ENGINEERING SCAFFOLD AND SOLUBLE CUES FOR CELL-INSTRUCTION

BY

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DISsertation

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Abstract

The ultimate aim of this work is to design and engineer an integrated tissue engineering approach that combines microenvironmental scaffold cues with soluble factor cues so as to enhance cell-instruction and tissue regeneration. In order for this aim to be accomplished, we started off by separately examining the control of microenvironmental cues and the regulation of soluble factor cues both *in vitro* and *in vivo*.

In the earlier parts of the research, we modified the microenvironmental scaffold cues of collagen-based scaffolds through various approaches. These approaches included (i) covalent cross-linking of collagen scaffolds to increase their bulk stiffness (chapter 4), (ii) enzymatic degradation of covalently cross-linked collagen scaffolds to decrease their bulk stiffness (chapter 5), and (iii) regulation of collagen fiber structure and rigidity through control of the thermodynamic driving force for collagen self-assembly (chapter 6). Through the various modifications, we were able to generate a range of stiffness in the physiologically relevant range and further regulate the malignancy of hepatocellular carcinoma cells and fibroblasts with these cell-instructive scaffolds.

In the later part of this research, we fabricated stiff and metastable poly(ethylene glycol diacrylate)-polyethylenimine hydrogels for the release of cytokines *in vivo* (chapter 7). The high stiffness of the material, attained from the highly branched architecture of polyethylenimine, allowed the hydrogel to release encapsulated substances independent of local tissue pressures. The decoupled control of stiffness and degradation rate was also achieved by tuning the relative numbers of acrylate and protonated amine
groups in the fabricated hydrogels. Following synthesis, the hydrogels were extensively characterized in terms of their mechanical properties, degradation, cytotoxicity, \textit{in vitro} and \textit{in vivo} drug release. This hydrogel system was also successfully used as an injectable depot for the controlled release of granulocyte colony stimulating factor in porcine models. Although the hydrogel system was only tested with bovine serum albumin and granulocyte colony stimulating factor, we expect this customizable and user-friendly platform to be readily applied to other cytokines.

The separate investigations of microenvironmental scaffold cues and soluble factors cues covered in this thesis would provide an important stepping stone for the subsequent combination of these cues in integrated tissue regeneration and cell-instructive applications. As this research only serves as groundwork in the proposed integrated strategy, new issues and challenges are expected to arise. This area will be examined by other members of the laboratories.
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<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>α-SMA</td>
<td>A-Smooth Muscle Actin</td>
</tr>
<tr>
<td>ΔH</td>
<td>Transition enthalpy of water</td>
</tr>
<tr>
<td>ℵ</td>
<td>Mesh size</td>
</tr>
<tr>
<td>κ</td>
<td>Average bending rigidities</td>
</tr>
<tr>
<td>ρ₀</td>
<td>Density of polymer</td>
</tr>
<tr>
<td>ρₛ</td>
<td>Density of solvent</td>
</tr>
<tr>
<td>τ</td>
<td>Recovery time constant</td>
</tr>
<tr>
<td>ν</td>
<td>Specific volume of collagen</td>
</tr>
<tr>
<td>A</td>
<td>Initial fluorescence</td>
</tr>
<tr>
<td>Aᵢ</td>
<td>Total surface area of collagen fiber</td>
</tr>
<tr>
<td>B</td>
<td>Modified Bessel function</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CAM</td>
<td>Chicken chorioallantoic membrane</td>
</tr>
<tr>
<td>dᵢ</td>
<td>Predicted collagen fiber diameter</td>
</tr>
<tr>
<td>D</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimeter</td>
</tr>
<tr>
<td>E</td>
<td>Compressive Young’s modulus</td>
</tr>
</tbody>
</table>
ECM Extracellular matrix

$F$ Recovery of fluorescence intensity

$F$ Fluorescence intensity

FAs Focal adhesion complexes

FDA Food and drug administration

FTIR Fourier transform infrared spectroscopy

$G'$ Storage modulus

GAG Glycosaminoglycan

GCSF Granulocyte colony stimulating factor

HCC Hepatocellular carcinoma

$k_1$ Degradation rate

$k_2$ Protein release rate

$K$ Boltzmann constant

$I$ Second moment of inertia

$M$ Release exponent

$m_c$ Mass of collagen

$M_\infty$ Total amount of protein released

MRI Magnetic resonance imaging

$M_t$ Cumulative amount of protein released at time, $t$

MMP Matrix metalloproteinase

$M_{\text{PEG1-COL}}$ Mass ratio of PEG-diNHS to collagen

$M_{\text{PEG2-COL}}$ Mass ratio of PEG to collagen

MSCs Mesenchymal stem cells

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

$N_0$ Initial number of cross-links
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>$N$</td>
<td>Swelling exponent</td>
</tr>
<tr>
<td>$n_{bi}$</td>
<td>Number of moles of bound water determined from DSC</td>
</tr>
<tr>
<td>$n_{ci}$</td>
<td>Number of moles of water bound to collagen fibers</td>
</tr>
<tr>
<td>$n_{pi}$</td>
<td>Number of moles of water bound to PEG chains</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethylene imine)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PEG-diNHS</td>
<td>Poly(ethylene glycol) di-(succinic acid N-hydroxysuccinimidyl ester)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PNIPAM</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>$Q$</td>
<td>Degree of swelling</td>
</tr>
<tr>
<td>$Q_0$</td>
<td>Initial swelling ratio</td>
</tr>
<tr>
<td>$Q_m$</td>
<td>Swelling ratio at time, $t$</td>
</tr>
<tr>
<td>$R$</td>
<td>Radius of fibrils</td>
</tr>
<tr>
<td>$R$</td>
<td>Radius of fibers</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>$S$</td>
<td>Shear modulus</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SHG</td>
<td>Second-harmonic generation</td>
</tr>
<tr>
<td>$T$</td>
<td>Temperature</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>$W_{fb}$</td>
<td>Freezable bound water</td>
</tr>
<tr>
<td>$W_{ff}$</td>
<td>Freezable free water</td>
</tr>
<tr>
<td>$W_{ft}$</td>
<td>Total freezable water</td>
</tr>
</tbody>
</table>
1 Introduction

*The motivation, hypotheses and the objectives for this work will be presented in this chapter.*
1.1 Motivation

Integrated tissue regeneration and engineering approaches which combine appropriate scaffold cues with tailored release of soluble factors show great potential in the area of regenerative medicine. However, the design and implementation of such integrated approaches remained hindered by numerous challenges in the respective areas of scaffold fabrication and controlled delivery vehicle design. While there had been ample evidence highlighting the importance of microenvironmental cues on cell phenotypes and activities, the reproduction of such cues in a three dimensional context still posed a challenge due to various confounding requirements in the tissue culture scaffolds. This had motivated our efforts to address some of the issues in scaffold design and to fabricate novel scaffolds. In overcoming some of the challenges and successfully recapitulating various microenvironmental cues, greater control of cell phenotypes would be enabled.

On top of the microenvironmental cues, this study further looked into the design of tailored controlled release vehicles to enable better regulation of local soluble cues. Through the customization of both scaffold and soluble cues, we aimed to achieve greater control of key cellular events for different tissue engineering applications.

1.2 Hypotheses

The key hypotheses in this work are defined as such:

1) To meet the specialized requirements of cell-instructive tissue engineering scaffolds, we hypothesized that collagen hydrogels can be imparted with suitable mechanical and structural cues for cell-instruction.
2) These cues can be introduced through different means *i.e.* chemical modification, enzymatic degradation, and tuning the thermodynamic driving force for collagen self-assembly.

3) The modified scaffolds, presenting key attributes required for cell instruction, can be further used to direct specific cell activities and phenotypes in different cell types.

4) It was further hypothesized that hydrogels can be imparted with specific material properties to allow optimal control of soluble factor release *in vivo*. The hydrogels should ideally be rigid to prevent deformation and uncontrolled drug release due to local tissue pressures, but degrade at a controllable rate to ensure sustained drug release during the therapeutic window.

### 1.3 Objectives

To investigate the above hypotheses, we devised the following objectives:

1) Design and characterize chemically-modified collagen hydrogels with tunable stiffness:
   a) Formulate collagen hydrogels with different stiffness
   b) Investigate material properties of the chemically-modified collagen hydrogels

2) Investigate effect of increasing stiffness on malignancy of a model cell line (hepatocellular carcinoma):
   a) Investigate effect of increasing stiffness on cell morphology
   b) Investigate effect of increasing stiffness on cell function
   c) Investigate effect of increasing stiffness on *in vivo* angiogenic activities
3) Decrease stiffness of chemically cross-linked collagen hydrogels through enzymatic degradation:
   a) Decrease stiffness of cross-linked collagen hydrogel through degradation by matrix metalloproteinases
   b) Investigate material properties of the degraded hydrogels

4) Investigate effect of decreasing stiffness on malignancy of a model cell line (hepatocellular carcinoma):
   a) Investigate effect of decreasing stiffness on cell morphology
   b) Investigate effect of decreasing stiffness on cell function

5) Design and characterize structurally- and mechanically-modified collagen hydrogels generated by varying thermodynamic driving force during collagen self-assembly:
   a) Formulate collagen hydrogels with different structural and mechanical properties by varying thermodynamic driving force
   b) Investigate material properties of the structurally- and mechanically-modified collagen hydrogels

6) Investigate effect of varying structural and mechanical properties on phenotype of model cell line (fibroblasts):
   a) Investigate effect of varying structural and mechanical properties on cell morphology
   b) Investigate effect of varying structural and mechanical properties on cell function
7) Design and characterize poly(ethylene glycol diacrylate)-polyethylenimine hydrogels with tunable mechanical and drug release properties
   
a) Formulate hydrogels with different mechanical and drug release properties
   
b) Testing material properties of hydrogels *in vitro*
   
c) Testing material properties of hydrogels *in vivo*
2 Literature review

This chapter describes the promise and issues faced in tissue engineering and tissue regeneration, with specific focus placed on cell-instructive scaffolds and controlled delivery vehicles.
2.1 Tissue engineering approaches

Annually, millions of people suffer from loss of organ function due to accidents and organ failure. However, the mainstream method of treatment, organ transplant, is greatly limited by the shortage of available donor organs. In view of this shortage, tissue engineering and tissue regeneration have emerged as promising alternative strategies.[1, 2] Tissue engineering involves the engineering and repair of tissues and organs.[2] An overview of the main features in engineered tissues is presented in the following figure (Fig. 2.1). The general approach in tissue engineering involves the harvesting of cells from a donor. These cells are usually expanded in vitro to generate more cells or differentiated through exposure to specific cytokines to yield desired cell types. These cells are then combined with various scaffolds in the absence or presence of additional growth factors to form the synthetic engineered tissue or organ.[3] The engineered product is then re-implanted into the body as a substitute for the failed tissue or organ.

![Figure 2.1 Conventional cycle in tissue engineering.](image)

Several factors are crucial in determining the success of a tissue engineering approach. These include the availability and viability of donor cells, the bioavailability
and the activity of the delivered growth factors, and the ability of the scaffold to support function and integration with local tissue.[1-3] A great deal of research has been undertaken in each of these areas. In this literature review, cell-instructive tissue engineering scaffolds will be covered in the earlier sections, and the delivery of cytokines to further augment tissue regeneration will be covered in the later sections.

2.2 Cell-instructive tissue engineering scaffolds

Traditionally, tissue engineering scaffolds have been designed with the sole purpose of providing structure and support for cell delivery. It has since become apparent that engineered scaffolds can be designed to carry out many other functions in the body.[1, 3, 4] With the right topographical cues, scaffolds can be used to direct cell organization.[3, 5] Not only that, the local availability of bioactive molecules and cytokines can be regulated through encapsulation or tethering.[1] Recent studies have also demonstrated the crucial role of scaffold mechanics in regulating cell signaling and cell cycle.[6] Apart from use in tissue engineering and regeneration applications, these scaffolds are also increasingly used for both cell culture and fundamental science research, in attempts to recapitulate native responses ex vivo.[7] In addition, these scaffolds also serve to bridge the gap between simple two dimensional (2D) tissue culture plates and complicated animal studies as their geometric complexities and intricacies provide a more realistic approximation to native structures.[7]

Due to the polymeric nature of the extracellular matrix (ECM), a wide variety of synthetic and naturally-derived polymeric materials have been utilized in scaffold fabrication. These polymers are processed by a wide variety of methods such to form different types of scaffolds such as foams, meshes, beads and hydrogels to name a few.[1,
In general, stiffer scaffold materials have been used for regeneration of harder tissues such as bone and cartilage.[8, 10] These scaffolds are usually made from hydrolytically degradable materials such as poly(lactide-co-glycolide) (PLGA) and are typically processed under harsh conditions in the organic solvents before subsequent cell seeding.[11] For the repair of softer tissues and organs, hydrogels are preferred due to their structural similarity to native ECM and their mild processing conditions.[1, 4, 7]

### 2.3 Hydrogel scaffolds

Hydrogels, which comprise of cross-linked hydrophilic polymeric networks, are said to have much structural similarity to native ECM components. Thus, they provide a better mimic of key ECM attributes such as growth factor transport and retention.[1, 4, 7] Furthermore, these materials generally facilitate transport of oxygen, nutrients and waste products, and are easily customizable to present different bioactive motifs and adhesion sites.[1, 4, 7] The reactions to form hydrogels are also usually mild and cell-compatible.[9] This allows easy encapsulation of cells within hydrogel scaffolds to provide three-dimensional (3D) stimulation. In addition, most hydrogels or their pre-gel solutions are injectable for minimally invasive surgical techniques.[9] They can also be used to fill tissue defects. Finally, the mechanical properties of certain hydrogels also mirror that of softer tissues in the body, thus allowing mechanotransduction in vivo to be reproduced.[7]

Hydrogels can be broadly classified into two categories – promoting and permissive hydrogels.[1, 7] Promoting hydrogels are capable of promoting cell function, viability and proliferation without the need for additional processing.[1, 7] These hydrogels are usually derived from biological sources and thus contain endogenous cues.
such as growth factors and cell-adhesion sites. Promoting hydrogels include native ECM components such as laminin, fibrin, collagen, Matrigel, and hyaluronic acid. [1, 7] Although these materials promote good cell function, their inherent complexity may render them unsuitable for fundamental scientific studies. Furthermore, as these hydrogel materials are often obtained from animal sources, there exist batch-to-batch variability and concerns of xenozoonosis. In addition, it might be difficult to customize the inherent properties of these natural materials to cater to specific applications.[1, 7]

Permissive hydrogels, on the other hand, maintain the viability of encapsulated cells but might result in compromised cell proliferation, function and spreading. Such gels are usually generated from synthetic polymers such as poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA).[1, 7] Although these materials do not inherently promote cell function, they can be customizable to present appropriate ligands for cell adhesion and cell-signaling.[7, 12] There is also no concern for xenogeneic contamination with these permissive hydrogels. Furthermore, synthetic permissive hydrogels are highly reproducible and customizable as compared to the naturally-derived hydrogels. However, many features might have to be incorporated into the permissive gels before they can possess levels of promoting functions that are similar to the naturally-derived hydrogels.

### 2.4 Hydrogel scaffold design considerations

To generate hydrogels scaffolds for 3D cell cultures, the hydrogels should ideally be both promoting of cellular activities and customizable to meet specific requirements of the study. To bridge the gap between natural and synthetic hydrogel materials, two different approaches have been adopted. The first approach involves modification of synthetic hydrogels through the addition of various cell-promoting factors such as cell-
adhesive peptide sequences and ECM components e.g. collagen and laminin. In the past few years, a large array of cues has been incorporated into PEG-based hydrogels to generate a milieu of specialized hydrogels, thus demonstrating the versatility of this approach.[12] While this method of adding specific cues on demand produces highly customizable hydrogels scaffolds, opponents of the approach argue that these matrices may still not fully recapitulate the cell-promoting functions of naturally-derived hydrogels due to the inherent complexities in the latter.[13] Furthermore, as a number of naturally-derived gels such as collagen and hyaluronic acid have already been approved by the United States Food and Drug Administration (FDA) for medical use, the efforts to reproduce these materials with synthetic hydrogels are criticized by some as re-inventing the wheel.[14-16]

The second approach involves the modification of natural hydrogel materials through various means to impart specific structural and mechanical characteristics.[17] This method may require less effort as fewer attributes have to be imparted to these inherently promoting materials. However, due to the complexities of the natural hydrogels, it can be difficult to predict the final material properties, thus requiring numerous iterations of trial and error before the desired product can be obtained. Furthermore, unwanted changes to material properties such as loss of bioactive moieties might result from the modification of the natural hydrogels.

Regardless of the approach, there are several key requirements that should be present in all good 3D cell culture hydrogels for both tissue regeneration and ex vivo applications. These attributes are (i) well-defined stiffness that is controllable within the physiological range, (ii) presence of cell-adhesion moieties to promote good cell adhesion,
(iii) good permeability to oxygen and nutrients, and (iv) non-toxicity to maintain cell viabilities.[7] Depending on the final intended application, other attributes might also be important. For example, if the hydrogel is intended to allow active remodeling by cells, the material should be cleavable by endogenous matrix metalloproteinase (MMP) produced by the cells; or if the hydrogel is required to gel in situ at a defect site, it should gel under mild conditions within suitable time frames.[1, 7]

Unfortunately, a great obstacle in the design and customization of hydrogel scaffolds lie in the interdependency of the various hydrogel properties. For instance, it may be common to use a variety of cross-linking reactions to alter the mechanical properties of hydrogels. However, such reactions may be accompanied by the undesired reduction in hydrogel permeability which might then result in reduced cell viability (Fig. 2.2). The alternative approach of changing total polymer concentration is likewise accompanied by similar reduction in hydrogel permeability.[18] In addition, the varying of total polymer concentration might also alter the concentration of bioactive components such as cell-adhesion sites in the hydrogel, thus leading to an unintended change in cell signaling.[9, 19, 20]
Figure 2.2 Interdependency of hydrogels stiffness and permeability. In conventional hydrogels, increasing stiffness by either (a) increasing cross-linking density or (b) increasing total polymer concentration result in the simultaneous reduction in permeability.

These obstacles have greatly limited research in this area. Owing to the conflicting requirements in 3D hydrogel design, many experiments involving hydrogels have been confined to the 2D configuration where issues concerning material permeability and cell viability are greatly simplified. However, it is now well-established that there might be limited connection between observations in the 2D and 3D configurations.[7] Cell culture on 2D substrates has now been shown to produce vast differences in cell morphology, phenotype and gene expression (Fig. 2.3). In 2D culture, cells’ interactions with the scaffold are polarized and are limited to a single plane.[7] The cells are also not exposed to any local gradients of soluble factors that are usually present 3D within matrices. Moreover, the migration of cells and interaction between neighboring cells on a 2D substrate is also altered.[7] As such, the 2D format provides a poor approximation to physiological conditions, and observations made in this configuration should not be generalized to cells in vivo and in 3D hydrogels.
Figure 2.3 Culture of liver cancer cells in 2D and 3D. (a) When the liver cancer cells are cultured in 2D, they spread and proliferate extensively to form cell layers. (b) When the liver cancer cells are cultured in 3D matrices with liver-like stiffness, the cells are well-organized into spheroids with suppressed proliferation. (Scale bars represent 50 µm)

Albeit there are still many new studies adopting the 2D culture format, increasing efforts have gone into surmounting the challenges in 3D hydrogel design. For instance, the dependency between hydrogel stiffness and permeability had recently been decoupled by introducing pendant chains into PEG-based polymer network.[19] By varying the ratio of the pendant PEG chains to bi-functional PEG, our group was able to vary the modulus of the hydrogel while minimally changing its permeability. In another work to decouple polymer stiffness and hydrogel swelling ratios, it was found that the incorporation of methacrylic alginate into a poly(ethylene glycol) dicarlylate (PEGDA) hydrogel allowed the stiffness to be modulated while minimizing changes to the hydrogels’ swelling ratios.[20] These innovative ways of decoupling interdependent parameters contribute significantly to the tool sets for customizing 3D hydrogels.

