and specified for FEM analysis. The vector plot is presented for a comparison with Figure 10C. Dotted lines are the boundary of chondrocytes cluster. Scale bar, 20 μm .

PIV is implemented through the method of digital image correlation to quantitatively measure displacement of the microbeads at different time points. A free program MatPIV distributed under the GNU general public license was adopted for our study. This program has functions to remove the spurious vectors and interpolate missing vectors by Kriging method. Both vectors are due to the background noises or defocus of images. The image for displacement field computed by PIV is shown in **Figure 3.10 C** by arrows for the pair of fluorescence images in **Figure 3.10 B**, which is ready for force simulation as the input data.

3.3.6 Finite element method analysis of surface tension under ‘edge flourish’ phenomenon

The thin layer of PAG sheet with a layer of embedded microbeads on the bottom part serves as the markers to determine the surface deformation of the agarose hydrogel caused by the ‘edge flourish’ phenomenon. The thin layer itself does not contribute much to the surface mechanical properties of agarose hydrogel because the thickness is in micro-scale (6.5 μm) and has similar compliance as agarose hydrogel. Ergo, the displacement of microbeads from the bottom of PAG thin sheet can be used as the

direct indicators of agarose hydrogel surface deformation.

The deformation and stress analysis of PAG sheet is implemented by the FEM software ANSYS® 11. Starting with the geometry of agarose hydrogel with a dimension of $446\ \mu\text{m} \times 328\ \mu\text{m} \times 400\ \mu\text{m}$, the whole domain (agarose hydrogel) is discretized by isoparametric, 20-node brick elements. The boundary conditions are specified displacements from PIV results, as shown in **Figure 3.10 C**, at corresponding nodes on the surface of agarose hydrogel, whereas the nodes on the bottom of substrate are fixed because agarose hydrogel is immobilized on the coverslip. These boundary conditions represent a stratified media where a layer of soft homogeneous agarose hydrogel rigidly bonded to the infinitely stiff coverslip. The specified displacement field on the xy plane is shown in **Figure 3.10 D**, where the vector plot is presented in comparison with the PIV calculated bead displacement field as shown in **Figure 3.10 C**. The vector plot is particularly illustrative for the identicalness of xy displacements between the two fluorescent images taken from the time interval of 44 hours. In other words, we successfully demonstrate that the planar deformation of gel which is quantitatively characterized by the PIV image processing of microbeads' movement can be directly used as input (boundary conditions) for subsequent FEM analysis.

Using finite element method, the mechanical responses of the agarose hydrogel under the influences of the embedded chondrocytes were predicted and investigated. With the boundary conditions specified as mentioned above, the stresses and strains

can be analyzed subsequently. **Figure 3.11** demonstrates the results of four stresses on the agarose hydrogel surface under the ‘edge flourish’ phenomenon. A linear elastic model with a Young’s modulus of 36.13 kPa and 0.5 Poisson’s ratio was used to simulate the stresses from the given displacement vector map from PIV result. These stresses further reinforced the previously described observation of the ‘edge flourish’ phenomenon, where the encapsulated chondrocytes close to the edge of the hydrogel have the tendency of protruding out of the hydrogel surface.

Figure 3.11 A shows the calculated von Mises stress with the presence of a chondrocyte cluster in dotted lines. The FEM model predicted that the agarose hydrogel surface experienced highest degree of stress at the center of the chondrocytes cluster with the maximum stress of 0.86 kPa. The von Mises stress gradient in Fig. 11A is in the radial direction with higher stress values at the cell center and lower values at the boundary of cells. Upon closer inspection of the three components of stress in all three directions, it was evident that the maximum von Mises stresses at the center of chondrocytes were mainly originating from the stresses acting along the normal direction (Z). It can be noticed that in **Figure 3.11 B**, a *negative* normal stress ($-\sigma_{zz}$) is presenting at the centre of the chondrocyte cluster and a *positive* normal stress ($+\sigma_{zz}$) is surrounding the edge of the chondrocytes cluster. Since a *negative* and *positive* σ_{zz} represent the upwards and downward stresses experienced by the agarose hydrogel surface respectively, this figure visibly demonstrates that the agarose hydrogel surface

becomes bulged at the center of chondrocytes cluster. This result is consistent with the defined ‘edge flourish’ phenomenon, where the chondrocytes cluster is supposed to have protruded out of the agarose hydrogel surface and pushed the hydrogel surface upwards. The outward forces at the center of the cluster, as generated by chondrocytes cluster, were leveled by the inwards forces around the edge of the cells in order to maintain the static condition. The corresponding shear stresses σ_{xz} and σ_{yz} are shown in **Figure 3.11 C and D**. Directions of shear stresses are indicated by arrows shown in the figures, which are as expected from the outwards deformation of hydrogel surface and closely related to the displacement vector map obtained from the PIV result (**Figure 3.10 C**).

Figure 3.11 Simulated stress contour on agarose hydrogel surface using the linear

elastic model ($E= 36.13$ kPa) in FEM analysis. **(A)** is the von Mises stress, **(B)** is z-component stress σ_{zz} , **(C)** is σ_{xz} shear stress, and **(D)** is σ_{yz} shear stress. Dotted lines are the boundary of chondrocytes cluster. Scale bar, 20 μm .

The migration of chondrocytes in a confined microenvironment was observed in the studies of tissue healing [245]. The Young's modulus of the encapsulated chondrocytes in an alginate hydrogel was taken as 3.2kPa based under static compression test [246] with a Poisson's ratio of 0.4 [247].

3.4 Conclusion

In conclusion, through in-depth analysis of the coincidental captured EF phenomenon during routine work with plain cell-laden agarose hydrogels, a clearer and more comprehensive understanding of PTCC/EF mechanism was built with the aid of successive mechanical modeling and mathematical algorithm. It is shown that EF, of the same nature as PTCC, is driven by the cell oriented outgrowth occurring at the certain site of hydrogels under critical thickness. Since natural chondrocytes proliferate at a robust pace, they are responsive to the asymmetric pressure coming from the gel surface and finally form an EF layer outside the gel bulk. The EF mechanism is also adaptable to the PTCC strategy based on the MCG system; to some extent, such kind of hydrogels is playing a physical bioresponsive role without any further biochemical factors incorporated. By exploring the mechanism of EF to serve as explanation for the driving of PTCC, the PTCC phenomenon is better understood and thus the PTCC

approach has acquired a comparable consolidated theoretical basis from a biomechanical angle, and may potentially be equally applied to other kinds of hydrogels under the same mechanism for cartilage tissue engineering.