When designing hydrogels matrices for 3D culture, it is essential to keep in mind the key requirements for 3D cell culture scaffolds, specific considerations for the study at hand, and the cells’ native microenvironment. For basic science studies investigating the
cell-ECM interactions, it may be crucial to have good control over specific parameters of interests while minimally affecting other parameters. However, for tissue regeneration applications, it is advantageous to mimic the native ECM and promote cell-mediated matrix remodeling. All-in-all, it is paramount for tissue engineers to be equipped with a sound understanding of the native ECM before embarking on hydrogel scaffold design and fabrication.

2.5 Native ECM

Before designing 3D hydrogel scaffolds, it is essential to have a sound understanding of the native ECM in terms of its unique structure and function. The ECM comprises an intricate network of proteins such as collagen, fibronectin and laminin, and polysaccharides such as glycosaminoglycan (GAG) (Fig. 2.4).[21, 22] The major component of the ECM is triple-helical collagen fibrils. Depending on the tissue location, collagen within the ECM is further processed to impart requisite properties. For instance, collagen within tendons assemble into rope-like structures which provide tremendous strength, and collagen fibrils in skin and tendon undergo increased cross-linking to provide greater tensile strength.[21, 23, 24] Another key component in ECM is fibronectin. Fibronectin is a multi-domain glycoprotein. The domains include the heparin-binding domain, the collagen-binding domain, and the well-known peptide sequence Arginine-Glycine-Aspartic Acid or RGD that is responsible for cell-binding. Fibronectin is not only important for cell-adhesion, the location and distribution of fibronectin can also guide cell migration.[21, 25] The ECM is also composed of the basal lamina which is formed primarily by laminin. One essential function of the basal lamina is to act as a selective barrier to cells. For instance, the basal lamina beneath the
epithelium permits the movement of macrophages, lymphocytes and nerve processes through it but blocks the transport of fibroblasts. The basal lamina also plays key roles in regulating the function of synapses and neuromuscular junctions.[21] Other than the protein constituents, the ECM also contains hydrophilic GAG chains. These chains regulate the turgor pressure of the ECM through their specific swelling.[21, 26] They also regulate the presentation of bioactive molecules to cells through local sequestration and sieving action.

**Figure 2.4 The ECM is an intricate network of proteins and polysaccharides.** The proteins in the ECM include collagen, fibronectin and laminin. The cells interact with the ECM through integrin receptors. [22]

The ECM is a dynamic structure that is in constant communication with the cells. It is remodeled by adjacent cells through cell-exerted forces, and is also continually renewed through well-regulated processes of matrix degradation and synthesis.[21, 23] At the same time, the ECM provides a myriad of cues to direct organization of cellular
cytoskeleton, cell signaling, and function. Through the study and understanding of cell-ECM interactions, the design of cell-instructive hydrogel scaffolds can be further improved and optimized.

2.6 Cell-ECM interactions

The cell surface possesses two classes of receptors for interaction with the ECM. These receptors are the non-integrin and integrin receptors. The non-integrin receptors include a number of laminin-binding proteins and proteoglycans such as CD 44 and syndecan. CD 44 is able to bind to a variety of ECM components including type I collagen, type IV collagen, and GAG; while syndecan is able to bind with collagen, fibronectin and growth factors such as basic fibroblast growth factor (bFGF).[27] Expression changes of certain non-integrin receptors have been associated with changes in cell adhesion, migration, morphology, and cell differentiation. The integrin receptors are a subset of the glycoprotein receptors.[27] These receptors are made up of the non-covalent association of α and β subunits. In mammals, eighteen α subunits and eight β subunits have been characterized. This allows a wide variety of integrin receptors to be formed from different combinations of α and β subunits. α subunits recognize different short sequences present in the ECM and are responsible for ligand specificity. β subunits, on the other hand, are said to have limited ligand specificity and are more essential for cytoskeletal association and specific intracellular changes. [27, 28]

As cells bind to ECM components through different integrin or non-integrin receptors, changes in their intracellular domains occur. This in turn mediates transformations in the cytoskeleton near the adhesion sites. Subsequently, other intracellular proteins may be roped in to form focal adhesion complexes (FAs).[29]
formation of such FAs can then further promote longer range cytoskeleton rearrangement to alter cell shape or chromatin configuration. As a result of cell-binding to specific ECM components, different cascades resulting in altered gene expression, proliferation or differentiation may be triggered.[29]

Many studies have focused on relating changes in ECM properties to different cellular responses. The ECM cues that are most commonly investigated in these studies are surface chemistry, topographical features, and mechanical properties.[6, 30-32] These cues can also be applied in combination within complex 3D environments. Such investigations of different cell-instructive ECM cues may shed light on the complex changes in ECM and cells during both physiological processes (such as cell differentiation and wound healing) and pathological changes (such as malignant transformations). The studies will also facilitate the design of specialized hydrogel matrices for directing specific cell responses such as cell proliferation or differentiation.

2.7 Scaffold-directed cell responses

As mentioned, the roles of different scaffold cues (such as surface chemistry, topography, and mechanical property) on cell behaviors have been the subject of much investigation.[6, 30-32] Due to the inherent complexities on the native ECM, many of these studies were conducted using customized synthetic scaffolds. The study of surface chemistry-cell relationships has been made possible by many enabling technologies and has led to substantial useful outcomes. The invention of new conjugation strategies has made the grafting of different moieties on various substrates possible; and the inclusion of flexible tethers has allowed cells to remodel covalently conjugated signaling molecules.[30] The screening of different surface-conjugated molecules has led to the
discovery of many active molecules capable of supporting cell growth and cell spreading. These molecules include peptide sequences, such as the well-known RGD sequence, and carbohydrates such as galactose.[30] Such moieties are now incorporated into many cell culture scaffolds to impart cell-adhesive functions. Another example of a key discovery in this area is the applicability of thermo-responsive poly(N-isopropylacrylamide) (PNIPAM) in cell culture.[33] By changing the ambient temperature, this polymer allows the attachment and detachment of cells through the changes in the polymer’s wettability. This polymer system is now widely used in the generation of cell sheets for tissue regeneration.[33]

With respect to topographical cues, nano- and micro-scale topographical features such as grooves and pillars have been patterned onto different substrate surfaces.[31, 32] These topographical features may also be accompanied by further chemical modifications. In general, the features may be broadly classified into two categories, namely, anisotropic and isotropic patterns.[31, 32] Anisotropic patterns such as grooves tend to result in pronounced changes in cell shape. These changes in cell shape may in turn induce polarized cytoskeletal tensions and may lead to subsequent changes in nuclear shapes and cell migration tendencies.[31, 32] It has been shown in different studies that the up-regulation of cytoskeletal tension on these anisotropic substrates enhanced the differentiation potential of mesenchymal stem cells (MSCs) into elongated cell lineages such as neurons and osteoblasts.[34, 35] When cells are cultured on isotropic patterns, they tend to undergo less obvious changes in shape. However, these cells might still show pronounced differences in several cell functions such as adhesion, proliferation and differentiation.[34, 36] It was found that when neural stem cells were cultured on
isotropic fiber meshes with decreasing fiber diameters from 1452 nm to 283 nm, there was a corresponding increase in proliferation and decrease in aggregation.[37] The culture on the fibers of different diameters also resulted in different differentiation characteristics. When cultured on the smallest fibers, the cells had a higher propensity to differentiate into oligodendrocytes.[37] However, when grown on fiber with intermediate thickness, the cells had higher propensity to differentiate into neurons.

Of the different scaffold cues, the effect of scaffold mechanical property has generated the most interest among cell biologists. While the chemical and topographical cues under investigation are usually synthetic in nature, mechanical property of the native ECM has been observed to vary according to tissue location, physiological states and pathological states. In the body, the stiffness of the ECM may vary up to several orders of magnitude from the brain to mineralized bone.[6, 38] As there are many methods to quantify and control the mechanical properties of both natural ECM and synthetic scaffolds, matrix rigidity can serve as a useful design parameter for the design of cell-instructive hydrogels.[23] To better utilize matrix rigidity as a scaffold cue in cell-instruction, the reciprocity between matrix mechanics and cellular activities should be thoroughly studied.

2.8 Effect of matrix stiffness

Cells probe and sense the elasticity of their substrates through different means. In load-bearing tissues such as bones, cells undergo mechanical stress and strain from the ECM, in the form of outside-in simulation.[6, 39] At the same time, cells in other softer tissues may also detect ECM mechanics through inside-out simulation, by pulling on their substrates.[6, 39] In this mode, forces generated by cells’ myosin are transmitted
through FAs to tug on the surrounding matrix. These FAs are integrin clusters that physically link the cells’ actin cytoskeleton to the ECM. According to the different resistances sensed by the cells, their cytoskeleton is further reorganized to regulate cell shape.[40] Concurrently, the activation state of FA kinases might be altered to regulate downstream cellular processes such as proliferation and differentiation.[41] Recent data has shown that while cells experiencing tensional stresses exhibited increased proliferation, compression tended to suppress proliferation and slow growths.[42-44] Such mechanical feedback helps in maintaining regular tissue shape and tissue homeostasis. The role of stiffness in regulating differentiation has also been highlighted in numerous studies. In embryogenesis, key changes leading from convergence and extension movements during the gastrulation of *Xenopus laevis* were shown to only occur when the mesoderm and notochord cell layers were stiff enough to withstand buckling.[45] In a separate study, it was also found that gene expression during early embryonic development was significantly altered by substrate stiffness. On stiffer substrates, several genes implicated in early mesoderm differentiation such as *Foxa2* and *Brachyury* were up-regulated as substrate stiffness increased from 41kPa to 2.7 MPa.[46] There was also a subsequent up-regulation of osteogenic differentiation of the mesoderm on the stiffer 2D substrates.

Other than regulating key cellular processes, aberrant mechanical stimuli are also associated with abnormal signaling in different diseases such as cancer and fibrosis. This area has evoked great interest as greater understanding of the reciprocal relationship between tissue stiffness and disease state might yield novel preventive measures and alternative therapeutic approaches.[44, 47]
2.9  Effect of matrix stiffness on cancer cells

In recent years, there is increasing evidence that tumor malignancy is not only directed by genetic mutations in the cells but also by the cells’ microenvironment. Tumors are usually characterized by their increased local stiffness which renders them palpable or detectable through transient elastography.[48] The stiffer tumor tissue is often accompanied by increased collagen cross-linking and collagen deposition.[49, 50] Cancer cells are also shown to possess increased sensitivity to substrate stiffness as compared to their non-malignant counterparts.[51] This increased sensitivity is mediated by increased activation of Rho-dependent actin-myosin contraction and enhanced actin assembly.[44] In addition to its role in regulating cellular tension, Rho activation has been linked to increased cell proliferation in many cancers. Conversely, the inhibition of the Rho-dependent actin-myosin contraction through genetic manipulation or pharmacological action significantly reduced cellular tension and resulted in less malignant phenotypes.[51]

To investigate the effects of substrate stiffness on cancer malignancy, a milieu of *in vitro* and *in vivo* experiments had been carried out both in the 2D and 3D format. In the 2D configuration, it was observed that for certain cancer cells, such as breast and lung cancer cells, increased matrix stiffness tended to result in increased proliferation and more malignant phenotypes.[52, 53] This could be attributed to the integrin clustering in the 2D configuration which resulted in the formation of FAs, subsequently leading to the downstream activation of Rho. However, in the 3D configuration, mixed effects have been observed. While several studies reported similar increase in malignancy with increasing stiffness, other studies observed reduction in cell proliferation and increase in
apoptosis when matrix stiffness was increased.[51, 54] The diverse effects could be attributed to the difference in ranges of mechanical properties being investigated. Alternatively, due to the diversity of the 3D matrices under investigation, these matrices could also provide vastly different mechanical cues to the entrapped cells as a result of the inherent differences in matrix structure and organization.[51, 54] The findings from these studies in the 3D configuration seemed to suggest that while increased tensional strains led to increased proliferation and cell migration, high compressive strains tended to result in suppressed proliferation and increased apoptosis. Such regulation is not unlike the tissue homeostasis observed in oogenesis.[42, 43]

These studies, coupled with the observation that tumor tissues generally possess increased stiffness, have led to the conjecture that tumors in the body could have arose from abnormal increase in the ECM stiffness.[55] However, emerging research has also demonstrated that conditional activation of Rho-associated protein kinase (ROCK) in cancer cells subsequently led to increased collagen deposition and increased stiffness of the surrounding ECM.[55] Hence, although tumors are often observed with increased ECM stiffness, it is still debated if the increased ECM stiffness caused tumors or *vice versa*. However, from the numerous in *in vitro* and *in vivo* experiments, it appeared that any departure from normal matrix stiffness likely increased the propensity for malignant transformations through aberrant cell-signaling.

### 2.10 Effect of matrix stiffness on fibroblasts

Apart from cancer, profound changes to local tissue stiffness have also been observed in tissue fibrosis or pathological cirrhosis.[56] Such observations are related to the activation of resident fibroblasts. When subjected to different stimuli, the fibroblasts
undergo various phenotypic and morphological transformations. These activated fibroblasts termed protomyofibroblasts, developed stress fibers, showed up-regulated expression of actin, and developed stronger cell-cell interactions.[57, 58] Through these concerted changes, the protomyofibroblasts formed bundles that had much higher contractile function than the non-activated counterparts. Further activation subsequently resulted in the transformation into myofibroblasts, characterized by the expression of α-smooth muscle actin (α-SMA) and the exhibition of even greater contractile activities. [57, 58]

Both in vitro and in vivo experiments have demonstrated the strong link between local mechanical stiffness and the activation of residing fibroblasts.[47, 56] During tissue injuries, the local disruption of the ECM can also lead to the indirect up-regulation of cellular tension, which in turn brings about fibroblasts activation. Alternatively, the fibroblasts may be activated by cytokines which are locally released from inflammatory cells during tissue injury. One major activator of fibroblasts in pulmonary fibrosis is transforming growth factor-β (TGF-β).[59] The activated protomyofibroblasts and myofibroblasts subsequently remodel the ECM through increased matrix synthesis, deposition, and increased contraction.[58, 60] Such fibroblast-mediated remodeling serves to speed up wound healing by closing up gaps in the ECM. Under normal conditions, the reconstructed ECM takes over the mechanical load and shields the protomyofibroblasts or myofibroblasts from additional stress.[61] This stress release subsequently promotes apoptosis of the myofibroblasts to prevent excessive contraction and abnormal stiffening of the local tissues, thus restoring normal tissue function.[61]
Several factors, however, could result in the failure of the myofibroblasts to undergo timely apoptosis. These include persistent up-regulation of inflammatory cytokines due to repeated injuries or over-stiffening of the local ECM.[62] These factors have been identified as the culprit for abnormal fibrosis or cirrhosis, resulting in tissue damage and ultimately tissue failure. Recent studies have shown the reduction of intracellular tension to be promising for managing the activation state and subsequent activities of fibroblasts. This can be achieved by increasing ECM compliance or by blocking cell-ECM interactions.[63] However, many of these studies were conducted in the 2D dimension. To further refine these strategies, the development of better in vivo and 3D in vitro models will provide a better understanding of pathologic fibrosis. In addition, it is also necessary to generate more refined methods to accurately quantify the stiffness of tissues and fabricated scaffolds at different length-scales.

2.11 Quantification of matrix stiffness

While there is increasing awareness of how scaffold and ECM stiffness may affect cell phenotypes in physiological and pathological processes, the different studies employ diverse strategies to quantify the matrix stiffness and differ on the stiffness values reported. The local stiffness of the ECM or scaffold is usually quantified by the bulk elastic modulus of the material.[6] The elastic modulus quantifies a material’s intrinsic resistance to stress and is defined as the slope of a stress-strain curve in the elastic deformation regime. A stiffer material will have a higher elastic modulus while a softer material will have a lower elastic modulus.

A variety of macro-scale and micro-scale techniques have been developed to quantify the mechanical properties of scaffolds and soft tissues.[6, 64] The most
commonly used methods include macro-scale methods implemented by applying a compressive or tensile force and then measuring the relative change in length or strain. Such approaches are commonly used due to ease of measurement and the availability of the machines.[6, 64] However, these methods only capture the macroscopic or bulk material stiffness and tend to overlook the microscopic features and subtleties in the native ECM and in synthesized scaffolds.[6, 64] In particular, the length and time scales that are relevant to cell-sensing are often not captured in macro-scale measurements. To obtain mechanical properties in a more physiologically relevant length-scale, alternative methods such as measurement with atomic force microscopy, micro-, and nano-indenters have also been used.[64] However, these methods may also be accompanied by other disadvantages such as specialized equipment and high cost. Due to the innate softness of biological samples, the capillary forces experienced by the tips or indenters might also exceed the forces associated with the indentation of the sample.[64] Furthermore, due to the sensitivity and compliance of biological samples, the measurement errors are also compounded by variations in sample preparation methods. For example, the reported elastic moduli of brain tissue might vary by a factor of 2 or more depending on the method of preparation.[65] An alternative method of determining local or micro-scale mechanical properties would be to obtain these properties through available models or equations. For example, with the storage modulus and mesh size of a polymer network, it is possible to calculate the rigidity of the individual fibers from the MacKintosh model.[66] Nevertheless, such methods are usually subjected to various assumptions and thus are only good for providing estimates.
Although it remains a challenge to obtain accurate and consistent mechanical measurements of soft tissues and biological samples, the key message is that there are inherent differences between the mechanical properties of different tissues such as bone, brain, muscles, fat, and liver.[38] These differences could attribute to phenotypic differences of the different cell types.[67] Due to the possible variations generated by different methods of sample preparation and measurements, it is ideal to use a standard method of sample preparation and measurement when comparing different conditions. In addition, when interpreting stiffness values published in the literature, one should remember that different methods of measurements often yield different values. Furthermore, it is important to keep in mind that the macro-scale methods of measuring mechanical properties tend not to account for micro-scale features in the cell-sensing length-scales, such as stiffness gradients and differences in local rigidities. Having discussed how tissue and scaffold stiffness might be quantified, the various methods for controlling scaffold mechanical properties will next be discussed.