Chapter 4 Living Tissue Based Scaffolding System for Cartilage Tissue Engineering

4.1 Introduction

In tissue engineering, synthetic biomaterials play essential roles in scaffolding and accommodating the growth and development of neo-tissues [210-212], during which, however, a concomitant clearance of these constructional materials poses another major challenge of the mission [214-215]. Many attempts for engineered cartilage have contained cell seeding in 3D scaffold that is natural or synthetic [248-249]. Ideally, during tissue regeneration, the scaffold, no matter made of synthetic or natural material, should degrade and provide space for cell growth and deposition of ECM proteins secreted by the cells. Although extensive effort has been spent on adjusting the degradation rate of all different kinds of materials to meet this requirement, it remains a challenge for the scaffold degradation to match the tissue regeneration rate. Further, the degradation of synthetic material such as polylactide usually generates acidic byproducts which may affect the cell fate.

In our previous chapters, we have demonstrated that EF-inspired PTCC strategy was successfully implemented within hydrogel bulk and PTCC-MCG structured scaffold to partially solve the abovementioned problem by providing significant space for cell proliferation and ECM secretion. Meanwhile biomechanical analysis provided

comprehensive evidence that the phenomenon of PTCC/EF is activated by cell oriented outgrowth and not cell migration. Here, we would like to take this advantage again by removing the remaining scaffold material to induce a second round of PTCC phenomenon.

In the study of this chapter, the aim is to create true man-made cartilage tissue in a test tube. We hypothesize to develop and apply an innovative living scaffold that is completely free of synthetic material components to enable phenotypically *bona fide* pure neo-tissue formation for artificial grafting. Technically, two steps of processes are to be performed. 1) PTCC in hydrogel scaffold: microspherical cavities are created in cell-encapsulating hydrogel (typically alginate) bulk via a retarded melting of co-encapsulated gelatin microspheres. By proliferative expansion and biomechanical direction, the encapsulated cell (typically chondrocytes) colonies adjacent to the gel/cavity interface spontaneously outgrow the hydrogel phase and sprout into the cavities, where neo-tissue islets are formed to fill up the vacancy and further expand throughout the whole system. 2) LS formation and hydrogel removal: expansion and fusion of PTCC induced neo-tissue islets within hydrogel bulk constructs a macroscopic framework of a LS that maintains the overall shape and size during removal of hydrogel components by a dissolving treatment (typically with sodium citrate). Successful fabrication of LS, as a porous scaffold made of pure and living cartilaginous tissue, can be further explored and applied to develop macroscopic true

cartilage grafts. The detailed overview of the entire process is illustrated in **Figure 4.1**.

This PTCC-LS system is designed as a physical bioresponsive system with which, besides a contribution to cell therapy and regenerative medicine, more profound insights about interactions between cells and hydrogel substrates can be pursued; and technically, brand new methodology for culturing and amplification of non-anchorage dependent cells are provided. In virtue of the nature as artificial ‘natural’ hard tissues produced *in vitro*, the fabrication of a LS system can supply transplantable grafts for clinical demands while bypassing the side-effects from engineering process, such as residues of synthetic materials or incompatibility of engineered grafts with the host. This technology holds great potential in the development of regenerative medicine.

Figure 4.1 Map of the work including finished work in this chapter (circled in solid)

line with gray background) and future work (circled in dotted line with white background).

4.2 Materials and methods

4.2.1 Construction of LS system

Gelatin microspheres (same source as described in Chapter 2) were fabricated following traditional double emulsion oil/water/oil emulsion method. After the microspheres were obtained, they were sieved to select sphere sizes in the range of 150-180 μm for introduction of cavities. In order to stabilize the microspheres and to control the dissolution process of gelatin, the microspheres were surface-crosslinked by N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC).

The chondrocytes harvested from the same source as detailed earlier were suspended in alginate solution (1.2% in 0.15M NaCl) containing surface-crosslinked microspheres at room temperature. After shaking gently, the mixture was injected into a mold and exposed to calcium chloride for gelation. The construct was then maintained in media for up to 35 days. The MCG construct was established via a 2 days' delayed dissolution of water-soluble gelatin microspheres, creating cavities and therefore multiple gel edges within the gel bulk. The outgrowth from the edges will later develop into neo-tissue islets when all the cavities are filled by densely populated cells and their endogenous ECM (**Figure 4.2**). Further culturing of the MCG construct revealed the strong tendency of expansion from the islets, which led to fusion of these islets with each other or scattered cells, giving rise to a maximally synthetic

material-free 3D macroscopic tissue that showed desirable morphologies similar to native cartilage. The final step in attaining a fully material-free LS scaffold is the removal of the alginate gel via sodium citrate treatment as adopted from the ARC method [190]. For a clear distinction between PTCC and LS samples, the timeline was re-named starting from day -35 where day 0 was the day of alginate removal.

Figure 4.2 Demonstration of construction of EF-derived LS system by adopting PTCC strategy. PTCC is to realize EF phenomenon inside hydrogel, and based on PTCC outcome LS can be formed and explored for further tissue development.

4.2.2 Optimization of LS development

In order to explore LS performance, a batch of LS was re-seeded with Passage 1 chondrocytes for refilling the vacancies left by alginate gel. Briefly, there are two pathways to realize this: 1) once LS is formed, the construct is transferred to another new well pre-coated with plain agarose gel and directly seeded with chondrocytes; 2) once LS is formed, the construct is transferred to a cell culture insert (BD Falcon) installed on top of 15 ml centrifuge tube. After centrifugation of the tube to remove extra surface medium of LS, the insert with LS is placed in a new well and receives additional chondrocytes seeding. These two other kinds of LS were named as ‘LS_w direct-adding’ and ‘LS_w insert-adding’ compared to initial ‘LS_w/o adding’, which were all cultured in CCM medium. By altering post-treatment method of cell adding onto newly formed LS, a better LS can be achieved. GFP-infected chondrocytes [250] were also used to evaluate whether the cell reseeded was successful.

Moreover, variation of culture medium was also attempted for enhancing the performance of LS, by changing the concentration of serum which is the essential component of culture medium. CCM medium with 50% fetal bovine serum was used for both ‘LS_w/o adding’ and ‘LS_w insert-adding’. The corresponding constructs were named ‘LS_w/o adding (HS)’ and ‘LS_w insert-adding (HS)’ respectively

4.2.3 Nude mice animal experimentation

After comparing the different kinds of LS’s, the superior LS constructs collected at

different time points of differentiation were implanted subcutaneously in the dorsum of athymic nude mice, followed by the same procedure described in Chapter 2 (as seen in Section 2.2.9, Page 41).

4.2.4 General bio-analytical methods and approaches

4.2.4.1 Biochemical assays

After frozen at -20°C and lyophilized for 24 hours, specimens were digested with 1 mL papain solution (0.3 mg/mL, with 0.1 mM disodium ethylenediaminetetra acetic acid (EDTA) and 0.2 mM dithiothreitol (DTT) as solvent) for 24 hours. Cell content in the specimens was determined by DNA quantification in digested solution using Hoechst 33258 dye assay (7.7 pg per cell). The measurement of collagen content was the same with the gelatin determination method following proline/hydroxyproline assay.

4.2.4.2 (Immuno-) Histology

Samples from each time point (day -23, -17, -11, -0, +0, +18, +35 and +70 for in vitro, 3 weeks and 5 weeks after implantation for in vivo) were fixed in 4% (w/v) neutral buffered paraformaldehyde for 2 days. The fixed specimens were then embedded in paraffin and cross sectioned using a microtome. Sections from all groups were subsequently stained with H&E, Safranin O and Masson's trichrome. For immunofluorescent staining, the sectioned specimens are incubated with 10% goat

blocking serum (w/v, in PBS) for 20 minutes to suppress non-specific binding of IgG. To evaluate chondrogenic favorable or undesirable markers, the specimens was immune-stained for COL II and COL I respectively following the method as detailed in Chapter 2 (see Section 2.2.7, Page 40).

Table 4.1 PCR primers used in this study.

Genes	Primer sequence (both 5' – 3') [a]
TBP1	F: AACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG
COL II [227]	F: GCTATGGAGATGACAACCTGGCTC; R: CACTTACCGGTGTGTTTCGTGCAG
Aggrecan [227]	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC
RhoA	F: AGCTGGGCAGGAAGATTATG R: TGTGCTCATCATTCCGAAGA
Integrin β 1	F: TGCCAAATCATGTGGAGAATGTAT R: GTCTGTGGCTCCCCTGATCTTA
Sox9	F: GCTGGCGGATCAGTACCC R: CGCGGCTGGTACTTGTA
COMP	F: GGCACATTCCACGTGAACA R: GGTTTGCCTGCCAGTATGTC
COL I [251]	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCCTT

[a] F and R stand for 'forward' and 'reverse' sequence, respectively.

4.2.4.3 Real time RT-PCR

RNA from the living scaffold was extracted using the combination of TRIzol® (Invitrogen) and RNeasy® Mini Plant Kit (Qiagen, Düsseldorf, Germany) [213]. The

acquired RNA samples were treated with RNase-free DNase I and converted to cDNA for the subsequent PCR experiments. For real-time quantitative PCR, the relative gene expression values were obtained following the described method in Chapter 2 (Section 2.2.5, Page 38). All the primers used were described as above.

4.2.5 Dynamic mechanical analysis (DMA)

An RSA III Rheometrics System Analyzer (TA instruments) in parallel-plate configuration was used for DMA. Samples of a certain size except native cartilage were incubated in PBS overnight. Mechanical spectrometry was carried out using dynamic frequency sweep with frequencies ranging from 0.1 Hz up to 1 Hz at 37°C and with a strain amplitude of 5%, which was in the linear region of viscoelasticity. Both auto-tension and auto-strain adjustment were applied. Force ramped from 0.001 N to 0.2 N (depending on the sample strength), and the maximum allowed strain was set at 10%. The storage modulus (E') and loss modulus (E'') of the samples were measured. The phase angle delta ($\tan_{\text{delta}}$) was computed from $\tan_{\text{delta}} = E''/E'$.

4.2.6 Statistical analysis

Biochemical and gene expression data from various constructs were compared with equal incubation time. Where appropriate, ANOVA was performed to analyze results and a $P < 0.05$ with 3 specimens in each group was considered to indicate a statistically

significant difference. Data are presented as mean $\pm$ SD.

4.3 Results

4.3.1 Formation and microstructure of alginate-based PTCC constructs

The corresponding procedure and architecture for the alginate-based PTCC construct are depicted in **Figure 4.3-4.6**, proving again the superiority of the PTCC-MCG construct compared to common non-PTCC hydrogels. The PTCC constructs were cultured in fetal bovine serum supplemented DMEM under regular cell culture conditions (37 °C, 5% CO₂). When the cells settled down in the gel bulk, the co-encapsulated gelatin microspheres, which had been preserved by moderate surface crosslinking, underwent a spontaneous melting vanishment after a certain lag period. The culturing was continued for another few weeks under the same conditions, during which the EF phenomenon began to occur visibly on the gel-cavity interface around day -23 (as shown in **Figure 4.3**).

Figure 4.3 Alginate-based PTCC construct development traced by Live/Dead-staining.

The inter-phase escaping cells were growing out of the gel bulk and became re-entrapped inside and/or on the inner edge of the cavities, from where scaffold-free neo-tissues were developed. Given the much greater total specific interface areas of the cavities than that of the general outer surface of the whole construct, the EF effect occurred significantly earlier and more aggressively in the cavities; more importantly, the EF phenomenon became controllable and also applicable for *in vivo* conditions.

After prolonged culture, the EF extended to refill the in-gel cavities with neo-tissues by which spherical neo-tissue islets became formed throughout the construct. By further culturing, these scaffold-free islets acted as neo-tissue cores

demonstrating strong a tendency to expansion and fusion with each other and also with the localized ‘neo-tissue rings’ around the isolated individual cells in gel bulk. Focusing on the cavitation-induced neo-tissue generation, the images indicated a morphologically *bona fide* cartilaginous phenotype and the quantitative biochemical and gene expression data suggested a significantly higher chondrocytic proliferation and cartilaginous ECM production. As shown in **Figure 4.3** and **Figure 4.4**, PTCC strategy propels the formation of a fused tissue construct at day +0, which is the LS sample. Interestingly, compared to its precursor PTCC construct or plain non-PTCC, the LS sample produces more ECMs such as GAG and collagen as shown in **Figure 4.5** on the biochemical level, which may due to the produced extra free growth space [252].

(A)

(B)

Figure 4.4 Alginate-based PTCC construct development traced by (A) H&E staining and (B) Safranin-O staining.