2.12 Scaffold modification to control stiffness

With increasing understanding of how mechanical properties regulate cellular processes, many studies have started investigating cell-instructive scaffolds with tunable mechanical properties. A wide variety of techniques has been devised to both increase and decrease elastic moduli of hydrogel scaffolds. One common method for increasing elastic modulus involves increasing the concentration of the bulk constituting polymer.[19, 20] For example the elastic moduli of agarose or collagen gels are typically controlled by varying agarose and collagen concentrations respectively. With this method, it is easy to generate a spectrum of mechanical properties. However, one major
concern is the significant decrease in material permeability with increasing polymer concentration.[19, 20] While this does not pose a major concern when cells are cultured in the 2D format, decrease in material permeability in 3D scaffolds may restrict the transport of nutrients and oxygen to cells. Furthermore, when natural polymers such as collagen are used, changing the concentration of collagen also means simultaneous changes in bioactive motifs such as cell-binding sites. These changes might alter cell activity and complicate scientific studies.[68]

An alternative method for controlling hydrogel stiffness would be to regulate the cross-linking density of the hydrogel network whilst maintaining constant total polymer concentration. This can be implemented in various ways. For example, a greater number of reactive functional groups can be grafted onto the polymer backbone chain to allow a greater degree of cross-linking.[69] By varying the degree of substitution on a polymer backbone chain, a spectrum of cross-linking densities and elastic moduli can be generated. In the case of photo-polymerizable hydrogels, the degree of cross-linking can also be regulated by varying the initiator concentration and the irradiation duration and intensity.[70] This method is easily implemented without the need for prior chemical modification. Alternatively, the cross-linking density of a hydrogel network can also be controlled by regulating the cross-linker concentrations. A large variety of cross-linkers are now available for reaction with different end groups. These include homobifunctional cross-linkers, hetero-bifunctional cross-linkers, and multi-arm cross-linkers. Cross-linkers of different lengths have also been used to generate zero-length cross-links, short bridges, or long bridges.[71, 72] Apart from chemical cross-linkers, enzymes e.g. lysyl oxidase and transglutaminase, and biochemicals e.g. ribose have also been used to
cross-link natural polymers such as collagen.[73-75] Whilst the cross-linking approach allows stiffness to be tuned without changing the concentration of the main constituting polymer, other concerns such as possible cytotoxicity of initiator, cross-linker or irradiation should also be considered. Prior to implementation, there needs to be thorough investigation to ensure that the chosen method of cross-linking does not significantly affect the viability of the cells. This is especially important for 3D in situ cell encapsulation. Furthermore, the permeability of the hydrogel scaffolds might still be significantly decreased if the cross-linking density is too high.[9, 19, 20] Hence, this is another key parameter that should be examined. The permeability of hydrogels usually depends on the polymer concentration, cross-linking density, length of polymer precursor, and nature of polymer.[76] It should be noted that hydrogels made from shorter synthetic polymers such as PEG ($M_w < 5000$ Da) tend to have smaller mesh sizes and lower permeability while hydrogels from natural polymers such as collagen tend to have larger mesh sizes and higher permeability.[76] While cross-linking with enzymatic and biochemical cross-linkers are associated with lower cytoxicity, it should also be noted that these methods often generate a smaller range of stiffness and may yield less consistent results.

In addition to the cross-linking of polymeric hydrogels to increase the overall elastic moduli, the local moduli of fibrous hydrogels can also be tuned by controlling the local diameters and rigidities of constituting fibers.[77] One common way of varying the fiber diameter of hydrogel is to use electrospinning. In this method, various parameters such as the flow rate and charge can be varied to control the final fiber sizes.[78] However, with this method, it might be challenging to encourage cell migration into the
electrospun scaffolds for subsequent cell culture.[79, 80] Alternatively, electrospinning can also be used for the direct encapsulation of cells within the fibers through the incorporation of cells into the pre-polymer solution.[81] Another approach for varying the diameters of self-assembling fibrous hydrogels is to change self-assembly conditions such as temperature and pH.[32] While these methods have been used to successfully vary the fiber diameters of self-assembling fibrous gels such as collagen gels, there has been limited application to *in situ* cell encapsulation as the varying temperature and pH might significantly alter cell viability and activities. As such, new methods to control fiber diameters of fibrous gels have to be examined.

Other than these common methods to increase scaffold rigidity, several approaches for softening scaffolds have also been proposed. The gradual degradation or softening of pre-formed scaffolds is often important in *in vivo* applications so as to allow the regenerating host tissues to gradually take over and perform their functions.[82] This gradual softening or degradation can be achieved by employing degradable natural polymers or by adding cleavable sequences such as MMP-cleavable peptide sequences to synthetic hydrogels.[82] In more recent years, the softening of hydrogels on demand has also been explored. Cell-encapsulating hydrogels that can be softened on demand can serve as useful models allowing the study of dynamic responses such as proliferation and cell migration in response to introduced changes in tissue stiffness. The softening of pre-formed scaffolds on demand can be easily achieved through the enzymatic degradation of degradable hydrogels or by using readily cleavable bonds such as disulfide bridges.[83] In addition, pH-sensitive polymers have been incorporated into hydrogels to allow tuning of mechanical properties with changes in pH.[84] More recently, photodegradable
groups were also incorporated into PEG hydrogels to allow for both spatial and temporal regulation of hydrogel stiffness by using controlled laser pulses.[85] Although there are various approaches to soften pre-formed hydrogels, it is essential to ensure that the approaches used for softening, be it enzymatic degradation, reduction of disulfide bridges, or photo-degradation, do not affect cell viability or activity.

As discussed, there are many different approaches to control both the overall and local stiffness of hydrogel scaffolds. Each of these methods presents their unique advantages and disadvantages. However, when choosing appropriate modification methods and hydrogel platforms, it is essential to also consider to ultimate application of the hydrogel scaffold. In this study, the hydrogel scaffolds will be designed for use ex vivo as models for studying cell-scaffold relationships, and also in vivo as cell-support system. To meet the varied requirements, we will be using collagen-based hydrogel scaffolds. The main features and advantages of collagen-based hydrogels will next be discussed.

2.13 Collagen-based hydrogel scaffolds

Collagen is widely used for many biomedical applications as it is the most abundant protein in mammalian tissues and also the main component of natural ECM.[86] Bovine collagen has also been approved by the FDA for various invasive and non-invasive medical applications.[16] Acid-solubilized collagen strands can self-aggregate to form stable fibrils and fibers.[87] These fibers can further entangle to form collagen hydrogels. Due to their bio-active and cell-adhesion sequences, collagen hydrogels are natural promoters of cell-activities. Furthermore, the mechanical properties of collagen hydrogels can be increased by introducing various chemical cross-linkers (e.g. bi-
functional cross-linkers and glutaldehyde), biomolecules (e.g. ribose), by crosslinking with physical treatments (e.g. UV irradiation, freeze-drying, heating), or by cross-linking with enzymes (e.g. lysyl oxidase).[88-91] The stiffness or collagen hydrogels can also be decreased through enzymatic degradation by MMPs or collagenase.[21] Furthermore, the fibrous structure of collagen hydrogels can also be tuned by altering the self-assembly conditions such as temperature and pH.[77] Due to the customizability and cell-promoting activities of collagen, collagen-based hydrogels will be modified by different strategies to generate various microenvironmental cues in cell-instructive scaffolds.

Following the earlier discussions of cell-instructive scaffolds, the second component of tissue engineering i.e. soluble cues will be discussed in the coming section.

2.14 Controlled delivery vehicles used in tissue engineering

As mentioned in the earlier sections of this chapter, the success of tissue engineering and regeneration strategies is dependent on three different factors i.e. the cells, the scaffold and the soluble factors. [1-3] While the focus in the earlier sections was primarily on the scaffolds, we now switch gears to the provision of soluble factors to regulate tissue regeneration. Tissue regeneration usually takes place in many steps. For example, the cascade of events required for endogenous liver regeneration is regulated by a myriad of soluble factors including hepatocyte growth factor, epidermal growth factor, urokinase-type-plasminogen-activator, TGF-β1, epiregulin, tumor necrosis factor-α, norepinephrine, α-2-macroglobulin.[92] These factors are tightly regulated in terms of their spatial and temporal presentation to ensure that each of the steps in the regeneration cascade is performed correctly.
The different classes of soluble proteins and peptides that are used for tissue regeneration and tissue engineering applications include growth factors, anti-inflammatory factors, anti-apoptotic factors, chemokines, colony stimulating factors and MMPs.[93-98] Growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and bone morphogenic protein (BMP) have been used to stimulate the local vascularization, growth, proliferation and differentiation of target tissues.[93, 98] Both anti-inflammatory factors and anti-apoptosis factors such as IL-10 and BH-4 anti-apoptotic peptides have been extensively employed to promote the survival of regenerating tissues at sites of injuries.[94, 95] Chemokines, also known as chemotactic cytokines, play an important role of recruiting endogenous stem and progenitor cells to carry out repair of the damaged tissues.[96] The recruited stem and progenitor cells may also secrete various cytokines to promote tissue repair. Colony-stimulating factors such as the granulocyte colony stimulating factor (GCSF) stimulate the bone marrow to produce granulocytes and stem cells and to subsequently release them into the circulating blood. [96] The circulating stem cells and granulocytes are then recruited to the sites of tissue injuries by the locally up-regulated chemokines. Finally, MMPs may be delivered to promote remodeling of damaged tissues, a key step in the regeneration process.[97] Apart from these proteins and peptides, a variety of genes have also been delivered in to carry out some of the functions described above.

A wide variety of delivery vehicles have been designed to deliver the various cytokines. The most commonly used delivery vehicles include polymeric microcarriers such as PLGA microparticles.[99] To encapsulate soluble factors into these particles, the soluble factors are often dissolved in the aqueous phase while the polymers are dissolved
in the organic phase.[99] Phase separation subsequently allows the encapsulation of the soluble factors within the polymeric shells. Following fabrication, the microcarriers are readily injectable into the target site due to their micron size. These particles are highly customizable and can be made into core-shell particles to sequentially release different soluble factors.[100] The encapsulated molecules are then slowly released for periods of weeks to months through diffusion out of the microcarriers, and through the degradation of the polymeric shells.

Another class of commonly used delivery vehicle is the hydrogels.[101-103] With hydrogels, the soluble factors may be incorporated directly into polymer precursors before cross-linking of the hydrogel network is induced.[102, 103] This method of encapsulation is usually applied when the cross-linking reaction to form hydrogel network is relatively mild. Another way of encapsulating soluble factors into the hydrogel network involves swelling of pre-formed dehydrated hydrogel network in a small volume of the soluble factors solution.[103] In this method, the bioactivity of the encapsulated substances is preserved independent of the initial cross-linking reaction. The drug-loaded hydrogels can be introduced into the body through direct injection of the soft gel or through the injection of the drug-loaded hydrogel precursors before inducing hydrogel formation in situ. The encapsulated substances are usually released over hours to weeks depending on the type of hydrogels used. The release of encapsulated substances is dependent on the swelling of the hydrogel and the degradation of the hydrogel network.[102, 103] To increase retention time of the encapsulated soluble factors, they can be further conjugated to the polymer network through physical interactions or covalent tethering.[102, 103] One of the main drawbacks of the hydrogel
delivery system is its low modulus. Due to the low modulus of the hydrogel systems, the gels might be compressed by local tissue pressures or by the movement of the patient. This might result in premature release of the encapsulated substances.

While nanoparticles have been extensively researched for anti-cancer therapies, they have only been recently applied to tissue regeneration applications. The use of nanoparticles has been shown to increase the plasma half-lives of soluble proteins, and the conjugation of targeting ligands onto nanoparticles can allow their targeted delivery to specific cells.[104] Owing to the size of nanoparticles, they readily enter target cells by endocytosis.[105] There are many different forms of nanoparticles that have been used for delivery for drugs and proteins. These include liposomes, polymeric particles, metallic particles, and self-assembled particles.[106-109] In a recent study, it was demonstrated that nanoparticles made from self-assembled fusion proteins comprising of elastin-like peptides and keratinocyte growth factor were able to localize to wounded dermis for wound-healing and skin regeneration.[109]

The diverse range of delivery vehicles, with their unique attributes, provides a multitude of options for the delivery of various proteins and genes. With these controlled delivery platforms, the provision of key molecules can be customized to meet the needs of important tissue regeneration events. The combination of the controlled delivery platforms with tissue engineering scaffolds is likely to generate a synergistic effect on tissue regeneration and tissue engineering applications. In the next section, the integrated application of scaffold cues and soluble factor cues will be discussed.
2.15 Integrated tissue engineering approaches

In the earlier sections, we have discussed how both mechanical and soluble cues are important in regulating cellular activities and tissue repair. In integrated tissue engineering approaches, scaffold cues are integrated with soluble factors cues to synergistically enhance tissue repair and regeneration.[110, 111] This integrated tissue engineering approaches may take several forms. In the simplest form of such integrated tissue engineering approaches, delivery vehicles such as growth factor-loaded microspheres have been incorporated into a variety of different scaffolds such as collagen sponges, silk fibroin scaffolds and poly(L-lactic acid) nano fibrous scaffolds to increase tissue vascularization and neogenesis in vivo.[112-114] In the various studies, it was demonstrated that tissue healing was enhanced with the integrated platform as compared to control conditions with only the scaffold or the microparticles. The incorporation of microparticles into these various scaffolds is relatively straightforward and usually involved the mixing of the microparticles with scaffold polymer precursors.[110, 112, 113] This strategy also allowed the scaffolds’ surface properties and soluble factor release kinetics to be tuned independently with different combinations of scaffolds and delivery vehicles. However, despite the seemingly straightforward procedures, the incorporation of delivery vehicles into scaffolds is not without challenges. For, instance, the use of incompatible materials may result in phase separation between delivery vehicle and scaffold. Also, the addition of microparticles may complicate the fabrication of tissue engineering scaffolds. This is especially true for fabrication processes that involve small openings and fine nozzles. For example, the addition of microspheres into pre-polymer solutions might hinder the electro-spinning of fibrous scaffolds. Furthermore,
the material properties such as porosity and elastic modulus of the integrated construct might also be different from that of the blank scaffold. Not only that, the release kinetics of encapsulated drugs might also be altered due to the presence of an additional barrier i.e. the scaffold. As many of these challenges can be circumvented through careful design and experimentation, it is crucial for researchers to be aware of these issues when incorporating delivery vehicles into existing scaffolds.

Another approach to integrate soluble factor cues and scaffold mechanical cues would be to introduce the soluble factors directly into or onto the scaffolds. For example, BMP-2 has been directly incorporated into poly(L-lactic acid) (PLLA) solutions for fabrication of electrospun fibrous scaffolds. In the study, it was shown that the addition of BMP-2 significantly improved the regeneration of a bone defect as compared to a blank PLLA fibrous scaffold. Although the bypass of delivery vehicles omits several associated problems such as phase separation and clogging of nozzles, other concerns such as loss of protein bioactivity and limited control of protein release come into the picture.

While most integrated tissue regeneration approaches involve the local combination of both soluble and mechanical cues, several studies have also introduced soluble and scaffold cues at different sites. For instance, in one instance, the local delivery of tissue-promoting scaffolds at the infarct area had been coupled with the remote delivery of colony stimulating factors. In this study, the injection of colony stimulating factors promoted the production of stem cells in the bone marrow and the subsequent release of these cells into the circulating blood in a process termed stem cell mobilization. These mobilized cells then preferentially migrate to the site of tissue injury
to augment tissue repair and regeneration. While there are few prior studies adopting this strategy, such an approach is slowly gaining ground as it harnesses the body’s natural regeneration capacity.

The design and application of these integrated platforms demonstrate significant progress in multi-disciplinary efforts integrating the areas of biology and biomaterial engineering. It also represents a greater understanding and mimicry of the body’s natural regeneration processes. The early research in these integrated approaches have yielded promising results and revealed many new challenges to be overcome. With further research and refinement, it is the hope that these platforms will eventually provide solutions to different medical challenges.
3 Materials and method

This chapter describes all the materials, methods and the equipment used in this work.

The overall experimental strategy in this work is presented in Fig. 3.1.
3.1 Overview of experimental scheme

The experimental scheme in this work is presented in the overview (Fig. 3.1). The experiments were divided into two sections. The first section involved generation of the cell-instructive scaffolds while the second section involved the synthesis of the controlled delivery vehicles.

To generate the cell-instructive scaffolds, collagen gels were modified by different approaches. The first approach involved cross-linking of collagen gel with bi-functional PEG cross-linkers to increase bulk gel stiffness (Chapter 3.3). In the second approach, the covalently cross-linked collagen gels were softened by partial digestion with MMPs (Chapter 3.18). The final approach involved modification of the local rigidity of collagen fibers by changing the fiber bundle thickness. This was achieved through regulation of the collagen gel structures by tuning thermodynamic driving force during collagen self-assembly (Chapter 3.3). The modified cell-instructive collagen scaffolds were further used to control the malignancy of hepatocellular carcinoma cells (HCCs) (Chapter 3.11) and to control the activation state of fibroblasts (Chapter 3.12).

The controlled delivery vehicles investigated in the second section were PEGDA-poly(ethylene imine) (PEI) hydrogels synthesized through Michael reaction between PEGDA and PEI (Chapter 3.21). These hydrogels were extensively tested before they were finally evaluated in vivo in a porcine model.

In the subsequent chapters, all materials were obtained from Sigma-Aldrich unless otherwise specified.
Figure 3.1 Overall scheme of experiments. (i) Scaffold cues were regulated through the design and synthesis of cell-instructive collagen gels. The collagen gels were modified by three different approaches. The first method involved cross-linking of the collagen gel to increase bulk gel stiffness. The second method involved partial digestion of cross-linked collagen gel to decrease bulk gel stiffness and the third method involved the increase of fiber thickness to increase the local rigidity of the fiber bundles. The scaffolds were subsequently used to regulate malignancy of hepatocellular carcinoma cells and to control the activation state of fibroblasts. (ii) Soluble factor cues were regulated through the design and synthesis of PEGDA-PEI controlled delivery hydrogels formed through the Michael reaction.

3.2 Chemical cross-linking of collagen gels

Pure collagen gels were synthesized by mixing pre-chilled collagen I solution (3 mg/ml, Puramatrix), Dulbecco’s Modified Eagle Medium (DMEM) (Cellgro), and reconstituting solution in a ratio of 8:4:1. The reconstituting solution was a mixture of 0.26 M sodium hydrogen carbonate, 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.04 N sodium hydroxide. This mixture was subsequently incubated at 37 °C for 4 hours to form 3D collagen hydrogels. The chemically cross-linked collagen gels were designated collagen-PEG hydrogels. To form collagen-PEG hydrogels, poly(ethylene glycol) di-(succinic acid N-hydroxy succinimidyl ester) (PEG-diNHS) (Mₜ 446.36) was dissolved in dimethyl sulfoxide at a concentration
of 50 mg/ml. The solution was then added to the collagen/DMEM/reconstituting solution mixture at a final PEG-diNHS to collagen mass ratio ($M_{PEG_{1-COL}}$) of 0.17 ($E_0 = 2.3$ kPa) or 0.33 ($E_0 = 4.0$ kPa) before incubation at 37 °C for 4 hours.