Figure 4.5 Biochemical assays indicating alginate-based PTCC chondral phenotype

Figure 4.6 Gene expression indicating alginate-based PTCC cell proliferative profile and chondrocytic phenotype

4.3.2 Formation of LS construct

Figure 4.7 shows the photo evidence that depicts how to obtain LS. **Figure 4.8-4.9** quantitatively and qualitatively proves the superiority of LS compared to its precursor PTCC sample and also describes the growth of LS according to the different post-treatments. Gene expression results showed that the LS construct continued with the secretion of normal chondrocyte-specific markers, and re-seeding chondrocytes into LS was shown to increase the gene expression level which can be seen obviously in integrin $\beta 1$ expression. It will be clear that re-seeding cells helped to increase cell-cell communication. Aside from this effect, re-seeding of cells also contributed to strengthen the ECM secretion as can be deduced from the histological and biochemical results as shown in **Figure 4.9**. Generally, the realized LS constructs were relatively close to natural cartilage. The preliminary studies above proved the feasibility of the formation of LS construct through PTCC-MCG system.

Figure 4.7 Photo evidence of the LS construct achievement. By detaching alginate by sodium citrate, PTCC sample transformed to LS construct which remained integral, while the non-PTCC sample became weakly visible with only few EF aggregates left.

Figure 4.8 Gene expression indicating LS cell proliferative profile and chondrocytic phenotype.

**GFP-labeled
cells added into
LS @Day+7**

Living Scaffold (LS)

Figure 4.9 Overall analysis of LS constructs development that experienced different kinds of post-treatment. The upper four staining results showed the (Immuno-)histochemical illustration of LS development. The lower left positive GFP staining confirmed successful extra cell adding as an alternative for LS development. The right three column data showed the biochemical assay of LS normalized to the construct volume for more significant comparison with native cartilage. And the macrophotograph at lower right side further verified the structure of LS further comparable to native cartilage.

By successfully seeding chondrocytes into LS (confirmed by the fluorescent image of GFP labeling in **Figure 4.9**), LS experiencing an insert-adding pathway behave much better than the plain LS or LS through direct addition of cells, and one more similar to native cartilage with half the amount of ECM density. It is possible that the insert pathway did not only make LS more absorbable by simple centrifugation, but also that the cell seeding space is narrowed in case of seeded cell loss. Noticeably, from the staining results shown in **Figure 4.9**, cells in LS constructs grow not so robust at day +70, which is more apparent in common long-term cell culture within material-based scaffold *in vitro* [253]. Take PTCC sample without gel-elution treatment for example, the shortage caused by long-term cell culture can be seen evidently in **Figure 4.10**. Hence, it is indicated that the appropriate time for LS implantation is before day +35.

Figure 4.10 (Immuno-) histological staining of long-term cultured MCG scaffold. The lowest two images were taken by optical microscope without staining.

4.3.3 Screening of various LS constructs compared to native cartilage

As shown in **Figure 4.8** and **Figure 4.9**, 'LS_w insert-adding' sample behave much better than the other two kinds of LS's. In comparison to samples cultured in more nutritive medium, long-term culturing PTCC and non-PTCC samples, 'LS_w insert-adding' sample perform more perfectly and mimics the native cartilage closely, as seen in **Figure 4.11-4.13**. From the comparison between the 'LS_w insert-adding' sample and the other four controls at the biochemical, gene expression and histological level, the 'LS_w insert-adding' sample secreted ECMs such as GAG and collagen

(calculated in density unit) at least twice more than the controls, maintained chondrocyte-specific gene markers and apparently presented histologically a more favoring chondrocytic phenotype. A higher serum concentration in the culture medium did not have a positive effect on LS.

Figure 4.11 ECM secretion of LS constructs compared with control samples.

Figure 4.12 Gene expression of LS constructs compared with control samples.

It is expected that LS constructs perform better than PTCC or non-PTCC samples,

since LS gets rid of the foreign material by merely keeping its natural cell-secreted ECMs that provides chondrocytes a familiar feeling like their original residence in native cartilage. Notably, cultivating LS in medium with a higher concentration of serum seems not to be a smarter choice. The LS undergoing this culture condition does not show a more positive chondrocytic phenotype and even begin to secrete COL I (as seen in **Figure 4.12 and Figure 4.13 E**), which is undesirable for cartilage. Because cartilage fibrosis might be the potential cue for this proven [254]. Therefore, LS with insert-adding was used for further trials including *in vivo* experimental tests.

(A)

(B)

(C)

(D)

(E)

Figure 4.13 Histological staining of LS constructs compared with control samples. (A) ~ (E) represents H&E, Safranin O, Masson's trichrome, immuno-COL II, immuno-COL I staining respectively.

4.3.4 Morphology and mechanical test of LS constructs

Figure 4.14 and Figure 4.15 showed the SEM and mechanical results of the LS construct. Therein, the used LS construct for test was plain LS without extra cell post-seeding. The results that the tested LS was more like native cartilage indirectly predicted superior 'LS_w insert-adding' would inherit similar *bona fide* performance and thus was confirmed again to be used as the kind of LS for *in vivo* experiment.

Figure 4.14 SEM of LS construct compared to native cartilage.

Figure 4.15 Mechanical test of LS construct compared to native cartilage.

4.3.5 *In vivo* evaluation of LS construct

As shown in **Figure 4.16-4.20**, the LS construct exhibited better chondrocytic phenotype than other controls. Interestingly, LS implanted at day +35 grew a bit better than that implanted at day +14, which indicated that the suitable time point for LS implantation will be around day +14 to day +35. H&E staining showed that the cells were distributed evenly in LS. Safranin-O and Masson staining together demonstrated that LS secreted abundant ECM *in vivo*. Immunohistochemical staining proved that the collagen secreted by LS was mostly made of COL II meeting cartilage-required property. Notably, except for similar proteoglycan results, LS even performed more positive histological staining than xenogenous native cartilage when both of them were implanted subcutaneously into nude mice.

In Vivo - Histochemistry illustration (H&E)

200 μm

Figure 4.16 H&E histological staining of LS construct compared to native cartilage for *in vivo* nude mice experiment, showing the distribution of cell nucleus and its cytoplasm.

In Vivo - Histochemistry illustration (Safranin-O)

Figure 4.17 Safranin-O histological staining of LS construct compared to native cartilage for *in vivo* nude mice experiment, showing the amount of proteoglycan deposition around cells.

In Vivo - Histochemistry illustration (Masson's trichrome)

Figure 4.18 Masson's trichrome histological staining of LS construct compared to native cartilage for *in vivo* nude mice experiment, showing the amount of total collagen secretion of cells.

In Vivo - Histochemistry Illustration (Col II *immuno-staining*)

Figure 4.19 COL II

immune-histological staining of LS construct compared to native cartilage for *in vivo* nude mice experiment, showing the amount of desirable COL II secreted by cells.