3.3 Increasing fiber rigidity of collagen gel through thermodynamic control

Pure collagen gels were synthesized as described in Chapter 3.2. For the modification of collagen fiber rigidity, the size of fiber bundles was modified by thermodynamic control through the addition of PEG ($M_w$ 7500). Unlike the chemically cross-linked collagen gel, the PEG used for thermodynamic control did not have any reactive functional groups. In this approach, the collagen gels modified through thermodynamic control were designated collagen/PEG gels. To form the collagen/PEG gels, the PEG was dissolved in DI water at a concentration of 300 mg/ml. The solution was then added to the collagen/phosphate buffered saline (PBS)/reconstituting solution mixture at a final PEG to collagen mass ratio ($M_{PEG_{2-COL}}$) of 5, 10, or 25 before incubation at 37 °C for 1 hour. In the above pre-gel solutions, the PBS volumes were adjusted to maintain constant total volume.

3.4 Scanning electron microscope imaging of collagen gels

Collagen and collagen/PEG gels were imaged by scanning electron microscope (SEM) to visualize the structural differences. To maintain the structural integrity of the hydrogels, the hydrogels were subjected to serial dehydration using increasing concentrations of ethanol. The dehydrated samples were then dried with CO$_2$ in a critical point dryer (Balzers CPD 030) and sputter coated with platinum (JFC-1300, JEOL) before imaging with the SEM (JSM-5600VL, JEOL). ImageJ software was used for the quantification of fiber diameters and the number of fibers measured per sample was no
less than 100. The mesh size of the respective collagen hydrogels were measured using four images per condition with ImageJ. The images were thresholded at 90 on an 8-bit image to distinguish surface fibers from fibers in deeper planes. The images were then processed with the invert function and the particle analysis function was used to find and measure particles greater than 4 pixels.

3.5 Mechanical characterization of collagen gels

Collagen and collagen-PEG gels were cast in molds with thickness of 1 mm and diameter of 5 mm for measurement of the compressive Young’s modulus ($E$). The hydrogels were compressed with a mechanical testing system (MTS Insight) at a rate of 0.1 mm/min and the resultant stress was determined. $E$ was determined from the slope of the linear curve between stress and strain at the first 10% of strain. Four samples were measured per condition.

The storage moduli ($G'$) of the collagen and collagen/PEG gels were measured by an ARG2 rheometer (TA Instruments). The gels were allowed to form by pipetting the respective pre-gel solutions between parallel 40 mm steel plates. Following incubation at 37 °C, measurements were carried out using a pre-optimized setting with a frequency sweep characterization from 0.1 – 100 rad/s at 5% strain.

The average bending rigidities ($\kappa$) of the fibers in collagen and collagen/PEG gels were calculated by using the MacKintosh model from the following equation[66]:

$$\kappa_{\text{actual}} = \sqrt{G'\xi^5 kT}$$  \hspace{1cm} (1)
where $G'$ is the storage modulus in Pa, $\xi$ is the mesh size in $\mu$m, $k$ is the Boltzmann constant and $T$ is the temperature in K.

The theoretical $\kappa$ was further calculated by assuming the fibers to be either continuous materials made up of fibrils with strong inter-fibril covalent cross-links, or bundles of free-sliding fibrils with no inter-fibril interactions[119]. Theoretical $\kappa$ was evaluated by using the following equation:[119]

$$k_{\text{theoretical}} = EI \quad (2)$$

, where $E$ is the Young’s Modulus of collagen fibers obtained from literature in MPa and $I$ is the geometry-dependent second moment of inertia.

When the collagen fibers were assumed to be continuous materials made up with strong covalent cross-links, $I_{\text{covalent}}$ was given by:

$$I_{\text{covalent}} = \frac{\pi R^4}{4} \quad (3)$$

, where $R$ is the radius of the fiber in $\mu$m.

When the collagen fibers were assumed to be bundles of free-sliding fibrils with no inter-fibril interactions, $I_{\text{free}}$ was given by:

$$I_{\text{free}} = \frac{N(\pi r^4)}{4} \quad (4)$$

, where $r$ is the radius of the constituting fibrils in $\mu$m and $N$ is the number of fibrils in a single fiber cross-section.
3.6 Permeability assay of collagen gels

Permeabilities of collagen and collagen-PEG gels were determined by determining diffusivities of macromolecules within the gels. This was carried out by using fluorescence recovery after photobleaching (FRAP). The FRAP experiments were performed on a multiphoton confocal microscope equipped with a FRAP module (Zeiss LSM 710). Fluorescein isothiocyanate (FITC)-dextran (40kDa) was incorporated into the gels as a fluorescent probe. Three randomly selected circular spots, each with a diameter of 95 μm, were photo-bleached with a short-pulsed 488 nm laser. The bleaching iterations were adjusted so that the resulting fluorescence from the spot became approximately 60% of the original fluorescence. The recovery of fluorescence intensity over time was measured, and the curve was fitted with the following equation:

$$f(t) = A + B \exp\left(-\frac{t}{2r}\right)$$  \hspace{1cm} (5)

where $f(t) = \frac{F(t)}{F_0}$, $F_0$ is the initial fluorescence before bleaching, $F(t)$ is the fluorescence intensity over time, $\tau$ is the recovery time constant, $A$ is the initial fluorescence, and $B$ is a modified Bessel function.

The diffusion coefficients ($D$) of FITC-dextran within the respective gels were then calculated with the following equation:

$$D = \frac{r^2}{4\tau}$$  \hspace{1cm} (6)

where $D$ is the diffusion coefficient of FITC-dextran (cm$^2$/sec) and $r$ is the radius of the cylindrical bleached spot.
3.7 Second-harmonic-generation confocal imaging of collagen gels

Unfixed, hydrated collagen and collagen-PEG gels were imaged with a multi-photon confocal microscope (Zeiss LSM 710) using the 900 nm laser. SHG signals generated from the collagen-based gels were collected at half the excitation wavelength. The SHG imaging was carried out using the 25x lens with water immersion. Five images were obtained at 12 μm intervals in the z-direction. These images were then projected onto a single image using ImageJ software.

3.8 Fourier transform infrared spectroscopy analysis of collagen gels

Collagen and collagen/PEG gels were transferred to crystalline sample holders and analyzed with FTIR (Shimadzu FTIR-8400) for studying of amide bands positions (band positions A, B, I and II) in the absence and presence of PEG.

3.9 Differential scanning calorimetric analysis of collagen gels

The water content in the collagen and collagen/PEG gels was determined by a differential scanning calorimeter (DSC). Water within the gel that melted at 0°C was defined as free water while water that did not melt or melted below 0°C due to interactions with the material was defined as bound water. The amount of bound water in the collagen and collagen/PEG gels was calculated by examining the transition enthalpy of water in the respective pre-gels and gels. To do so, the respective pre-gels and gels were heated in the DSC at a rate of 5 °C min⁻¹ from -30 °C to 30 °C and the total amount of bound water were calculated. In the DSC experiment, both freezing free water (Wff) and freezing bound water (Wfb) was regarded as freezable water (Wf). Wff and Wfb can be calculated from the following equations: [120]
\[ W_{ff} = \frac{q_{T=0}}{\Delta H} \]  

\[ W_{fb} = \frac{q_{T<0}}{\Delta H} \]

where \( W_{ff} \) is freezable free water, \( W_{fb} \) is freezable bound water, \( \Delta H \) is the transition enthalpy of water, \( q_{T=0} \) is the amount of heat loss at 0 °C during melting as measured with the DSC and \( q_{T<0} \) is the amount of heat loss at temperatures less than 0 °C during melting as measured with the DSC.

### 3.10 Calculation of theoretical fiber diameters in collagen/PEG gels

The theoretical fiber diameters at different \( M_{PEG2-COL} \) were predicted by considering the amounts of bound water and the theoretical amounts of water bound to the PEG chains. In these calculations, it was assumed that the moles of bound water bound to collagen fibers (\( n_{ci} \)) would be directly proportional to the total surface area of the formed fiber bundles (\( A_i \)) (where subscript \( i \) denotes the respective \( M_{PEG2-COL} \)). By estimating \( n_{ci} \) for different \( M_{PEG-COL} \), it was then possible to calculate the respective \( A_i \) and thus the predicted fiber diameters (\( d_i \)). A good agreement between predicted and actual \( d_i \) would indicate that that the structures of the PEG-containing collagen gels were controlled by the amounts of bound water i.e. the relative water entropies. For pure collagen gel (\( M_{PEG-COL} = 0 \)) with known mean fiber diameter, \( D \):

\[ n_{c0} = n_{bw0} \]  

where \( n_{bw0} \) is the total moles of bound water in collagen gel determined from DSC measurements.
Following some algebraic manipulation, the total surface area of the fibers in pure collagen gel could be simplified into the following equation:

\[ A_0 = \frac{4mcv}{D} \]  

where \( m_c \) is the known mass of collagen in mg, \( v \) is the known specific volume of collagen in \( \mu m^3/mg \) and \( D \) is the actual mean fiber diameter in \( \mu m \).

Assuming direct proportionality of \( n_{ci} \) to \( A_i \),

\[ \frac{n_{ci}}{A_i} = \frac{n_{c0}}{A_0} = \frac{n_{c5}}{A_5} = \frac{n_{c10}}{A_{10}} = \frac{n_{c25}}{A_{25}} \]  

where subscript \( i \) denotes the respective \( M_{PEG2-COL} \).

The moles of water bound to collagen fibers in the respective conditions can be readily calculated from the following equation:

\[ n_{ci} = n_{bwi} - n_{pi} \]  

for \( i = 5, 10, 25 \) where \( n_{bwi} \) is the total moles of bound water in the respective gels determined from DSC measurements and \( n_{pi} \) is the moles of water bound to PEG chains (calculated from mass of PEG by assuming 3 water/ether group)[121].

Thus, the predicted diameters of the fibers in the respective gels could be calculated from the following equations:

\[ A_i = \frac{A_0}{n_{c0}} \times (n_{bwi} - n_{pi}) \]  

\[ d_i = \frac{4mcv}{A_i} \]
, (for $i = 5, 10, 25$).

### 3.11 Cell seeding of HepG2

Human hepatocyte cell lines HepG2 (ATCC) was cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin (PS, Gibco). The cells were maintained in T-75 flasks at 37 °C in a 5% CO$_2$ and 95% relative humidity environment. The medium was renewed once every 3 days and the cells were passaged once a week. For encapsulation of the cells in collagen and collagen-PEG gels, cells were trypsinized and re-suspended in DMEM. The cell suspension was then mixed with collagen and reconstituting solutions with a ratio of 4:8:1 for the pure collagen hydrogel; 0.17 and 0.33 mg PEG-diNHS/mg collagen were added to generate collagen-PEG hydrogels with different elastic moduli. The final cell density in all gel conditions was kept constant at $1 \times 10^3$ cells/μL. 50 μL of the pre-gel solution was then dispensed into each well of a 96 well plate, with each well containing a total of $5 \times 10^4$ cells. After incubation for 4 hours to allow hydrogel formation, 100 μL of cell culture media was added to each well. Media was exchanged with fresh media every three days.

To generate spheroids by the rocking method, $2 \times 10^4$ HepG2 cells were seeded in 35 mm petri dishes. The cells were rocked on a plate shaker at 60 rpm overnight. The spheroids formed were then isolated by gentle centrifugation and were further encapsulated in collagen or collagen-PEG gels for 3 days.

### 3.12 Cell seeding of fibroblasts

Mouse fibroblast cell lines L929 (ATCC) was cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) PS. The cells were maintained in T-75 flasks at 37
°C in a 5% CO₂ and 95% relative humidity environment. The medium was renewed once every 3 days and the cells were passaged once a week. Prior to cell encapsulation, cells were trypsinized and re-suspended in DMEM. The cell suspension was then mixed with collagen and reconstituting solutions in the ratios described earlier to form PEG-free pure collagen hydrogels or collagen/PEG hydrogels. The final cell density in all gel conditions was kept constant at 500 cells/µL. 80 µL of the pre-gel solution was then dispensed into each well of a Transwell-24 well plate (Corning). After incubation for an hour to allow hydrogel formation, the samples were rinsed thrice by pipetting PBS into the lower chamber of each well and allowing the solutions to gradually flow into the transwell inserts. This was to prevent accidental disruption of the fragile gels. Following which, 1500 µL of DMEM containing FBS and PS was added to the lower chamber of each well. Media was exchanged with fresh media once every three days.

For control experiments, L929 cells encapsulated in pure collagen gels were subsequently incubated in the both PEG-free and PEG-containing media for a period of 3 days. The cells were cultured in PEG-free media for another 3 days before their morphologies were studied.

3.13 Cytotoxicity assays

To evaluate the cytotoxicity of PEG-diNHS and PEG, HepG2 and L929 cell lines were plated in 96 well plates and incubated with DMEM containing PEG-diNHS and PEG respectively. The concentrations of PEG-diNHS and PEG were similar to the concentrations used in the gels. The viability of the cells were evaluated after 2 days by the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s protocol.
3.14 Total DNA quantification

Cell proliferation of HepG2 was determined by counting number of cells through total DNA assays. At fixed time points, gels were degraded through the addition of 100 μg/mL collagenase (Invitrogen). De-ionized water was then added to the isolated cells to bring about osmotic lysis of the cells. The cell membranes were further disrupted by subjecting the lysed cells to 3 freeze-thaw cycles. The samples were then incubated for 30 minutes with 20 μL of 10 μg/mL bisbenzimide in pH 7.4 buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 M sodium chloride. The fluorescence intensities, which are proportional to the total amount of DNA, were then determined using a microplate reader at 356 nm excitation and 470 nm emission and compared to a standard curve.

3.15 Immunofluorescent staining and confocal imaging

For confocal imaging of the HepG2 cells, HepG2 cells were fixed with 4 % paraformaldehyde and permeabilized with 0.5 % Triton-X after 9 days of culture. The permeabilized cells were then blocked with 2 % (w/v) bovine serum albumin and 5 % (v/v) fetal calf serum (Gibco). Following blocking, the cells were incubated with nuclear stain, 4',6-diamidino-2-phenylindole (DAPI, Invitrogen), and their respective antibodies. For imaging of actin filaments, phalloidin-Alexa 488(Invitrogen) was used. For imaging of β1-integrin, mouse monoclonal β1-integrin antibody and goat polyclonal antibody conjugated with Cy5 (Abcam) were used as the primary and secondary antibodies respectively. For imaging of E-cadherin, rabbit monoclonal E-cadherin antibody conjugated with Alexa 555 (Cell Signaling Technologies) was used. After staining with
the respective antibodies, the samples were washed with phosphate buffered saline and imaged with the multi-photon confocal microscope (Zeiss LSM 710) using the 2-photon mode for phalloidin and the single-photon mode for β1-integrin and E-cadherin imaging. For quantification of the size of nuclei and spheroids, confocal images of cells encapsulated in various gels were obtained at a magnification of 20× and the dimensions of each aggregate or nucleus were measured by using ImageJ software. For quantification of the E-cadherin expression level, confocal images of cells encapsulated in various gels were obtained at a magnification of 20× and the integrated pixel intensities of E-cadherin signals obtained from ImageJ were normalized by the total area of cells.

For confocal imaging of L929 cells, the gels were fixed and blocked as before following 10 days of culture. Following blocking, the cells were incubated with nuclear stain, Hoechst and Phalloidin-Tetramethylrhodamine B isothiocyanate for imaging of actin filaments. After the staining, the samples were washed with PBS and imaged with the confocal microscope (Nikon C1). For quantification of the actin level, confocal images of cells encapsulated in various gels were obtained at a magnification of 20× and the integrated pixel intensities of actin obtained from ImageJ were normalized by the total volume of cells.

3.16 Cytochrome P450 assay

For quantitative analysis of cytochrome P450 activity at different time points, cell culture media was aspirated at various time points and the cells were rinsed twice with PBS solution. 100 µL of PBS containing 5 µM 7-ethoxyresorufin and 20µM dicumarol
was added to the well before incubation in the dark for 30 minutes. The fluorescence of the resorufin-containing supernatant was then determined with a microplate reader at 535 nm excitation and 595 nm emission. The cytochrome P450 detoxification activity of the different conditions were normalized with cell numbers obtained from the total DNA assay at different time points.

For live imaging of cytochrome P450 activity, the cells in the various gels were incubated with 2 ml phenol-free DMEM containing 20 µM 7-ethoxyresorufin, 25 µM dicumarol, and 2 mM probenecid for 5 minutes. Confocal microscopy was carried out using a 514 nm laser and emissions above 570 nm were collected. Pinhole size was set at 100 µm with a pixel dwell time of 1.6 µs. The same settings were used to image and process all samples. The fluorescence intensity was then converted to pseudo-color with ImageJ with a 256 color map.

3.17 Urea detoxification assay

The detoxification of ammonia by the HepG2 cells was quantified by a urea detoxification assay. For quantification of urea levels at different time points, phenol-free cell culture media was aspirated and quantified with a colorimetric urea detection kit (QuantiChrom) according to the manufacturer’s instructions. Absorbance was determined at 475 nm with a microplate reader and the values obtained were compared with a standard curve. The urea detoxification activity was normalized by the cell number at different time points.
3.18 Matrix metalloproteinase degradation assay

MMP-1 (Calbiochem) was activated by p-Aminophenylmercuric Acetate (APMA, Calbiochem) immediately prior to use. Briefly, 12 μL of 50mM of APMA in 0.5 N NaOH and 30 μL of MMP-1 were added to 90 μL of buffer solution (pH 7.5) comprised of 25 mM HEPES, 5 mM calcium chloride, and 20 % (v/v) glycerol. The mixture was incubated at 37 ℃ for 2 hours for MMP-1 activation, after which the activated MMP-1 solution was brought up to 3 mL with DMEM solution. The resulting solution was then added to collagen-PEG gels at a 2:1 volumetric ratio. The gels were incubated for 0 h, 1 h, 3 h, and 5 h respectively at 37 ℃ before 3 rounds of washing with sterile PBS. The washed gels were subsequently immersed in DMEM containing 10 % fetal bovine serum (FBS, Gibco) to inhibit any traces of MMP-1. The MMP-1 treated gels were subsequently characterized through mechanical characterizations, permeability assays and SHG imaging as described above.

To investigate effect of MMP-1 treatment on collagen-PEG encapsulated HepG2 cells, HepG2 cells were cultured in collagen-PEG gels as described above. Following 5 days of culturing, the collagen-PEG gels were exposed to MMP-1 for 0 h, 1 h, 3 h and 5 h respectively as described above. Following exposure to MMP-1, the gels and cells were washed three times with sterile PBS and the HepG2 cells were furthered cultured for 4 days. The cells were then examined with confocal imaging and cytochrome P450 assay as described above.
3.19 Evaluation of angiogenic activity

VEGF secretion was quantified by analyzing collected cell culture media with a VEGF ELISA kit (R&D Systems). Absorbance was determined with a microplate reader at 570 nm with wavelength correction at 450 nm. The values obtained were compared with a standard curve.