In Vivo - Histochemistry illustration (Col I immuno-staining)

Figure 4.20 COL I immuno-histological staining of LS construct compared to native cartilage for *in vivo* nude mice experiment, showing the amount of unfavorable COL I secreted by cells.

4.4 Discussion

The intrinsic drawback of a scaffold is that its degradation cannot easily match the tissue regeneration rate and even the degradation products of some scaffolds will be harmful for the encapsulated cells. Either natural or synthetic scaffolds, no matter they are in the form of hydrogels or not, are required to be degradable along with cell proliferation and biocompatible with neo-tissue formation. Investigation on a perfect scaffold that can concomitantly degrade with cell growth but favorably leave harmless degradation products, has been still challenging tissue engineers till now [255-256]. As described in Chapter 2, in order to overcome the deficiency of cell affinity and also significantly improve the living space for the engineered neo-tissues, while still reserve all the beneficial talents of injectable hydrogels, we have developed a novel MCG scaffolding system as well as a novel PTCC strategy to enable *bona fide* neo-tissue self-formation in artificial graft.

The MCG structured scaffold can solve the scaffold-faced problem to a large extent by promoting chondrocyte proliferation and secretion more effectively; meanwhile it can degrade *per se* at a faster pace (**as seen in Figure 2.10**). Although it can be expected that finally the synthetic material part inside MCG would be wiped off, the period of scaffold existence is still a bit long that is not good for cell and tissue development. As seen in **Figure 4.10**, cells did not grow so well after long-term culturing inside MCG scaffold. The existing scaffold incompetence to perfectly match

tissue regeneration speed along with its degradation rate is always undesirable for tissue engineering aim.

Therefore, in this chapter, we further proceed to design a totally scaffold material-free LS containing of solely transplanted chondrocytes and their endogenous cartilaginous ECM based on the PTCC-MCG system. The development of LS from MCG-based cell-laden alginate hydrogel construct begins with PTCC that leads to neo-tissue islets filling up the voids in the MCG construct as shown in **Figure 4.2**. These islets display further proliferative expansion and form a connected and integrated neo-tissue network of inter-penetrating the gel (alginate) scaffold. Therefore, the removal of alginate scaffold by sodium citrate dissolution treatment does not disturb the integrity of the system and eventually generates a LS construct made of pure and living cartilaginous neo-tissues (as seen in **Figure 4.8** and **Figure 4.9**). At the same time, the advantage of hydrogels – injectability - is preserved in the development of a material-free engineered cartilage tissue, whose size can be customized into the defect site.

Replacing agarose with alginate in constructing the MCG system, it was found that the agarose-based PTCC strategy was reproducible in an alginate-based system, as seen in **Figure 4.3-4.4**. The sequence of events was similar to that in agarose-based strategy (**Figure 2.7**), with the invasion of chondrocytes into the cavities 12 days (day -23) after being in culture. The cavities were completely filled up with neo-tissue by

the fourth week (day -11), and the neo-tissue islets became cores that continued to expand beyond the cavities. These neo-tissue islets fused with each other by 35 days (day 0) in culture to finally form a structure whose integrity was no longer dependent on alginate - the PTCC sample at day +0 showed that the construct did not disintegrate after sodium citrate treatment, unlike that of a homogeneously seeded non-PTCC construct (**Figure 4.7**). After removal of alginate, the remaining construct consists only of chondrocytes and their endogenously secreted ECM network.

The PTCC constructs' superiority over non-PTCC constructs were further proven by the gene expression profile and the biochemical assay results (**Figures 4.5** and **Figure 4.6** respectively). The consistently higher expression profiles of all cartilaginous genes in PTCC constructs as compared to non-PTCC constructs confirmed that the PTCC system is more promising towards engineering cartilage tissue than conventional cell encapsulation techniques. Across the time period of 35 days, there is an increasing trend of cartilaginous gene expression, but a decrease is observed for both PTCC and non-PTCC constructs on day +35 (**Figure 4.12**). This observation can be explained: chondrocytes begin to resume their chondrogenic phenotype in 3D culture after monolayer culture expansion; they proliferate and produce more ECM as they penetrate and fill up the cavities via EF phenomenon; finally when the cavities are filled up and there is space constraint, cartilaginous gene expression is reduced, and growth and ECM production slows down. In the case of

COL I, the initially high expression values can be attributed to the dedifferentiation of chondrocytes in monolayer culture (similar to those in CC P1) and they have not completely redifferentiated within the short period of two days after 3D culture (day -33). However, over time, the values are constantly decreasing and approaching those of native cartilage. COL I gene expression is undesired in hyaline cartilage, and its much lower expression levels (as compared to the cartilaginous genes) are reassuring; it confirms that in 3D culture, chondrocytes retain their cartilaginous phenotype. It is notable that COL I gene expression values in PTCC constructs are lower than those in non-PTCC constructs from day -28 onwards, showing that PTCC constructs do favor the hyaline cartilage phenotype better than the latter. Collectively, in terms of the gene expression profiles at day -0, PTCC constructs' quality was not comparable to native cartilage, although they have values more similar to it as compared to non-PTCC constructs. The lack of space prevents further growth and ECM production of encapsulated chondrocytes; this is a typical problem faced by conventional tissue engineering technique of cell encapsulation within a scaffold.

Hence, to create more space, alginate needed to be removed. A second round of active proliferation can be induced as more space is opened for cells to proliferate into (**Figure 4.14**). Its removal from PTCC constructs at day 0 via sodium citrate treatment, as described in the ARC method by Masuda *et al* [190], resulted in a material-free 3D construct (**Figure 4.7**). This structure of living cells, held together by its secreted ECM,

is hence termed LS. The biochemical assay results in **Figure 4.5** showed that ECM content and secretion was consistently higher in PTCC constructs than in non-PTCC constructs; the PTCC strategy is superior compared to conventional cell encapsulation technique. However, PTCC constructs were unable to produce as much ECM as native cartilage but is expected: apart from the neo-islets, the gel bulk did not have high cell and hence ECM densities. After sodium citrate treatment, the ECM content per unit dry weight increases (comparing between LS day +1 and PTCC day -0) due to the reduction of dry weight as alginate is removed. However, LS constructs showed higher secretion values at day +1 than PTCC constructs; this is because chondrocytes within the LS are allowed to expand and secrete more ECM as they try to fill up the spaces left behind by alginate. Through the biochemical assay results, LS construct showed the most promising results out of the three types of constructs, because its values were the closest to those of native cartilage.

To further ascertain the claim that removal of alginate was inevitable for the constructs to grow well, PTCC constructs were cultured for a prolonged period of 70 days without alginate removal. The histological illustrations in **Figure 4.10** showed the poor quality of constructs in prolonged culture - a drastic difference from native cartilage's illustrations - hence proving that alginate removal was necessary. The sparse distribution of chondrocytes may have been the result of widespread cell death within the constructs, as the neo-tissue islets seen in **Figure 4.3** could not be observed in

Figure 4.10. A plausible cause is that as spaces within the PTCC construct are filled up with neo-tissue, the diffusion of nutrients into and waste out of it becomes harder to the extent that cells deep inside the construct have insufficient nutrients and start to die. The removal of alginate was then proven to be inevitable in order for the existing chondrocytes to survive and further grow. The time point to remove alginate was decided to be after 35 days of culture *in vitro*, when gene expression levels and biochemical assay data were highest.

The problem of space constraint due to slow scaffold degradation as compared to growth of new tissue also leads to the low cell density within the engineered cartilage tissue, which is another commonly faced issue in tissue engineering as well. Although LS strategy had done away with the problem of space constraint, its histology was not reflective of that in native cartilage (comparing histology of 'LS_w/o adding' in **Figure 4.9** and native cartilage in **Figure 4.10** respectively). Native cartilage is a stiff and compact tissue, without any unfilled spaces within. The voids in LS present even after 35 days of culture hence required an improvisation to LS - increasing the cell numbers so as to allow more proliferation of cells and their secreted ECM.

Reseeding chondrocytes onto the LS proved to be successful as seen in **Figure 4.9**. Reseeded chondrocytes (tagged with GFP) stained green throughout the LS construct and were able to attach and survive within. The ECM in the LS construct, being naturally present in native cartilage for cell anchoring, hereby offers anchors for

reseeded cells to attach on. As such, optimization of the chondrocyte reseeding was essential in order to maximize the number of chondrocytes attaching onto the LS construct and thereby minimizing wastage of chondrocytes. The two methods of reseeding chondrocytes via directly adding and adding into a cell culture insert containing the LS construct were proven to be advantageous - their histological illustrations, biochemical assay data and gene expression profiles generally showed more cartilaginous ECM and genes expressed than LS constructs with no reseeded chondrocytes (**Figures 4.8-4.9**).

In **Figure 4.8**, histological illustrations showed that there were fewer voids in reseeded LS constructs than those without reseeding i.e. “LS_w/o adding”; qualitatively, there is even and more compact distribution of cells and ECM in the reseeded LS constructs. By increasing the number of chondrocytes through reseeding, the new chondrocytes are able to proliferate and fill up the spaces left behind by alginate more quickly than if the original number of chondrocytes already present inside the LS construct were to fill up the voids. The observed decrease in quality of constructs by D +70 can be due to the lack of diffusion of nutrients and waste as the scaffolds are filled up completely with cells and ECM after day +35. Without sufficient nutrients reaching the cells inside the construct in long-term *in vitro* culture, the cells started to die. As for biochemical assay data in **Figure 4.8**, ECM content stabilized in the long run (from day +18 onwards); the threshold for ECM content within the

scaffold is reached. The gene expression profiles in **Figure 4.8** were mostly acceptable - cartilaginous genes were more highly expressed and had levels resembling native cartilage in constructs with reseeded chondrocytes. The cell proliferation marker, RhoA, was lowly expressed in reseeded constructs than the control, but can be accounted for - without reseeded chondrocytes, the LS control had more space for the original chondrocytes to fill up, and hence they had to proliferate more quickly by producing RhoA.

However, comparing amongst the three types of LS tested (i.e. 'LS_w/o adding', 'LS_w direct-adding' and 'LS_w insert-adding'), the method of using a cell culture insert showed the most satisfactory results, bearing most similarity to native cartilage in ECM content and gene expression levels (**Figure 4.8** and **Figure 4.9** respectively). Its H&E stained samples showed even cell distribution with the least unstained portions. Likewise for the ECM content, the smooth distribution mimicked that of native cartilage. By using cell culture inserts, the reseeded chondrocytes can be concentrated within a small area, as compared to the direct adding of cells into a well. Hence, there is much higher probability that chondrocytes in the medium settle in and attach onto the ECM of the LS construct. With more chondrocytes in the "LS_w insert-adding" than in "LS_w direct-adding" constructs, voids can be filled up more quickly through chondrocyte proliferation (both from reseeded ones and LS neo-tissue cores) and its correspondingly secreted ECM.

As mentioned previously, across the board, the decrease in ECM content and secretion values in all LS constructs, whether with reseeded chondrocytes or not, by day +70 (**Figures 4.9**) can be attributed to the long-term *in vitro* culture conditions being unsuitable for cells. By day +70, cells have been cultured *in vitro* for about 120 days in total (from the time of harvest from bovine articular cartilage). Hence, the LS constructs cannot be grown in *in vitro* conditions for such a long period of time as their quality will decrease; the goal of creating a cartilage-like tissue will not be reached in such conditions. Even at day +35, although samples had the closest histology, ECM secretion and gene expression values to native cartilage, the results were still inferior to native cartilage (**Figures 4.8-4.9**). In addition, the results from the variance of serum concentration of culture medium showed that higher serum concentration does worse for LS as compared to normal one (20% serum in medium) as seen in **Figure 4.11-4.13**. *In vivo* experiments using “LS_w insert-adding” constructs receiving normal CCM were hence carried out to test the development of the constructs *in vivo*.

In vivo experiments showed the superiority of LS constructs over PTCC constructs in terms of histology, especially observed in the stained samples in **Figures 4.18** and **Figure 4.19**. There are more unstained areas within the PTCC constructs e.g. samples at 3 weeks in **Figure 4.19** (green-stained COL II). This is due to the lack of cells and hence ECM within the alginate gel bulk, which was not removed. On the other hand, the LS constructs were visibly more compact in samples implanted at day +14 and day

+35 on the grounds that a second round of PTCC phenomenon has taken place and chondrocytes have taken over the spaces left behind by alginate.

Comparing between the implantation time points of samples, experiments showed that a better quality engineered cartilage construct can be produced from LS samples implanted 35 days after reseeding. Samples implanted at day +35 gave better histological staining results than those implanted at day +14, with day +1 implanted samples being the worst. This is generally observed in all stained images in **Figure 4.16-4.20**, but is most reflected in the H&E staining (**Figure 4.16**). The latter, although with comparable intensity of stain (and hence amount of ECM and cells) in general, had more voids within the non-PTCC samples. The spaces within the samples was not reflective of native cartilage, which is naturally compact; this can lead to inferior mechanical strength of the sample as well. The presence of voids was due to the inadequate space allowed for the encapsulated chondrocytes to grow. Control groups implanted at day +1 hence had the most voids, followed by those at day +14. When given sufficient time to take over the voids (35 days), the distribution of cells and ECM in the sample was shown to be much more uniform (**Figure 4.16-4.20**), a situation that echoed the histology of native cartilage. The qualitative results of COL I immunostaining in **Figure 4.20** is worthy of notice - there is a lack of COL I content in LS samples as compared to PTCC and non-PTCC samples, showing the cartilaginous phenotype of cells are better retained in the former, and hence LS's superiority to

PTCC and conventional (non-PTCC) constructs.