For evaluation of in vivo angiogenic activity, fertilized chicken eggs (Hy-Line W-36) were purchased from the Poultry Research Farm at the University of Illinois (Urbana, IL). The eggs were incubated at 37 °C with 5 % CO₂ and 65 % relative humidity. On the 8th day of gestation, a small hole (1 cm × 1 cm) was created on top of each eggshell. After an additional day of incubation to acclimatize the embryos, collagen and collagen-PEG gels containing 5 × 10⁴ cells, prepared four hours before, were implanted on top of the chicken chorioallantoic membranes (CAMs) (2 eggs per condition). The openings were covered with tape and the eggs were further incubated for 6 days. During this period, images of the CAM implantations were taken using a S6E stereomicroscope (Leica) linked with a D-Lux E Camera (Leica). All procedures were performed under sterile conditions. After the 6-day incubation, the membranes were fixed with 10% neutral buffered formalin (NBF) for 20 hours. The fixed membranes surrounding the gel disk (10 mm × 10 mm) were cut out and the collected tissues were processed by paraffin embedding and α-SMA staining to examine and quantify mature blood vessel formation. Images of the stained CAM cross-sections at the implant site were taken using a DIML inverted microscope (Leica) linked with a D-Lux E camera (Leica). The images were then examined using ImageJ software to determine overall blood vessel densities. The blood vessel densities were calculated as the average overall area of positively stained
blood vessels normalized to the total area of the tissue. The number of vessels and their corresponding diameters were also measured and normalized to the area of the CAM membranes.

3.20 Collagen gel contraction assay

To evaluate the contraction of collagen and collagen/PEG gels by the L929 fibroblasts, the final areas of the different gels were also tabulated after 10 days of culture and normalized by the initial gel areas to give the percentage gel shrinkage.

3.21 Synthesis of PEGDA-PEI hydrogels

The PEGDA-PEI hydrogels were prepared by mixing PEGDA (molecular weight (Mₘ) ≈ 400, Polysciences) and PEI (Mₘ ≈ 2000).[122] Both PEI with branched architecture and linear architecture were used. The mixtures of PEGDA and PEI were placed between two glass plates separated by a 1 mm-spacer. Hydrogel disks with diameters of 5 or 10 mm were punched out after the gel was formed. Pure PEGDA hydrogels were also formed by mixing aqueous solutions of PEGDA with 0.01 % Irgacure 2959 and subsequently exposing the PEGDA solutions to UV light for 10 minutes.

3.22 NMR characterization of PEGDA-PEI hydrogels

To examine the reaction between PEGDA and PEI, the ¹H-NMR spectra of polymer precursors, and the degraded fragments of PEGDA-PEI hydrogels were collected using NMR (500MHz, Varian).
3.23 Mechanical characterization of PEGDA-PEI hydrogels

The $E$ of the PEGDA-PEI hydrogel disks were measured by the MTS testing system as described above.

3.24 Swelling studies of PEGDA-PEI hydrogels

The initial swelling ratios of the hydrogels ($Q_0$) were quantified with the ratios between the mass of the hydrogel disks and that of the hydrogels dehydrated via lyophilization. The initial number of cross-links ($N_0$) was further calculated using the following equations according to rubber elasticity theory:[123]

\[
N_0 = \frac{SQ}{\frac{1}{RT}}
\]  \hspace{1cm} (15)

, where $S$ is the shear modulus calculated from the slope of stress versus ($\lambda^2 - \lambda$) assuming the hydrogels followed an affined network model ($\lambda$: ratio of deformed height to original height) and $Q$ is the degree of swelling calculated from Eq. (16):

\[
Q = \rho_p \left[ \frac{Q_0}{\rho_s} + \frac{1}{\rho_p} \right]
\]  \hspace{1cm} (16)

, where $Q$ is the degree of swelling, $\rho_s$ is the density of water, and $\rho_p$ is the density of polymer.

3.25 Degradation studies of PEGDA-PEI

To evaluate the degradation of PEGDA-PEI gels, hydrogels were incubated in 1 ml PBS (pH 7.4), and their changes in swelling ratios were recorded by measuring their masses until the disks were completely disintegrated. The swelling ratio at time, $t$, ($Q_m$) was normalized by the initial swelling ratio of the disks ($Q_0$). The increase in $Q_m$ of the
hydrogel over time was further fitted to Peppas’s model to calculate its degradation rate ($k_1$) and swelling exponent ($n$):[124, 125]

$$\frac{Q_m - Q_0}{Q_0} = k_1 t^n$$  \quad (17)

where $Q_0$ is the initial swelling ratio, $Q_m$ is the swelling ratio of hydrogel disks at time, $t$ (min).

The diffusivity ($D$) was further calculated from $k_1$ and $n$ using the following equation:[126]

$$D = \pi r^2 n \sqrt{\frac{k_1}{4}}$$  \quad (18)

where $D$ is the diffusion coefficient of water in cm$^2$s$^{-1}$, $r$ is the radius of the hydrogel disks in cm, and $k_1' = k_1(T)^n$, where $T$ is the time expressed in s.

3.26 Determination of number of unreacted amines

The number of unreacted amines after hydrogel formation through Michael reaction between PEI and PEGDA was investigated by reaction with 2, 4, 6-trinitrobenzenesulfonic acid (TNBS). Briefly, hydrogel disks were degraded by reaction with 1 M sodium hydroxide to expose the unreacted amine groups. The number of unreacted amines remaining after PEGDA-PEI crosslinking was then determined by a 15 minute-incubation with TNBS in the presence of sodium bicarbonate buffer (0.01M, pH 8.5). At the end of the assay, the absorbance was measured at 335 nm with a microplate reader (Synergy HT Multi-Mode Microplate Reader, Biotek Instruments), and the
absorbance was converted to the number of unreacted amines using a calibration curve generated with different concentrations of PEI.

3.27 Imaging of water diffusion into PEGDA-PEI hydrogels using magnetic resonance imaging (MRI)

Imaging of water diffusion through hydrogel disks was carried out using 600 MHz Varian Unity/Inova nuclear magnetic resonance (NMR) spectrometer (14.1 T magnet) at room temperature.[127-129] The maximum strength of the magnetic field gradient was 90 G cm\(^{-1}\). Each hydrogel disk (10 mm in diameter, 1 mm in thickness) was incubated in deionized water. Then, the cross-sectional images of hydrogel disks through the middle were taken every 25 minutes by placing the swollen hydrogels in glass bottles which were inserted into a Radio Frequency (RF) coil for the measurements. Spin echo multi-slice (SEMS) pulse sequence was used to acquire resonance data, which were then converted into water density maps using VNMR 6.1C software. For SEMS pulse sequence, the repetition time (T\(_R\)) of 2 s and the echo time (T\(_E\)) of 10 ms were used. The field of view (FOV) was 1.5 x 1.5 cm with the slice thickness of 1 mm, and the image matrix was 256 x 256 pixels. After acquiring the images, colors were added to the images to visualize the water density spectrum using ImageJ.

3.28 Cytotoxicity assay of PEGDA-PEI hydrogels

Toxicity of the PEGDA-PEI hydrogels was evaluated by implanting them on the CAMs of chicken embryos. Fertilized chicken eggs (Hy-Line W-36) were purchased from the Poultry Research Farm at the University of Illinois (Urbana, IL). The eggs were incubated at 37°C with 5% CO\(_2\). On the 8\(^{th}\) day of gestation, a small hole (1 cm x 1 cm) was created on top of each eggshell. After an additional day of incubation to acclimatize
the embryos, different hydrogel disks (5 mm diameter, 1 mm thickness) were implanted on top of the CAMs (5 eggs per condition). After incubation for 7 days, the membranes were fixed with 10% neutral buffered formalin (NBF) for 20 hours. The fixed membrane surrounding the gel disk (10 mm x 10 mm) was cut out. The collected tissue was processed for paraffin embedding, and the cross-sections were stained with standard Hematoxylin & Eosin (H&E) for histological analysis.

3.29 In vitro protein release assay of PEGDA-PEI hydrogels

Fluorescent bovine serum albumin (BSA, Invitrogen) was mixed with the aqueous PEI solutions prior to the cross-linking reaction with the PEGDA solutions. Hydrogel disks with diameters of 5 mm were punched out and incubated in PBS at 37 °C. The PBS was exchanged with fresh media on a daily basis until the hydrogel disk was disintegrated. The fluorescence intensity from the BSA was measured with a microplate reader (Synergy HT Multi-Mode Microplate Reader, Biotek Instruments), and the amount of BSA released was back-calculated from the intensity using a calibration curve. The masses of the BSA released following the initial lag period was further converted to the release rate \(k_2\) and release exponent \(m\) using Ritger-Peppas equation:[124]

\[
\frac{M_t}{M_\infty} = k_2 t^m
\]  

(19)

, where \(M_t\) is the cumulative amount of BSA released at time \(t\), \(M_\infty\) is the total amount of BSA released, \(k_2\) is the release constant, and \(m\) is the release exponent.

3.30 In vivo drug release assay of PEGDA-PEI hydrogels

To investigate in vivo drug release from PEGDA-PEI hydrogels in mice models, BSA was labeled with fluorescent IRDye® 800CW N-hydroxysuccinimide ester (Li-cor
Biosciences) according to the instructions provided in the IRDye Protein Labeling Kit (Li-cor Biosciences). 30 µl of the mixture of fluorescent BSA and PBS or 30 µl of the fluorescent BSA-encapsulated PEGDA-PEI pre-gel solution (i.e. 12.5 % PEGDA and 10 % PEI) was injected into the back of mice (3 months old female C57BL/6 mice). The mice were anesthetized at different time points, and the injection sites and ears of the mice were imaged with the Odyssey Infrared Imaging System with Mousepod (Li-cor Biosciences). The fluorescent intensities of the BSA in all the mice were processed using the same settings. The fluorescence images were further superimposed with photos of the actual mice for clearer visualization. All procedures on mice were performed with approval from the University of Illinois Institutional Animal Care and Use Committee (IACUC).

3.31 Stem cell mobilization with PEGDA-PEI hydrogels

To investigate the function of the PEGDA-PEI hydrogels, the hydrogels were used to mobilize stem cells in porcine models through release of porcine granulocyte colony stimulating factor (GCSF). The GCSF was dissolved in PBS or encapsulated in hydrogels before delivery into pigs (3 month old female white landrace pigs weighing approximately 30 kg each) via intramuscular injection. Conditions tested in this experiment included (i) Single bolus injection of 3 mL PBS containing 1.2 mg of GCSF (number of samples was one), (ii) Daily bolus injections of injection of 3 mL PBS containing 0.3 mg of GCSF (number of sample was one), (iii) single injection of 3 mL blank PEGDA-PEI hydrogel i.e. 20 % PEGDA and 3.5 % PEI (number of sample was one), and (iv) single injection of 3 mL of PEGDA-PEI hydrogel containing 1.2 mg of GCSF (number of sample was three). Blood samples were taken daily from the
brachiocehalic vein by atraumatic venipuncture and collected directly into EDTA-containing vacutainer tubes. Cell counts of the non-erythrocyte fraction were performed on a Beckman Coulter AcT10 hematology analyzer (Beckman Coulter). At the end of the experiment, animals were sedated with a telozal-xylazine-ketamine cocktail and euthanized by an intravenous overdose injection of sodium pentobarbital. All procedures on pigs were performed with approval from the University of Illinois Institutional Animal Care and Use Committee (IACUC).

3.32 Statistical analysis

Three samples were analyzed per condition unless otherwise specified. One-way ANOVA was used when the number of conditions was more than 2. Student t-tests were used to compare differences between pairs of conditions. Data was considered significant for $P$ values < 0.05.
4 Regulation of HCC malignancy through covalent modification of collagen scaffold

As discussed in chapter 2, 3D collagen scaffolds with tunable stiffness can serve as useful tools to study the effects of stiffness on cell malignancy. This chapter describes the alteration of collagen gel stiffness through covalent cross-linking with PEG-diNHS and the culture of HCC in pure collagen and cross-linked collagen (collagen-PEG) scaffolds. By controlling the bulk stiffness of the collagen hydrogels, we were able to control the malignancy of encapsulated HCC. The work in this chapter addresses objectives 1 and 2 described in chapter 1. This work has been published in Biomaterials.[118]
4.1 Tuning stiffness of collagen gels through chemical cross-linking

The elastic modulus \((E)\) of the collagen hydrogel was controlled by incorporating varying amounts of PEG-diNHS into the pre-gel solution of collagen with a pH of 3. The resultant collagen-PEG hydrogel was formed by adjusting the temperature and pH of the mixture of collagen and PEG-diNHS (Fig. 4.1). No gel was formed either by changing the temperature and pH of pure PEG-diNHS solution. The resulting hydrogel remained structurally stable over nine days. As expected, increasing the mass ratio between PEG-diNHS and collagen \((M_{PEG1-COL})\) from 0 to 0.33 linearly increased the \(E\) of the hydrogel from 0.7 kPa to 4.0 kPa as evaluated from the mechanical testing system (Fig. 4.2). Such variation in the elastic modulus was broad enough to encompass the stiffness of several soft tissues, including fat and liver. Note that 0.7 kPa corresponded to the \(E\) of fat tissue, while 4.0 kPa corresponded to that of healthy liver.[130, 131]

![Figure 4.1 Control of collagen gel stiffness with PEG-diNHS.](image)

The soft pure collagen gel was formed from self-association of collagen fibers. Individual single collagen fibers consisted of multiple helical bundles of collagen fibrils. These fibrils were prepared by increasing the pH of the collagen solution to induce self-assembly of collagen molecules. Stiffness of the collagen gel was increased by introducing covalent cross-links between collagen fibrils and PEG-diNHS to form collagen-PEG gels.
4.2 Second-harmonic-generation confocal imaging of cross-linked collagen gels

As the non-centrosymmetric collagen fibrils possessed first hyperpolarizabilities large enough for SHG microscopy, SHG imaging allowed visualization of collagen fibrils without fixation or staining.[77] As demonstrated by the positive SHG signals observed in all conditions, the supplemental chemical reaction between collagen and PEG-diNHS did not interfere with the formation of interconnected collagen fibrils (Fig. 4.3). The SHG analysis also revealed that increased cross-linking with PEG-diNHS increased the homogeneity of the collagen gels but decreased the mesh size from 3 to 1 µm, quantified by the average spacing between the fibers (Fig. 4.4).

Figure 4.2 Characterization of hydrogel stiffness. Increasing the mass ratio between collagen and PEG-diNHS ($M_{PEG1-COL}$) linearly increased the $E$ of the hydrogel. Differences between conditions were statistically significant. (*$P < 0.05$)

Figure 4.3 SHG images of gels prepared at different $M_{PEG1-COL}$ demonstrated that incorporation of PEG-diNHS in collagen gels resulted in a more homogeneous distribution of fibers. The inset in each image represented the magnified view of the region marked by the circle (scale bars represent 25 µm, inset magnified 2.5×).
4.3 Permeability assay of cross-linked collagen gels

One major concern with covalently cross-linking 3D hydrogel scaffolds was the decrease in gel permeability with increased cross-linking. Such decrease in gel permeability may result in reduced transport of nutrients and oxygen to the cells and result in compromised cell viability. To investigate if the permeability of the covalently cross-linked collagen-PEG gels was significantly decreased, the diffusivities of fluorescein-conjugated dextran with molecular weights of 40 kDa within the hydrogels were evaluated by the FRAP assay. In this assay, several spots on the dextran-containing hydrogels were photo-bleached and the time taken for the fluorescence to recover through diffusion of the dextran was monitored. From the FRAP assays, the diffusivity \( D \) of fluorescein-conjugated dextran within the hydrogel could be calculated (Fig. 4.4). \( D \), quantified with the FRAP assay, slightly decreased with increasing \( E \) of the hydrogels, but the difference between conditions was not statistically significant. In addition, the \( D \) were in the order of \( 10^{-7} \) cm\(^2\)/sec, which was similar to that in native ECM.[132] The insignificant decrease in the \( D \) of dextran could be attributed to the small changes in mesh size which were still within the same order of magnitude.
Figure 4.5 Change of permeability with stiffness. Increasing $E$ caused a limited decrease of the $D$ of fluorescent dextran within the hydrogel, as characterized with FRAP assay. Differences between conditions were not statistically significant.

4.4 Control of HCC morphology with bulk gel stiffness

The collagen-PEG hydrogels with varied $E$ but similar permeability were used to prepare 3D HCC spheroids. HepG2 cells, used as model HCCs, were encapsulated in the hydrogel via an in situ cross-linking reaction. According to experiments conducted using HepG2 cells cultured on a 2D cell culture plate, the difference in PEG-diNHS concentrations between conditions did not influence cellular viability or metabolic activity. Cells encapsulated in the different hydrogels proliferated to form spheroids over time. Interestingly, the frequency of the cell cycle, quantified by the increase of the cellular DNA mass, decreased with increasing $E$ of the hydrogel (Fig. 4.6). Within nine days, the cells encapsulated in the pure collagen gel with $E$ of 0.7 kPa formed large and loosely packed malignant spheroids with diameters ranging from 30 to 330 $\mu$m (Fig. 4.7). These cells also actively invaded the collagen gel, which is the typical behavior of malignant cancer cells. In contrast, cells cultured in the collagen-PEG hydrogel with $E$ of 4.0 kPa formed small and compact spheroids with diameters ranging from 20 to 60 $\mu$m (Fig. 4.7). The small spheroids had good cell-cell interaction and resembled hepatoids, clusters of normal hepatocytes formed in 3D matrices.
**Figure 4.6 Cell proliferation of HepG2.** The extent of increases in the intracellular DNA during 24 hours, which represents the frequency of cell division, was reduced with increasing $E$ at given time points. (bar represents the pure collagen gel with $E$ of 0.7 kPa, bar represents the collagen-PEG gel with $E$ of 2.3 kPa, and bar represents the collagen-PEG gel with $E$ of 4.0 kPa, *$P < 0.05$). The values and error bars represent averaged values of three samples and standard deviation, respectively.

**Figure 4.7 Morphology of HepG2.** Spheroids formed within the pure collagen gel ($E$ = 0.7 kPa; 1st row) were larger and more disorganized than those formed within the collagen-PEG gels ($E$ = 4.0 kPa; 2nd row). Intracellular actins were stained with phalloidin (green color), and nuclei were stained with DAPI (red color). Images in (b) are magnified views of spheroids shown in (a).
4.5 Control of HCC phenotype with bulk gel stiffness

Apart from the observable changes in spheroid morphology, hydrogel stiffness also influenced the morphology and gene expression of individual cells in the spheroids. The average nuclear diameter of HepG2 cells increased from 8 ± 0.9 to 10 ± 1.2 µm with increasing $E$, thus indicating increasing intracellular tension.[133, 134] In addition, the HepG2 cells in the small hepatoid-like spheroids formed within the stiffer collagen-PEG gel displayed down-regulated $\beta_1$-integrin expression and up-regulated E-cadherin expression compared to those in the larger spheroids developed within the softer collagen hydrogel (Fig. 4.8).

It had been shown in previous studies that malignant cancer cells expressed higher levels of the $\beta_1$-integrin. In these studies, blocking of $\beta_1$-integrin, mediator of cell adhesion to type I collagen, subsequently resulted in suppressed malignancy of the cancer cells and reverted the cells to a more benign phenotype.[135] On the other hand, E-cadherin receptors, which were essential for cell-cell adhesion, were often found to be down-regulated in cancerous cells.[136] The E-cadherin receptors had also been shown to be crucial for liver-specific activities.[137] Hence, decrease in E-cadherin expression was often associated with loss of hepatocyte-specific functions.