Comparing between *in vitro* and *in vivo* samples' histological stainings (**Figure 4.9** and **Figure 4.16-4.20**), *in vivo* implantation seemed to give a much better result by producing a much more compact engineered cartilage construct similar in histology to native cartilage. The results were expected as it is impossible to supply exactly all of the many biochemical signals and physical stimuli required for cartilage development in the incubator (*in vitro*), unlike that *in vivo*. The *in vivo* process is hence still essential for the development of LS into a cartilage-like tissue.

Based on the practiced studies above, the LS system, though, high in cell density, is still highly porous and relatively soft compared to the native cartilage. Native cartilage is stiff and compact tissue with essentially no pores. Consequently, this intrinsic property of native cartilage hinders it as appropriate cartilaginous implant candidate, and even native cartilage simply cultured *in vitro* for couples of days grows badly as reported in the literatures [257-258]; while the porous structure in the LS system favors efficient nutrients exchange throughout the entire construct. Moreover, the soft LS structure promotes integration at the graft-tissue interface and facilitates transplantation as it is easy for size and shape manipulation to fit the defect. For complete regeneration of cartilage, the LS system still requires the *in vivo* process so as to fully develop into a compact cartilage.

4.5 Conclusion

In conclusion, even though native cartilage is mechanically strong and compact, it is not suitable for the purpose of transplantation due to poor permeability and poor integration at interface. Porous PTCC-LS system as developed in this study is superior since it can achieve efficient nutrients exchange and good integration at the interface with surrounding tissue. The biochemical results showed LS system inherits the high ECM secretion ability from PTCC-MCG construct and further increases the ECM density significantly which is a critical factor for engineered tissue formation. The mechanical test showed LS system has the most similar pressure response to native cartilage. With these advantages, the results from transcriptional level illuminated that LS system not only maintains normal chondrocytic gene expression but also avoid undesirable COL I over-expression that might result from cartilage fibrosis. Hence, qualitative and quantitative results from *in vitro* and *in vivo* experiments all demonstrated PTCC-LS is a more promising and advanced system compared to PTCC-MCG system and holds the potential of exploiting for various extensive tissue engineering applications

Chapter 5 Conclusions and future work

5.1 Conclusions

Among the various applications in tissue engineering categorized to regeneration medicine, scaffold is one of the most pivotal elements and has been investigated and advanced during the last decades. The main two problems lying in scaffold design and application are as below: 1) there are limited space to accommodate encapsulated cell growth and cell-secreted ECMs deposition; 2) the scaffold degradation rate cannot process well in conformity with the speed of tissue regeneration. According to these two problems limiting the ultimate therapeutic effect of tissue engineered products, numerous solutions have attempted by material scientists, biochemist and other relevant researchers, however, breakthrough have not been achieved and necessitates further investigation.

In this Ph.D. project, from the beginning a MCG scaffolding system adopting PTCC strategy was proposed to partially figure out the highlighted problems, offering interesting insights into the beneficial results of creating extra more space for incorporated cell growth and neo-tissue formation. Based on the PTCC-MCG system, we then extensively utilized PTCC phenomenon to produce a PTCC-LS system, providing a feasible means for scaffold material removal with integral pure cell/ECMs hybrid construct left. The size of both the invented constructs can be controlled and the

thickness of them is much close to that of native cartilage. Therefore, theoretically the latter progressive work in this Ph.D. project can mostly resolve both the two problems meanwhile. The additional significance of PTCC-LS system is that once successful it can be directly applied on cartilage transplantation, cartilage regeneration and etc for those people with osteoarthritis. It is expected that LS construct will integrate with surround tissues better and further fill the injury site when it is indeed installed in a cartilage defect, which will be confirmed by future investigation on in situ bigger sized animal model.

The most essential finding from the above investigations is that the settlement and commitment of therapeutic cells and their further tissue formation in 3D hydrogel matrices are highly regulated by the PTCC strategy. Specifically, the enough accommodations are required for therapeutic cells to maintain their phenotype and normal functionalities. Therefore, for maximally performing scaffold advantages together with favouring cell and tissue development, researchers can manipulate to provide man-made ‘cavities’ within hydrogel matrices in order to facilitate the settlement and growth of encapsulated cells. Furthermore, only if within hydrogel network, the space requirement will be a lasting challenge even after adopting PTCC strategy. Therefore, the removal of hydrogel part will lead a second round of PTCC strategy and the encapsulated cells with their secreted ECM will embrace more unlimited space by this way, ultimately producing more cartilage-like tissue. This

conclusion is drawn from both engineering exploration and mechanism investigation in this dissertation.

Firstly, a PTCC-MCG system was established by co-encapsulating cells, hydrogel precursor with dissoluble microspheres to solve the spatial problem of scaffold application in tissue engineering. After hydrogels formed, the microspheres would melt off and there thus leave micro-cavities inside hydrogels. After a certain period of time, the fast proliferated cells were found to outgrow into the cavities and continue to proliferate and secrete ECM. Finally, neo-tissue formation was found in the cavities. On one hand, the *in vitro* and *in vivo* experimental results both showed the positive effects of MCG scaffolding methodology contributing to solve abovementioned problems associated with scaffold and benefitting encapsulated cells and formed tissue. On the other hand, the good results of MCG system owe to the PTCC strategy inspired by EF phenomenon. It was shown that PTCC and EF are essentially similar in nature and asymmetric pressure was found as driven force for PTCC/EF occurrence. Compared with other reported approaches targeting at bioresponsive hydrogels such as grafting peptide sequences into hydrogel bulks, our PTCC-MCG system would be a more promising physical bioresponsive tissue engineering scaffold.

Secondly, the biomechanical mechanism of PTCC was investigated by exploring cell biomechanology in the simplified PTCC construct - EF model. As PTCC and EF are the same phenomena in nature, the model for mechanism investigation can be

simplified to focus on EF only. Without introducing cavities inside hydrogels, the EF model returned to adopt plain hydrogels encapsulating therapeutic cells. We found that when cells are encapsulated in highly hydrated 3D network of hydrogels, the specific outgrowth of cells is required for their survival and proliferation. The fulfilment of such outgrowth – instead of being confined within hydrogel bulk – is the key for increasing cell population. Different types of mechanical modelling coincidentally proved that the mechanism of PTCC/EF is cell oriented outgrowth but not results from cell migration.