The HCC spheroids formed within collagen-PEG hydrogels with different $E$ also exhibited diverse levels of hepatocyte-specific activities, including those of cytochrome P450, and ammonia detoxification. The cytochrome P450 detoxification level was evaluated with the cellular capacity to convert non-fluorescent 7-ethoxyresorufin into
fluorescent resorufin, while the ammonia detoxification level was assessed with the cells’ ability to convert toxic ammonia to urea.

Figure 4.8 Cell surface receptors of HepG2. Spheroids grown in the collagen-PEG gels ($E = 4.0 \text{ kPa}$) displayed (a) lower $\beta_1$-integrin expression (yellow) and (b) higher level of E-cadherin (blue) compared with those grown in the pure collagen gel ($E = 0.7 \text{ kPa}$). Cell nuclei were stained with DAPI (red). (Scale bars represent 50 $\mu$m.)

The small hepatoid-like spheroids formed within the stiffer collagen-PEG hydrogels presented higher cytochrome P450 detoxification levels (Fig. 4.9) and greater ammonia detoxification activity (Fig. 4.10) compared to the large spheroids formed in the soft collagen hydrogels. These liver-specific activities were known to be inversely related to the cell’s degree of malignancy.[138] As such, the higher levels of liver-specific detoxification activities in the stiffer collagen-PEG gel ($E = 4.0 \text{ kPa}$) indicated
that the covalent cross-linking was not only able to change the morphology of the cancer cells but was also able to alter the cells’ phenotype.

Figure 4.9 Cytochrome P450 detoxification activities of HepG2. (a) Spheroids formed within the collagen-PEG gel ($E = 4.0$ kPa) presented higher cytochrome P450 detoxification activity than those cultured within the pure collagen gel ($E = 0.7$ kPa). The fluorescent images of the live cells were processed to present 256 pseudo-colors which represent the P450 activity level. (Scale bars represent 50 $\mu$m.) (b) The cytochrome P450 detoxification activity was elevated with increasing $E$ at given time points. (■) bar represents the pure collagen gel with $E$ of 0.7 kPa, (■) bar represents the collagen-PEG gel with $E$ of 2.3 kPa, and (■) bar represents the collagen-PEG gel with $E$ of 4.0 kPa.) The values and error bars represent averaged values for three samples and standard deviation, respectively.
Figure 4.10 Ammonia detoxification of HepG2. The ammonia detoxification level also increased with increasing $E$. (*$P < 0.05$. ▼ bar represents the pure collagen gel with $E$ of 0.7 kPa, ▼ bar represents the collagen-PEG gel with $E$ of 2.3 kPa, and ▼ bar represents the collagen-PEG gel with $E$ of 4.0 kPa.) The values and error bars represent averaged values for three samples and standard deviation, respectively.

In contrast, hydrogel stiffness could not control the intercellular organization and phenotype of pre-made tumor spheroids. In a control study, HepG2 cells suspended in media were constantly rocked overnight to generate spheroids. The resulting spheroids displayed a large variance in size and shape. Encapsulation of these pre-formed spheroids into hydrogels with varied $E$ did not cause any significant morphological changes in the spheroids, as confirmed by limited changes in intercellular organization. The spheroids also presented limited P450 detoxification activities, regardless of hydrogel stiffness. These results demonstrate that pre-made spheroids are less responsive to mechanical signals from the hydrogel matrix than those assembled in situ.

4.6 Control of HCC angiogenic propensity with bulk gel stiffness

HCC spheroids formed within hydrogels with differing $E$ also exhibited different levels of angiogenic activity, which is directly proportional to malignancy. Angiogenic activity was evaluated with the secretion of VEGF and in vivo blood vessel growth on embryonic CAMs. To determine the total amount of VEGF secreted by HCC
 encapsulated in different gels, the HCC-conditioned media were analyzed by a VEGF ELISA assay. Greater VEGF secretion was found in large HCC spheroids formed within the softer collagen gel as compared to those cultured within the stiffer gels (Fig. 4.11). To evaluate the in vivo angiogenic potential, the HCC spheroid-hydrogel constructs were implanted on CAMs of chicken embryos to examine their ability to stimulate the growth of vascular networks in vivo. Following 6 days of incubation, the density and size of vessels in the different conditions were quantified. As displayed in both the top view and the cross-section of CAMs stained with α-SMA antibody, the HCC spheroids formed within the soft collagen gel stimulated greater growth of blood vessels into the implants than those created within the stiffer collagen-PEG hydrogel (Fig. 4.12). Quantitative analysis of the images also confirmed that spheroids formed in the softer gel increased the size and number of blood vessels as compared to hepatoid-like spheroids formed in the stiffer hydrogel (Fig 4.12). Taken together, these results indicate that the larger spheroids in the softer collagen gel displayed higher angiogenic activities both in vitro and in vivo. The higher secretion of VEGF from the larger spheroids could be attributed to lowered oxygen tension due to increasing spheroid sizes.[139] VEGF, together with other angiogenic factors secreted by the cells subsequently increase local vessel formation. Previous studies have demonstrated that cancer cells with higher malignancies tended to promote greater degree of angiogenesis. Hence, these observations, like the earlier findings, implied that increasing stiffness of 3D matrix from the range of fat (0.7 kPa) to liver (4.0 kPa) suppressed malignancy of liver cancer cells.

One interesting finding from this in vivo study was the further change in morphology when hepatoids within collagen-PEG gels were tested in vivo. While these
hepatoids were initially observed as spheroids in the confocal images, they had transformed into polarized structures with lumens when implanted on the CAMs (Fig. 4.7 & Fig. 4.12). This change in morphology could indicate further differentiation of the HCCs towards hepatocyte phenotype, as primary hepatocytes are normally polarized in the liver and are arranged around the bile ducts.

![Figure 4.11 The VEGF secretion of HepG2.](image)

**Figure 4.11 The VEGF secretion of HepG2.** The cellular VEGF secretion level was reduced by increasing $E$ from 0.7 kPa to 4.0 kPa. ( ▅ bar represents the pure collagen gel with $E$ of 0.7 kPa, ▄ bar represents the collagen-PEG gel with $E$ of 2.3 kPa, and ▆ bar represents the collagen-PEG gel with $E$ of 4.0 kPa. *$P < 0.05$)**
Figure 4.12 Angiogenesis of HepG2. (a) HepG2 cells were encapsulated in pure collagen ($M_{\text{PEG1-COL}} = 0$) with $E$ of 0.7 kPa and collagen-PEG gels ($M_{\text{PEG1-COL}} = 0.33$) with $E$ of 4.0 kPa. Top view and cross-section images exhibit vascular networks formed near the gel constructs 6 days post-implantation. Blood vessels in the cross-section were stained by the antibody to $\alpha$-SMA (brown). The red arrows denote the blood vessels while the dotted circles denote the HepG2 cell clusters. HepG2 cells in collagen-PEG transformed into polarized structures with lumens when implanted on the CAMs. (Scale bars represent 50 $\mu$m.) (b) Blood vessel density (quantified by normalizing the area stained by $\alpha$-SMA antibody with total tissue area) was significantly higher with implantation of the malignant spheroids formed within the pure collagen gel than either the acellular collagen gel or the hepatoids in the collagen-PEG gel (*$P < 0.05$). (c) The malignant spheroids in the pure collagen gel also generated more and larger blood vessels. ( $\square$ bar represents the pure collagen gel with $E$ of 0.7 kPa, $\square$ bar represents the collagen-PEG gel with $E$ of 4.0 kPa, and $\square$ bar represents the acellular pure collagen gel with $E$ of 0.7 kPa.)
4.7 Discussion on regulation of HCC malignancy through covalent modification of collagen scaffold

Taken together, the experiments in this study successfully demonstrated a strategy to assemble 3D liver tumor spheroids with controlled intercellular organization, phenotype, and angiogenic activity. This successful 3D spheroid assembly was achieved with cell-instructive hydrogels formed by inter-fibril and inter-fiber crosslinking of collagen with PEG-diNHS to control its stiffness. The HCCs encapsulated in a fat-like soft hydrogel formed large and malignant spheroids characterized with active \( \beta 1 \)-integrin expression, cell proliferation, VEGF expression, and microvascular development, along with limited hepatocyte-like activities. In contrast, the cells encapsulated in a normal liver-like stiffer hydrogel formed small spheroids with suppressed malignancy, characterized by hepatocyte-like phenotypes and limited microvascular development. When these cells were implanted in vivo, they were further transformed into polarized luminal structures. This indicated greater resemblance to healthy hepatocytes.

The matrix design developed in this study allowed us to control the stiffness of a fibrillar matrix without significantly altering other matrix properties, including permeability and the number of cell adhesion motifs. The range of elastic moduli achieved with this approach was relevant to liver physiology, because it encompassed the elastic moduli of fat and normal liver tissue.[130, 131] The SHG images, which revealed the interconnected fibrous architecture of collagen-PEG hydrogels, showed that PEG molecules minimally influenced self-organization among collagen fibrils. The short PEG
cross-linker was chosen to mostly localize the chemical reaction between collagen and PEG-diNHS within the collagen fibers. This local stiffening of collagen fibers with PEG-diNHS only resulted in slight decrease in both the spacing between collagen fibers and the molecular diffusivity within the gel. The molecular diffusivity attained with the collagen-PEG hydrogels prepared in this study was similar to that within the native ECM. It was also larger by 2 to 4 orders of magnitude than the molecular diffusivity within the synthetic hydrogels commonly used for 3D cell culture.[140] Therefore, the effects of gel permeability on cellular activities should be minimal in this study.

We propose that the range of moduli achieved was vital to engineering tumor spheroids with various levels of malignancy, characterized by cell proliferation, detoxification activities, and proangiogenic activities. We found that the softer matrix, similar to fat tissue, facilitated cellular adhesion to the matrix, represented by increased \( \beta_1 \)-integrin levels, while the stiffer matrix, similar to normal liver, promoted cellular adhesion to neighboring cells, represented by higher E-cadherin levels. Therefore, we suggest that deviations from normal liver stiffness altered integrin signaling and resulted in downstream signaling changes. The deviation from normal liver stiffness also stimulated cell proliferation while down-regulating cellular ability to detoxify metabolites, characteristic of malignant phenotypes. The resulting large spheroids, formed in the softer matrix, likely experienced limited oxygen transport. This had been known to activate the cellular expression of VEGF driven by overexpression of hypoxia-inducible factors, which stimulated the development of tumor vascular networks in the spheroid-hydrogel implants. [139] In contrast, the cells in the small spheroids experienced only limited oxygen deprivation, leading to lower VEGF expression. This cellular
responsiveness to matrix stiffness was observed only when the cells were individually encapsulated in the hydrogels. The pre-made spheroids exhibited insensitivity to matrix stiffness.

Another interesting finding was that the cellular response to matrix stiffness reported in this study differed from previous studies. Certain studies conducted using breast cancer cells, previously reported that cells cultured on and within a gel stiffer than normal breast tissue exhibited an elevated malignant phenotype.[51] However, the $E$ of the normal breast tissue was closer to that of the pure collagen gel used in this study. Therefore, these opposing results may indicate that malignancy was not merely a function of increase or decrease in stiffness but was promoted by departure from normal tissue stiffness. However, this perspective needs to be explored more systematically in future studies. Another key difference between this and other studies was the difference in culture format. Many studies investigating the effect of matrix stiffness were conducted in the 2D configuration.[7] On 2D substrates, cells tended to experience greater tension with increasing moduli. When encapsulated in 3D gels however, the cells may experience greater compression instead of tension as a result of matrix stiffening. It had been shown in previous studies that increasing tension led to increased cell proliferation, while greater compression tended to suppress cell proliferation.[42-44] This highlighted the importance of generating 3D hydrogels with controllable stiffness such as the system presented in this chapter.
4.8 Conclusion on regulation of HCC malignancy through covalent modification of collagen scaffold

This study demonstrated a novel strategy to regulate cellular organization and phenotype of 3D liver tumor spheroids. This spheroid assembly was achieved with the cell-instructive hydrogels with controllable stiffness and limited changes in permeability. The tuned matrix stiffness allowed control of cellular adhesion to both matrix and neighboring cells and subsequently created 3D tumor spheroids with varied levels of malignancy. We believe that this material design strategy can be readily extended to a wide array of fibrous hydrogels formed by self-association of different molecules such as oligopeptides and proteins. In addition, the hydrogels developed in this study would be broadly useful to understand emergent behaviors of a wide range of stem and cancer cells. In the next chapter, we further investigated if the subsequent softening of stiff matrix led to changes in malignancy of encapsulated cells.
5 Regulation of HCC malignancy through MMP-1 degradation of cross-linked collagen

As demonstrated in chapter 4, increase of collagen hydrogel stiffness through covalent cross-linking suppressed malignancy of HCC. This chapter describes the reduction of hydrogel scaffold stiffness via MMP-1 degradation, resulting in the subsequent reversion of the HCC malignant phenotype. The work in this chapter addresses objectives 3 and 4 described in chapter 1.
5.1 Softening of cross-linked collagen gel through MMP-1 degradation

The 3D cell culture studies in the earlier chapter demonstrated that pure collagen gels with $E$ of 0.7 kPa created HCC spheroids with high malignancy while the collagen-PEG gel with $E$ of 4.0 kPa created HCC hepatoids with suppressed malignancy, as characterized by the changes in cell morphology, phenotype, and angiogenic activities. In this chapter, we investigated if the observed suppression of HCC malignancy by increasing matrix stiffness from fat-like range to liver-like range could be reverted with subsequent decrease in collagen-PEG stiffness.

In order to decrease $E$ of collagen-PEG hydrogels, we exposed the gels to low concentrations of MMP-1 for varied durations (Fig. 5.1). The concentrations of MMP-1 used for degradation had been shown in previous studies to only partially digest collagen.[141] Following formation of the collagen-PEG hydrogels as earlier described, the collagen-PEG gels were incubated in MMP-1 solutions for periods of 0, 1, 3, or 5 h to degrade the collagen-PEG gels to varying degrees. $E$ of the treated gels were then quantified by compressive mechanical testing as described earlier. As the period of MMP-1 exposure was increased from 0 h to 5 h, $E$ of the hydrogel was decreased from 4.0 to 0.5 kPa (Fig. 5.2). These results demonstrated that the collagen-PEG gel ($E = 4.0$ kPa) could be successfully softened to various degrees by varying digestion time. This variation range of $E$ ($E = 4.0$ kPa to $E = 0.5$ kPa) was also similar to that obtained by the covalent cross-linking of collagen ($E = 0.7$ kPa to $E = 4.0$ kPa). Thus, this method of hydrogel softening was suitable for returning the stiffness of the cross-linked gel to its pre-cross-linked range.
Figure 5.1 MMP-1 degradation of collagen gels. The collagen-PEG gel ($E = 4.0$ kPa) was exposed to MMP-1 for varied durations, in order to soften the gels. The concentrations of MMP-1 used were previously shown to only partially digest the collagen fibrils.[141]

Figure 5.2 Regulation of elastic modulus through MMP-1 degradation. Increasing duration of exposure to MMP-1 from 0 h to 5 h decreased elastic modulus ($E$) of the hydrogels from 4.0 kPa to 0.5 kPa. The differences between the conditions were statistically significant. (*$P < 0.0001$)
5.2 Second-harmonic-generation confocal imaging of degraded collagen gels

Apart from the mechanical characterization, the partial digestion of fibrils by MMP-1 degradation was also confirmed by SHG imaging. As before, collagen-PEG hydrogels exposed to different durations of MMP-1 were imaged with SHG confocal imaging to visualize the SHG fibrils. As the duration of exposure to MMP-1 increased, SHG signals corresponding to non-centrosymmetric fibrils decreased, implying loss of fibrils through enzymatic degradation (Fig. 5.3). This was accompanied by increase in the average spacing between fibrils from 1.2 to 2.1 µm (Fig. 5.4).

Figure 5.3 SHG images of MMP-1 degraded gels. SHG images of collagen-PEG gels following exposure to MMP-1 for varied durations. The SHG signals decreased with increasing exposure to MMP-1. The inset in each image represented the magnified view of the region marked by the circle (scale bars represent 25 µm, inset magnified 2.5×).

Figure 5.4 Average spacing between collagen fibrils following MMP-1 degradation increased from 1.2 µm to 2.1 µm as the duration of exposure of collagen-PEG gels to MMP-1 increased.
5.3 Permeability assay of degraded collagen gels

Due to the increased mesh size, the partial digestion of the collagen-PEG gels resulted in slight increase in the diffusion coefficients of dextran molecules from 15 to 23 \( \times 10^{-8} \) cm\(^2\)/sec, as quantified with the FRAP assay (Fig. 5.5). These changes were comparable to the change in permeability with covalent cross-linking of pure collagen gels. The range of diffusivities observed was in the same order of magnitude as the diffusivities of proteins within native ECMs.

![Graph showing change in permeability following MMP-1 degradation](image)

**Figure 5.5 Change of permeability following MMP-1 degradation.** The diffusivity of dextran (\(D\)) within the collagen-PEG gel with \(E\) of 4.0 kPa was increased with increase in MMP-1 exposure duration. (*\(P < 0.0001\))

5.4 Control of HCC morphology through MMP degradation of gel

We next examined whether the decrease of collagen-PEG hydrogel stiffness from 4.0 to 0.5 kPa would trigger changes in morphology of hepatoid-like spheroids, formed in the stiff collagen-PEG hydrogel. Following the formation of hepatoid-like spheroids in the stiff collagen-PEG hydrogel, the spheroid-hydrogel constructs were exposed to MMP-1 for varied durations (Fig. 5.6). These constructs were then returned to normal cell culture media free of MMP-1 and further cultured for 4 days to observe the effect of matrix softening through MMP-1 degradation on tissue morphology.
Within 4 days, the cells in the gel degraded for 5 h \((E = 0.5 \text{ kPa})\) invaded the matrix and proliferated extensively. This generated large interconnected cell clusters with average span of 335 \(\mu\text{m}\). In contrast, the hepatoids in the undigested collagen-PEG gel \((E = 4.0\text{kPa})\) retained their size and compact morphology (Fig. 5.7). The sizes of the cell clusters were proportional to the period of exposure to MMP-1.

![Scheme for MMP-1 degradation of HepG2-encapsulating collagen gels](image)

**Figure 5.6** Scheme for MMP-1 degradation of HepG2-encapsulating collagen gels. HCC spheroids were formed by encapsulation in collagen-PEG \((E = 4.0 \text{ kPa})\) for 5 days. Then, the cell-encapsulating collagen-PEG hydrogel was incubated with MMP-1 for 0, 1, 3 or 5 hours. The cells were further cultured in MMP-1-free media for 4 days.

![Morphology of HepG2 cells following MMP-1 degradation of collagen gels](image)

**Figure 5.7** Morphology of HepG2 cells following MMP-1 degradation of collagen gels. The hepatoid-like HCC spheroids formed within the collagen-PEG gel became larger and more disorganized over 4 days after exposure to MMP-1. The change in spheroid size and the degree of intercellular disorganization were dependent on the exposure duration to MMP-1.
intracellular actin was stained with fluorescent phalloidin (green) and nuclei were imaged with DAPI (red). (Scale bars represent 50 µm)

In order to examine if the differences observed were due to change in matrix properties and not a result of MMP-1 exposure, we incubated HepG2 cells cultured on tissue culture plate for varying durations with MMP-1. When cultured on the tissue culture plate, exposure to MMP-1 for 0 h or 5 h did not result in any changes in cellular organization and cell growth rate. This indicated that the changes observed were a result of changes in matrix properties.

**5.5 Control of HCC phenotype through MMP degradation of gel**

Apart from the changes in intercellular organization and cell proliferation with different degrees of collagen-PEG softening, the decrease in $E$ of the hydrogel from 4.0 to 0.5 kPa also decreased the average diameter of the cell nucleus from $10 \pm 1.2 \, \mu m$ to $8 \pm 0.8 \, \mu m$. This implied that the cells responded to the softening of the matrix by lowering their intracellular tension.[133, 134] The reduced hydrogel stiffness also elevated the cellular expression of $\beta 1$-integrin (Fig. 5.8) while decreasing E-cadherin expression (Fig. 5.8). As mentioned in the earlier chapter, higher $\beta 1$-integrin expression had been associated with increased malignancy, while decrease in E-cadherin expression had been associated with both malignant transformation and loss of hepatocyte-specific functions.[135-137]

To determine if the softening of collagen-PEG gel subsequently resulted in a change of hepatocyte-specific activity, the cytochrome P450 detoxification activities of the encapsulated HCC were subsequently examined. It was found that without exposure to MMP-1, the HCC encapsulated in collagen-PEG gel retained high levels of
hepatocyte-specific cytochrome P450 activities. However, when cultured in MMP-1-softened collagen-PEG gels, the HCC exhibited much lower levels of cytochrome P450 detoxification activities (Fig. 5.9). This indicated that the softening of the gel subsequently lowered the intracellular tension of the HCC and resulted in changes in gene expression and cellular activities.

**Figure 5.8 Cell surface receptors of HepG2 following MMP-1 degradation of collagen gels.** Exposure of the collagen-PEG gel with spheroids to MMP-1 for 5 hours (a) increased expression of β1-integrin (yellow) and (b) decreased cellular expression of E-cadherin (blue). (Scale bars represent 50 μm.)
Figure 5.9 Cytochrome P450 detoxification activities of HepG2 following MMP-1 degradation of collagen gels. Exposure of the collagen-PEG gel with spheroids to MMP-1 for 5 hours reduced the P450 detoxification activity (marked by 256 pseudo-color in images). (Scale bars represent 50 μm.)

5.6 Discussion on regulation of HCC malignancy through MMP-1 degradation of cross-linked collagen

The reduction of hydrogel stiffness was implemented by exposing the stiffer collagen-PEG hydrogel, within which the HCC spheroids with suppressed malignancy were formed, to MMP-1 for varied durations. The exposure of collagen-PEG hydrogel to MMP-1 resulted in partial digestion of the collagen-PEG gels as shown from the SHG images and achieved a range of matrix stiffness ($E = 0.5$ kPa) from normal liver stiffness ($E = 4.0$ kPa). The softening of 3D matrix through MMP-1 exposure in turn altered intracellular tension as shown by the change in average nucleus diameter and promoted β1-integrin expression.[133-135] We believe that these changes led to alteration of cell signaling and subsequently resulted in up-regulation of cell proliferation and reduction in hepatocyte-specific P450 activities.
The reversion of benign hepatoids in collagen-PEG gel ($E = 4.0$ kPa) to malignant phenotypes, following matrix softening further demonstrated that the cells are able to sense and respond to dynamic changes in their microenvironment. This implied that our versatile platform, which allowed temporal control of matrix stiffness, would be a highly useful platform for studying the dynamics of malignant transformation. Previous studies reported that local up-regulation of MMP may result in increase in cell malignancy.[142] However, the underlying mechanism had never been explained with respect to quantified changes in matrix stiffness. Coupled with control studies in which HCCs were directly exposed to MMP-1, our results demonstrated that the elevation of cancer malignancy with MMP exposure could be related to matrix softening induced-cell signaling changes of top of other suggested pathways.

5.7 Conclusion on regulation of HCC malignancy through MMP-1 degradation of cross-linked collagen

Overall, this chapter demonstrated that benign hepatoids encapsulated in 3D gels with normal liver stiffness ($E = 4.0$ kPa) could be transformed to malignant phenotypes through MMP-1-induced matrix softening. This was characterized by changes in cell morphology, proliferation and cell phenotype. As this method of matrix softening allowed the $E$ of the matrix and onset of softening to be controlled, this approach, together with the platform generated through covalent cross-linking of collagen, would serve as a highly useful tool for studying how dynamic changes in matrix stiffness affects cell malignancy.
6 Regulation of fibroblast activation state through thermodynamics-driven modification of collagen

As discussed in chapter 2, apart from the bulk material stiffness of 3D scaffolds, the microstructure of the 3D scaffolds can also alter cell-integrin signaling and local material stiffness. This chapter describes the control of collagen microstructure through the regulation of the thermodynamics-driving force during collagen self-assembly. The varied microstructure and fiber stiffness, generated by adding free PEG chains to form collagen/PEG gels, was subsequently used to control the activation state of encapsulated fibroblasts. The work in this chapter addresses objectives 5 and 6 described in chapter 1.
6.1 Increasing fiber diameter of collagen gel through thermodynamic control

In the earlier chapter, bulk stiffness and architecture of collagen gels were altered by chemical cross-linking of collagen gels and MMP-1 degradation of collagen-PEG gels. Here, we report another method to control collagen gel fiber structure – via incorporation of free PEG chains to generate collagen/PEG gels. Unlike previous studies where PEG was covalently conjugated to collagen to alter collagen gel properties, no chemical reaction between the collagen and PEG was required.

Using this approach, we prepared collagen gels with different mass ratio of PEG chains to collagen ($M_{PEG2-COL}$). Increasing $M_{PEG2-COL}$ from 0 to 25 led to a corresponding increase in mean size of fibers from 90 nm to 30 µm as shown in the SEM images (Fig 6.1). At $M_{PEG2-COL} = 25$, we were able to generate gels with collagen fibers having similar fiber diameters as perimysial collagen cables surrounding muscle fibers (Fig. 6.1).[143] These features were not reproducible by chemically cross-linked control gels formed through reaction with bi-functional PEG-diNHS (Fig. 6.1). The mean fiber diameters ($d$) increased in a fairly linear fashion with increasing PEG concentrations (Fig. 6.2). Similar to other studies, pure collagen gels ($M_{PEG2-COL} = 0$) comprised almost exclusively of small fibers with diameters of approximately 90 nm. These small fibers were present at all values of $M_{PEG2-COL}$. However, the proportion of small fibers decreased with increasing PEG concentration. As the total collagen concentration remained constant in all the conditions, the mesh size ($\xi$) of the gels increased with increasing fiber thickness (Fig. 6.3). However, the largest pores observed in the condition of $M_{PEG2-COL} = 25$ were less than 5 µm. This implied that cells are still able to interact with adjoining fibers.
Figure 6.1 SEM images of collagen gels. Increasing the mass ratio of PEG to collagen ($M_{PEG2-COL}$) resulted in the corresponding increase in thermodynamic driving force for self-association, thereby increasing the fiber diameters. These images were obtained with SEM following critical point drying of the respective gels. Pure collagen gels comprised exclusively of small fibers ($\approx 90$ nm) while the PEG-containing gels comprised of a mixture of large and small fibers. The fraction of small fibers decreased with increasing $M_{PEG2-COL}$. The collagen gels formed by thermodynamic control had distinct architecture as compared to chemically cross-linked control gels formed by reaction with PEG-diNHS cross-linkers as shown above. Also, the size of the collagen fiber bundles in $M_{PEG-COL} = 25$ resembled that of perimysial collagen cables found in muscle as shown. (*Image was obtained from study by Gillies, A. R. et. al.[143]) (Scale bars represent 1 µm, inset magnified 4×)

Figure 6.2 Change in collagen fiber diameters. The increase in $M_{PEG2-COL}$ from 0 to 25 resulted in a corresponding increase in the mean fiber diameter ($d$) from approximately 90 nm to 30 µm in a linear fashion. (Bars represent standard error. *Single-factor ANOVA test, $\alpha < 0.01$)
Figure 6.3 Change in mesh size of collagen gels. The increase in $M_{PEG2-COL}$ from 0 to 25 also resulted in a corresponding increase in the mesh size ($\xi$) from approximately 1.0 µm to 2.1 µm in a linear fashion. (Bars represent standard error. *Single-factor ANOVA test, $\alpha < 0.01$)

6.2 Mechanistic study of thermodynamic control

To investigate the validity of our proposed mechanism, we carried out further characterizations with FTIR and DSC. FTIR was used to investigate if there was direct hydrogen bonding between collagen and PEG, while DSC was used to determine the relative proportions of freezable free water and un-freezable bound water, both before and after gelation. A higher proportion of freezable free water would lead to a larger endothermic peak during melting of frozen water, while higher proportion of un-freezable bound water would lead to a smaller peak.[120] The ratios of free water to bound water provided an indication of the relative entropies of the systems. Through FTIR analysis, we found that there was no shift in the amide bands of collagen gels after the incorporation of PEG (Table 6.1), thus demonstrating limited hydrogen bonding between collagen and PEG. This lack of hydrogen bonding was also observed in previous studies involving polymer films cast from collagen and PEG.[144, 145]
Table 6.1 FTIR analysis of collagen and collagen/PEG gels. Amide bands of collagen showed limited shift after addition of PEG, thus indicating that hydrogen bonds were not formed between collagen and PEG.

From the results of the DSC characterization, it was apparent that increase in $M_{PEG2-COL}$ resulted in the corresponding reduction of the endothermic peak in the pre-gel solutions (Fig. 6.4). This indicated larger ratios of bound water due to the increase in amount of hydrophilic PEG chains. The increasing ratios of bound water to free water implied decreasing entropy with increasing $M_{PEG2-COL}$. However, following complete gelation, there were no longer differences in endothermic peaks with increase in $M_{PEG2-COL}$ (Fig. 6.4). This implied that the initial difference in entropies was normalized during the gelation process.

A theoretical model was proposed to explain the underlying mechanism of structural control by incorporation of free PEG chains. In pre-gel solutions, solubilized collagen molecules formed extensive hydrogen bonds with water. The water associated with collagen, termed bound water, decreased the overall entropy of the system due to their lowered motility. This unfavorable entropy provided a driving force for assembly of the solubilized collagen molecules into fibers bundles (Fig. 6.5). During the self-assembly of collagen, new hydrogen bonds between collagen were formed while hydrogen bonds between collagen and water were broken. As the gain in entropy due to the freeing of water far outweighed the loss of entropy due to collagen association, favorable entropy was restored. When long PEG chains were introduced into the pre-gel
solution, the amount of bound water was further increased through hydrogen bonding with the hydrophilic PEG. This resulted in even lower entropy in the pre-gel solutions, thus providing a larger driving force for collagen self-assembly. The result was the formation of larger fiber bundles so as to restore entropy (Fig. 6.5). With this proposed mechanism, the fiber diameter should be proportional to the mass ratio of $M_{PEG2-COL}$, thus providing a simple way of controlling collagen fiber size and density in situ (Fig. 6.6).

![Figure 6.4 DSC characterization of collagen gels.](image)

**Figure 6.4 DSC characterization of collagen gels.** Increasing $M_{PEG2-COL}$ from 0 (○) to 5 (●), 10 (△) and 25 (▲) resulted in the corresponding reduction of water’s melting endothermic peaks in pre-gel solutions. This indicated that there was a smaller portion of freezable free water and a larger proportion of un-freezable bound water. However, following complete self-association of the collagen fibrils to form fibers, the melting endotherms were uniform for all $M_{PEG2-COL}$ from 0 (■) to 5 (●), 10 (△) and 25 (▲). This indicated that the initial disparity in bound water fraction and entropy was normalized by the different degree of self-association between collagen fibrils.
Figure 6.5 Regulation of collagen fiber structure through thermodynamic control. (a) In pre-gel solutions, solubilized collagen molecules formed extensive hydrogen bonds (H-bonds) with water (blue spheres). The large proportion of H-bonded water, termed bound water (dotted), decreased the overall entropy of the system. This low entropy provided the thermodynamic driving force to bring about association of collagen fibrils into collagen fiber bundles. Collagen fiber formation subsequently decreased H-bonds with water, thereby reducing the fraction of bound water and restored overall entropy of the system. (b) With the incorporation of hydrophilic PEG chains (red spheres), the proportion of bound water in pre-gel solution further increased, creating a lower entropy. This provided a larger thermodynamic force for self-association of collagen fibrils in order for entropy to be restored. As a result, the size of the resultant collagen fibers increased.

Figure 6.6 Predicted change in fiber thickness with PEG content. Fiber thickness was expected to increase with increasing $M_{PEG-COL}$. As the total collagen content was kept constant, the mesh size was expected to increase with increasing fiber thickness. These trends were observed in the actual gels shown in Fig. 6.1.

By considering the measured amounts of bound water and the theoretical amount of water bound to the PEG chains, we were able to predict the change in collagen fibers
diameters with change in $M_{PEG\text{-}COL}$ (Fig. 6.7, details of calculation presented in chapter 3.10). The good agreement of actual fiber diameter with predicted fiber diameter further indicated that change in entropy due to binding state of water was the dominant mode of structural control.

**Figure 6.7 Theoretical fibers of collagen gels.** The theoretical fiber diameters were predicted by considering the amounts of bound water and the theoretical amounts of water bound to the PEG chains (•••). The good agreement of actual $d$ (○) and predicted $d$ indicated that the suggested mechanism was the dominant mode of structural control of the PEG-containing collagen gels.

### 6.3 Increasing fiber rigidity of collagen gel through thermodynamic control

We further investigated the effect of PEG incorporation on bulk and local fiber mechanical properties. The bulk mechanical properties were quantified through the measurement of storage moduli ($G'$) of the gels subjected to low-amplitude oscillatory shear deformation. As $M_{PEG\text{-}COL}$ was increased from 0 to 5 and 10, a corresponding decrease in $G'$ was observed (Fig. 6.8). However, further increase of $M_{PEG\text{-}COL}$ from 10 to 25 resulted in a subsequent increase of $G'$. This complex trend in $G'$ with varying $M_{PEG\text{-}COL}$ could be attributed to the simultaneous changes in fiber diameter and porosity.[119] According to the MacKintosh model, an increase in porosity was expected to result in the reduction of $G'$ while an increase in fiber diameter was expected to result
in the increase in $G'$.\[119\] The $G'$ of pure PEG solution was almost zero, indicating that the incorporated PEG chains did not participate directly in the gel mechanics (Fig. 6.8).

**Figure 6.8 Storage modulus ($G'$) of the collagen gels** displayed a complex trend with change in $M_{\text{PEG2-COL}}$. As $M_{\text{PEG2-COL}}$ was increased from 0 (-■-) to 5 (-●-) and 10 (-▲-), the $G'$ decreased to a minimum. However, further increase of $M_{\text{PEG2-COL}}$ to 25 (-▼-) resulted in an increase in $G'$. This complex relationship could be attributed to the concurrent changes in mesh size and single fiber rigidities with changes in $M_{\text{PEG2-COL}}$. Pure PEG solution (-◆-) had $G'$ of almost zero, indicating that the pure PEG solution did not participate in the gel mechanics.

The individual fiber stiffness were quantified by determining the fiber bending rigidity ($\kappa$), calculated from the MacKintosh model from $G'$ and $\xi$ (Fig. 6.9, equations presented in chapter 3.5).[66] As expected, the $\kappa$ values increased with $M_{\text{PEG2-COL}}$ due to increasing fiber diameters. These $\kappa$ values of the individual fibers fell within the theoretical $\kappa$ values calculated from their second moment of inertias. To obtain the theoretical $\kappa$ values, the fibers bundles were either assumed to be continuous materials with strong covalent cross-links between comprising fibrils or assumed to be loose bundles of fibrils with no inter-fibril interactions (Fig. 6.9, equations presented in chapter 3.5).[119] In the former scenario, the theoretical $\kappa$ was much higher and scaled with the fourth power of fiber diameters. However, in the latter case, individual fibrils were able to slide over each other, thus resulting in low $\kappa$ values. As the actual $\kappa$ values fell between the two extremes but were closer to the second scenario, it was expected that the
fibrils within each fiber bundle should be able to slide over each other to some degree. However, their movements were restricted by hydrogen bonding between fibrils.

Figure 6.9 Fiber bending rigidities ($\kappa$) of collagen gels were estimated from the $G'$ and mesh size ($\xi$) of the respective gels with the MacKintosh model (-0-). The values obtained were within the range of theoretical $\kappa$ calculated from the respective mean second moment of inertias. For the calculation of the theoretical $\kappa$, the fibers bundles were either assumed to be continuous materials made up of fibrils with strong covalent cross-links (---) or bundles of free-sliding fibrils with no inter-fibril interactions (•••). As the $\kappa$ values of the collagen gels fell between these two extreme models, it was expected that fibrils in the same fiber bundle were able to slide over each other to some degree. However, this movement could be restricted by hydrogen bonding between fibrils.

It had been shown in previous studies that cells were only able to sense the mechanical properties of the surrounding matrix within a couple of microns, thus indicating that encapsulated cells should be more greatly affected by local fiber mechanics than the overall mechanical properties.[146, 147] Apart from the generation of unique gel architecture, another notable feature of this mode of control was the increase in mesh size with individual fiber rigidity. The rigidity of hydrogels was conventionally increased by increasing either polymer concentration or cross-linking densities.[51, 148-150] This typically led to decrease in mesh size and could be associated with compromised mass transfer and reduced viability of encapsulated cells.[151] With the proposed method of gel synthesis, stiffer environments could be
introduced without reducing mesh size. We postulate that in future studies, it should also
be possible to vary fiber diameter while maintain constant mesh sizes by simultaneously
tuning both collagen and PEG concentrations.

6.4 Control of fibroblast morphology and phenotype with varied fiber rigidity

Fibroblasts were incorporated into the collagen gels modified with varied $M_{PEG2-coll}$ in order to examine the effects of modified fiber structures on cellular morphology and phenotype. The cells were loaded into the different hydrogels by mixing them with pre-gel solutions and subsequently incubating the mixtures at physiological temperature and pH to activate gel formation. As there were no covalent cross-linking between collagen and PEG, the gels were then washed to remove most of the PEG after gel formation. Following culture for 10 days, cell morphology of the fibroblasts in the respective gels was examined. The fibroblasts in pure collagen gels were spindle-shaped and formed an interconnected network with limited cell-cell adhesion. This morphology was commonly observed when fibroblasts were cultured in soft cell-adhesive hydrogels. Conversely, fibroblasts in the collagen/PEG gel with thick fibers formed bundles with extensive cell-cell contact (Fig. 6.10). These fibroblast bundles bear resemblance to contractile proto-myofibroblasts which were commonly found in healing wound tissues.[58, 152] Such morphology was not found in control gels formed by PEG-diNHS chemical cross-linking (Fig. 6.10). Apart from the change in appearance, the fibroblast in the collagen/PEG gels also displayed higher levels of actin, which was also commonly observed in proto-myofibroblasts (Fig. 6.11). [58, 152] At the end of the culture period, the contractile functions of the fibroblasts in pure collagen gels and muscle-like gels were further examined by using a common wound healing assay, where the degree of gel
shrinkage between different conditions were compared. After culture for 10 days, it was found that there was a greater shrinkage of the collagen/PEG gel as compared to its counterpart, indicating higher contractile function of the fibroblast bundles in the former (Fig. 6.12). Taken together, the results demonstrated that the gels formed by thermodynamic control with free PEG chains were effective in instructing cell phenotypes. By altering the fiber architecture, we were able to direct the fibroblast to adopt either a quiescent state or an activated, proto-myofibroblast state.