Thirdly and reversely, based on the successful establishment of PTCC-MCG system and intensive investigation of PTCC/EF mechanism, we proceeded to produce a scaffold-free construct aiming to solve the remaining problem existing in tissue engineering scaffold application by providing even more space for neo-tissue formation based on the PTCC-MCG system. We innovatively loaded removable hydrogels with MCG scaffolding system. Following with the reoccurrence of PTCC *bona fide* results in the newly-adopted hydrogels, the foreign hydrogel materials could be easily and gently removed without any harm to encapsulated cells and formed neo-tissue. Meanwhile, with hydrogel detachment a macroscopic genuine living engineered tissue was received as an integral which was named LS. By this way, the problem of scaffold degradation rate can be well fixed up, since LS does not have any natural or synthetic scaffolding materials *per se* but effectively maintain

macroscopically visible shape.

Inherited the advantageous features from invented MCG scaffolding technique that realizes novel PTCC strategy, PTCC-LS approach would emerge as a new strategy for therapeutic cell culture and tissue engineering especially in which the potential direct application of engineered cartilage implant to patients is of extremely high concern.

Admittedly, the obvious drawback of the invented constructs is that it takes time for cell culture before transplantation. Therefore, more efforts need to be made for shortening the preparation period in real clinical setting. Various conditions can be tried for accelerating PTCC process and LS construct stabilization. Besides, much simpler experimental methods are worthy of consideration. For example, uncrosslinked microspheres can be directly used for alginate since it can be gelated under low temperature which will not affect microspheres.

5.2 Future outlook

Towards the advancement of regenerative medicine and cell-therapeutic biotechnology, continued efforts are expected on expanding the application fields PTCC strategy for cell culture and tissue regeneration. Particularly, future study can be directed to several essential aspects including: i) extending the *in vivo* experimental evaluation to big animal model, ii) combining auxiliary strategies with PTCC-LS system for

endochondral osteogenesis or osteogenic angiogenesis (aspect i and ii can be seen in **Figure 4.1**), iii) exploring more sophisticated information of cell biology in PTCC strategy, iv) utilizing PTCC strategy on more different cell types including anchorage-dependent cells for its universality test.

Firstly, we have designed and constructed a kind of novel and genuine living tissue architecture purely with cells and their secreted matrices inside successfully, leading favourable *in vitro* and *in vivo* (nude mouse model) results. In pursuit of future clinical practice for human beings, it is still not enough for evaluating engineered tissue based on solely nude mouse experiment. Nude mouse normally receives implants subcutaneously but for in-situ implantation it inevitably lacks of operation space [259], especially for making cartilage defects. Therefore rabbit specie, which is bigger than mouse and musculoskeletal mature enough, is regarded as a good model for in-situ implantation. Hence, commonly adopted New Zealand white rabbit will be chosen for implantation of 3D LS constructs to detect the condition of cartilage regeneration locally. More importantly, the success of rabbit or bigger animal experiment will help solving the shortage of autologous chondrocytes. Since cartilage is avascular, aneural, and alymphatic tissue [13] which consists of only one cell type, chondrocytes, embedded within a rich ECM of proteins, xenografting may also do a good work for cartilage defect healing.

Secondly, regenerative functions of PTCC-LS system may be enhanced with the

guided endochondral osteogenesis or osteogenic angiogenesis, as illustrated in **Figure 4.1**. There are two pathways to realize the enhancement target: i) transdifferentiation of LS construct and ii) supplemental seeding of multi-/pluripotent stem cells (mouse ESCs and induced pluripotent stem cells (iPS)) on LS construct. For the first part, it is a hypothesis that chondrocytes in LS construct have sub-population namely chondroblasts remaining certain plasticity or stemness for differentiation [260], due to their ability to form colonies that is exclusive characteristic of stem cells and tumour cells. Accordingly, we propose to evaluate whether LS construct has the ability of transdifferentiating encapsulated cells into osteogenic lineages and address to further explore the potential of the PTCC-LS system inducing it toward angiogenesis. For the second part, mESCs and miPS are to be seeded on the LS construct to examine the potential of LS in supporting their growth and differentiation and resulting osteogenesis and angiogenesis. Both *in vitro* and *in vivo* tests would be carried out. Therefore, this PTCC-LS platform could not only encompass cartilaginous tissue regeneration but also mimic endo-chondral pathway as the primary developmental pathway of osteogenesis for regeneration.

Thirdly, for more profound scientific insights, substantial work remains to interpret cell biology in PTCC strategy. Besides asymmetric pressure that functions essentially in mediating PTCC, other mechanical stimuli for cell-ECM interaction and cell-cell communication have been witnessed active in regulating cell behaviours as

well [261-262]. Beyond the biomechanical factor, biochemical factor is equally important in determining cell fate [263-264]. All of the stimuli and factors might trigger cellular signal transduction [265], which cell proliferation fate and tissue regeneration rate. Therefore, interdisciplinary approaches ranging from genetic microarrays, physical modelling and electrochemical sensing are all helpful to conduct more comprehensive PTCC mechanism study.

Last but not the least, the remarkable PTCC strategy should be extended for application in other tissues for regeneration. Other types of cell for various therapeutic purposes can be employed. Aside from chondrocytes, a wide variety of anchor dependent cell species such as myoblasts, fibroblasts and endothelial cells could be employed as cell sources receiving PTCC strategy, for engineering corresponding tissues. Moreover, the progenitor cells, e.g. MSCs and ESCs, are also worth investigating their fate in PTCC-MCG system. Once they have the similar appealing growth trend like chondrocytes, in terms of the differentiation ability of stem cells into different lineages, the resulted PTCC-induced constructs could be smart and tuneable enough to adapt for different tissue regeneration needs. For example, BMPs, vascular endothelial growth factors (VEGFs) or neurotrophins could be incorporated the constructs to potentially accelerate osteogenesis, angiogenesis or neurogenesis respectively. Likewise, gene therapy could be also considered to involve for enhancing these purposes.

In conclusion, our investigation on the performance of PTCC-LS system *in situ* would continue and extend to bigger animals with body size comparable to human, which will eventually lead the bench research to bedside application for cartilage repair. The extension of PTCC strategy into other types of cells would also benefit other organ regeneration by diverse pathways/techniques described above.

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