![Figure 6.10 Morphology of fibroblasts.](image)

Fibroblasts cultured within the respective gels for 10 days were stained with Hoescht (red) to reveal the nuclei and with TRITC-phalloidin (green) to reveal the actin cytoskeleton. Fibroblasts in pure collagen gels ($M_{PEG2-COL} = 0$) were spindle-shape and formed interconnected networks with limited cell-cell contact between individual cells. This was typical of collagen cultured in soft, cell-adhesive hydrogels. However, when cultured in collagen/PEG gels ($M_{PEG2-COL} = 25$) with thick collagen fibers resembling muscular collagen cables, the fibroblasts aggregated to form fibroblast bundles with extensive cell-cell contact. These fibroblasts resembled proto-myofibroblasts. This phenotype was not observed in the PEG-diNHS cross-linked control collagen-PEG gels ($M_{PEG1-COL} = 0.33$). (Scale bars represent 50 µm)
Figure 6.11 Actin levels of fibroblasts. The fibroblasts in the muscle-like collagen/PEG gel ($M_{PEG2-COL} = 25$) had a higher level of normalized actin levels than fibroblasts cultured in pure collagen gel ($M_{PEG2-COL} = 0$). This up-regulation of actin was typical in activated fibroblasts or proto-myofibroblasts. (Bars represent standard error. *Student’s t-Test, $\alpha < 0.05$)

Figure 6.12 Contractile activity of fibroblasts. Following culture for 10 days, the fibroblasts in muscle-like collagen/PEG gels ($M_{PEG2-COL} = 25$) contracted the gels extensively while no contraction was observed in pure collagen gels ($M_{PEG2-COL} = 0$). This indicated that the fibroblasts in the muscle-like gels had a highly contractile phenotype as compared to their counterpart in pure collagen gels. (Bars represent standard error. *Student’s t-Test, $\alpha < 0.01$)

To confirm that the phenotypic changes observed were due to the differences in fiber structure rather than the initial presence of PEG, we further encapsulated fibroblasts in pure collagen gels and incubated the gels in PEG-containing media. The addition of PEG after stable gel formation did not result in differences in fibroblast morphology or metabolic activity as characterized by the MTT assay (Fig. 6.13 & 6.14). This demonstrated that the activation of the fibroblast was due to the change in fiber structure which took place during self-assembly rather than due to the presence of PEG. We
propose that the observed phenotypic changes could be attributed to the increased stiffness of the individual fibers in collagen/PEG gels. This observation was in line with previous studies which reported the activation of quiescent fibroblasts when cultured on substrates with increased rigidity.[153] However, unlike the 2D studies, our 3D configuration allowed extensive reorganization and formation of bundles with good cell-cell contacts.

![Figure 6.13 Effect of free PEG on fibroblast morphology.](image)

Fibroblast cells encapsulated in pure collagen gels were subsequently incubated in the both PEG-free and PEG-containing media for a period of 3 days. The cells were cultured in PEG-free media for another 3 days before their morphologies were studied. Fibroblasts in both conditions were spindle-shaped and had no observable differences, indicating that the differences in morphologies observed in the earlier experiments were due to change in fiber architectures and not due to the presence of PEG. (Scale bars represent 50 µm)

![Figure 6.14 Effect of free-PEG on fibroblast metabolic activity.](image)

Metabolic activity of fibroblast did not alter significantly after incubation with PEG solution for two days. (Bars represent standard error. Student’s t-Test, p = 0.27)
6.5 Discussion on regulation of fibroblast activation state through thermodynamics-driven modification of collagen

Here, we reported a novel method to generate unique collagen gel structures for cell instruction. This was achieved by directing collagen self-assembly with free hydrophilic PEG chains. With this method, the microstructures of three-dimensional collagen gels were readily controlled in terms of fiber size and density. Increasing PEG content during collagen gel formation resulted in increasing fiber size and mesh size. At $M_{PEG2-COL} = 25$, we were able to form large collagen fiber bundles resembling perimysial collagen cables in muscles. The collagen/PEG gels formed by non-covalent PEG modification had very different structures when compared to the collagen-PEG gels formed by PEG-diNHS cross-linking in chapter 4 due to the different underlying mechanisms of gel formation. With the method of collagen modification presented in this chapter, we observed an increased mesh size with increasing fiber rigidities. This was also different from the covalently cross-linked gel proposed in chapter 4. As such, this method of collagen modification provided the added advantage of increasing local rigidities of fiber bundles without any decrease of mass transfer rates. By using FTIR and DSC characterizations, we were able to provide evidence for the underlying mechanism of this PEG-mediated thermodynamic control. We were also able to predict the changes in fiber structure with initial PEG content.

Finally, we investigated the effects of these unique structures in cell-instructive collagen gels. When fibroblasts were cultured in collagen/PEG gels resembling perimysial collagen cables, pronounced phenotypic changes occurred and quiescent fibroblasts were activated to form contractile proto-myofibroblast bundles. These
morphological and phenotypic changes were not observed in Peg-diNHS cross-linked collagen-PEG gels. Through further calculations, it was found that in the covalently cross-linked collagen-PEG gels presented in Chapter 4, despite increase in bulk moduli from $E = 0.7$ kPa to $E = 4.0$ kPa, the fiber rigidity decreased approximately ten-fold. This could be attributed to the thinner collagen fibers due to the more homogenous distribution in the presence of the PEG-diNHS short cross-linkers. In the case of the collagen/PEG gel investigated in this chapter, despite a decrease in bulk storage modulus from $G' = 13.2$ Pa to $G' = 8.3$ Pa, fiber rigidity increased approximately nine-fold. The differences in local and bulk rigidities of collagen-PEG and collagen/PEG gels demonstrated that apart from the overall stiffness of 3D scaffolds, the microstructures of the scaffolds also play very important roles in regulating cell activities. This could probably be attributed to varied engagement of integrin with changes in gel microstructures. Taken together, the collagen-PEG scaffold presented in chapters 4 and 5, and the collagen/PEG scaffold presented in chapter 6 were useful for different cell instruction purposes.

6.6 Conclusion on regulation of fibroblast activation state through thermodynamics-driven modification of collagen

In summary, through the incorporation of free PEG chains, we were able to generate unique gel structures not reproducible by chemical cross-linking. We were also able to achieve increased substrate stiffness with increasing mesh size, which was opposite from the trend exhibited by conventional cross-linked gels. This study was also the first to demonstrate that thermodynamic control could be used to generate unique cell-instructive scaffolds. This mode of control could potentially be applied to other self-
assembling gels such as peptide and fibrin. Through the microstructuring mediated by thermodynamic control, we were able to provide the stimulation for fibroblast activation.

As this was only a preliminary study to examine the feasibility of PEG-mediated thermodynamic control for generation of cell-instructive collagen gels, further characterizations in terms of gene and protein expression have to be carried out to study the underlying mechanisms.
7 PEGDA-PEI hydrogels with tunable mechanical and drug release properties

On top of the microenvironmental cues covered in the earlier chapters, this chapter looks into the design of tailored drug release vehicles to enable better regulation of local soluble cues. This chapter presents a stiff and metastable poly(ethylene glycol) diacrylate (PEGDA)-poly(ethyleneimine) (PEI) hydrogel which exhibits an elastic modulus equivalent to bulk plastic materials, and controllable degradation rate independent of its initial elastic modulus. Such unique stiffness allowed the hydrogel to resist local tissue pressures while releasing encapsulated drugs in a timely fashion. With the PEGDA-PEI hydrogel, a single intramuscular administration of granulocyte colony stimulating factor (GCSF)-encapsulated hydrogel extended the mobilization of mononuclear cells to four days. Overall, the hydrogel created in this study will be useful for the controlled and sustained delivery of a wide array of drug molecules. This work covers objective 7 described in chapter 1. This work has been published in Biomaterials.[122]
7.1 Synthesis of PEGDA-PEI hydrogels

The PEGDA-PEI hydrogels were formed through Michael reactions between the acrylate groups in PEGDA and primary and secondary amine groups in branched PEI (Fig. 7.1). It was predicted that the highly branched PEI structure would contribute to the high stiffness of the PEGDA-PEI hydrogel, while the large number of protonatable amine groups on the PEI would contribute to water influx and timely drug release. To prepare the hydrogels, PEGDA and PEI in separate syringes were mixed through syringe connectors. Following mixing, the pre-gel solution was fluid and easily injectable. This PEGDA-PEI mixture then set within a minute upon mixing and expulsion. This allowed the PEGDA-PEI hydrogel to be retained at the site of injection.

The Michael reaction between the acrylate groups of PEGDA and the amine groups of PEI was confirmed by the disappearance of acrylate peaks at 6.2 ppm in the $^1$H-NMR spectra of the hydrogel and the disintegrated hydrogel fragments (Fig. 7.2). In contrast, mixtures of PEGDA and linear PEI did not form a hydrogel at similar concentrations. Previously, several studies had reported the use of Michael reaction between PEGDA and PEI to prepare polymeric gene carriers in the form of nanoparticles. However, this was the first study in which the Michael reaction between PEGDA and PEI was used to form a bulk hydrogel.
Figure 7.1 PEGDA-PEI hydrogels were formed by Michael-type cross-linking reactions between the amine groups of branched PEI and the acrylate groups of PEGDA. The highly branched structure of the PEI contributed to the high stiffness of the hydrogel while the protonated amines contributed to the high water influx.

Figure 7.2 $^1$H-NMR analysis of PEGDA-PEI hydrogels. $^1$H-NMR spectra of (a) pure PEGDA solution, (b) pure PEI solution, and (c) degradation products of the PEGDA-PEI hydrogel. The disappearance of peaks at 6.2 ppm in (c) indicated that acrylate groups in the PEGDA were consumed to form the hydrogel.
7.2 Tuning mechanical properties of PEGDA-PEI hydrogels

Most conventional hydrogels were soft and had limited mechanical stiffness. However, a certain level of load-bearing capabilities was essential to prevent pre-mature release of encapsulated substances due to local tissue pressures. The load-bearing capacities of the resulting hydrogels were evaluated by measuring the compressive elastic moduli ($E$) of the hydrogels formed with varied concentrations of PEGDA and PEI. The initial compressive moduli ($E_0$) of the PEGDA-PEI hydrogels were significantly increased with increasing concentrations of PEI at a constant PEGDA concentration of 20 %, or with increasing concentrations of PEGDA at a constant PEI concentration of 10 % (Fig. 7.3). Overall, $E_0$ of the PEGDA-PEI hydrogels were controlled from 1 to 8 MPa, which were one to two orders of magnitude higher than elastic moduli of the pure PEGDA hydrogels formed from a radical cross-linking reaction (Fig. 7.3). Strikingly, $E_0$ of the hydrogels consisting of 20 % PEGDA and 5 to 10 % PEI were comparable to the compressive modulus of poly(styrene) (~4 MPa) as provided by American Society for Testing Materials (ASTM). The initial number of cross-links ($N_0$) for the PEGDA-PEI hydrogels, calculated from the $E_0$ and the initial swelling ratios ($Q_0$) using rubber elasticity theory, was one order of magnitude higher than that of the pure PEGDA hydrogels formed from radical cross-linking reactions (Table 7.1).[123]
Figure 7.3 Initial compressive elastic moduli ($E_0$) of PEGDA-PEI hydrogels were increased with PEI concentration at a given PEGDA concentration of 20 % (▲). $E_0$ of the PEGDA-PEI hydrogels were also increased with PEGDA concentration at a given PEI concentration of 10 % (■), and the dependency of $E_0$ on the PEGDA concentration was more significant than the radically cross-linked PEGDA hydrogels (●). The $E_0$ value and error bar for each condition represent the average elastic modulus and the standard deviation from the measurements of four samples.

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Table 7.1 Cross-linking densities of PEGDA-PEI hydrogels. Initial number of cross-links ($N_0$) of the PEGDA-PEI hydrogels was one to two orders of magnitudes higher than that of the radically cross-linked PEGDA hydrogel.

7.3 Tuning degradation of PEGDA-PEI hydrogels

In parallel, degradation rates of the PEGDA-PEI hydrogels incubated in neutral PBS were quantified by measuring the increase in swelling ratio ($Q_m$) over time (Fig. 7.4). Initially, the swelling ratio increased without a decrease in solid mass. However, as the swelling ratios reached higher values of about 10, the solid mass of the hydrogel disks started to decrease significantly. Interestingly, the time required for complete structural
disintegration of the hydrogels decreased with increasing concentrations of PEI at a constant PEGDA concentration of 20 %, or with decreasing concentrations of PEGDA at a constant PEI concentration of 10 %. The dependency of $Q_m$ on incubation time was fitted to calculate the degradation rate ($k_1$), swelling exponent ($n$) and water diffusivity ($D$) (Table 7.2). Increasing the PEI concentration at a constant PEGDA concentration of 20 % increased $k_1$, $n$, and $D$. In contrast, increasing the PEGDA concentration at a constant PEI concentration of 10 % significantly decreased $k_1$, $n$ and $D$. These changes in $k_1$, $n$, and $D$, resulting from changes in PEGDA concentrations or PEI concentrations, were related to the number of unreacted amine groups of PEI (Fig. 7.5). Overall, increasing the PEGDA concentration at a constant PEI concentration resulted in an inverse dependency between $D$ and $E_0$, typical of conventional hydrogels (Fig. 7.6). Conversely, increasing the PEI concentration at a constant PEGDA concentration resulted in increases of both $D$ and $E_0$ of the hydrogel, which is distinct from conventional biodegradable hydrogels (Fig. 7.6).
Table 7.2 The swelling constants ($k_1$), swelling exponents ($n$), and water diffusion coefficients ($D$) for different PEGDA-PEI hydrogel formulations were determined from logarithmic plots of $Q_m$ vs $t$ ($r^2 \geq 0.97$). $k_1$, $n$ and $D$ decreased with increasing PEGDA concentration at a given PEI concentration of 10 %, while they increased with increasing PEI concentration at a given PEGDA concentration of 20 %. The moles of unreacted amines decreased with increasing PEGDA concentration at a given PEI concentration of 10 % and the moles of unreacted amines increased with increasing PEI concentration at a given PEGDA concentration of 20 %.

<table>
<thead>
<tr>
<th>PEGDA (%)</th>
<th>$k_1$</th>
<th>$N$</th>
<th>$D$ (10^{-5} \text{ cm}^2\text{s}^{-1})$</th>
<th>Unreacted Amines (10^{-6} \text{ moles})</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>0.32</td>
<td>0.97</td>
<td>94.8</td>
<td>1888</td>
</tr>
<tr>
<td>15</td>
<td>0.28</td>
<td>0.92</td>
<td>72.3</td>
<td>1569</td>
</tr>
<tr>
<td>20</td>
<td>0.21</td>
<td>0.86</td>
<td>42.8</td>
<td>1196</td>
</tr>
<tr>
<td>25</td>
<td>0.18</td>
<td>0.78</td>
<td>23.3</td>
<td>892</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PEI (%)</th>
<th>$k_1$</th>
<th>$N$</th>
<th>$D$ (10^{-5} \text{ cm}^2\text{s}^{-1})$</th>
<th>Unreacted Amines (10^{-6} \text{ moles})</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>0.05</td>
<td>0.66</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.09</td>
<td>0.83</td>
<td>7.0</td>
<td>9.4</td>
</tr>
<tr>
<td>10</td>
<td>0.21</td>
<td>0.86</td>
<td>42.8</td>
<td>1249</td>
</tr>
<tr>
<td>15</td>
<td>0.22</td>
<td>0.92</td>
<td>49.2</td>
<td>2041</td>
</tr>
</tbody>
</table>

Figure 7.4 The normalized swelling ratios ($Q_m$) of the PEGDA-PEI hydrogels was also modulated by the PEGDA concentration which was increased from 12.5 % (●), to 15% (★), 20 % (●) and 25 % (●) at a given PEI concentration of 10 %.
Figure 7.5 Degradation rates \( (k_1) \) of PEGDA-PEI hydrogels were increased with the number of unreacted amines, which were varied with PEI concentrations (■) or PEGDA concentrations (▲) of the PEGDA-PEI hydrogels.

Figure 7.6 Diffusivities within PEGDA-PEI hydrogels. Increasing the PEI concentration of the hydrogels at a given PEGDA concentration of 20 % resulted in the increase of both \( E_0 \) and \( D \) (■). In contrast, increasing the PEGDA concentration of the hydrogels at a given PEI concentration of 10 % led to the inverse dependency between \( D \) and \( E_0 \) (▲).

The degradation process of the PEGDA-PEI hydrogel was further examined with MRI, which enabled the visualization of water protons that were bound to ("bound water"), and free from ("free water") the hydrogels (Fig. 7.7).[127] It had been demonstrated that “bound water” presented much stronger signal intensity than “free water”. Following imaging of the hydrogels, the resonance intensities in the MRI was converted to the color density map as shown in the scale bar (Fig. 7.7). The pure
PEGDA hydrogel undergoing swelling in PBS showed a gradual increase of “bound water” volume as confirmed with the increase of yellow color intensity. In contrast, the PEGDA-PEI hydrogel which rapidly swelled in PBS showed an initial increase “bound water” volume followed by the increase of the “free water” volume, as exhibited by the increase and subsequent decrease of yellow color intensity. The increase of the ‘free water’ demonstrated the much higher water diffusivities in PEGDA-PEI hydrogels as compared to pure PEGDA hydrogels. The rate of increase in the “free water” volume became larger with an increase in PEI concentration, which illustrated that the PEI drove the faster structural disintegration of the hydrogel by facilitating the accumulation of “free water” in the hydrogel.
Figure 7.7 MRI imaging of PEGDA-PEI hydrogels. Water sorption processes into (i) a hydrogel solely consisting of 20% PEGDA, (ii) a hydrogel consisting of 20% PEGDA and 3.5% PEI, and (iii) a hydrogel consisting of 20% PEGDA and 10% PEI were monitored using MRI. The MRI images were taken every 25 minutes after incubating the gel in deionized water. White bars under the gel represent the original lengths of the hydrogels (10 mm). The dotted lines in the MRI images represent the periphery of the hydrogels. Scale bar on the bottom indicates the degree of interaction between water and hydrogels. A high intensity of yellow color denotes strongly “bound” water molecules, whereas a low intensity of yellow color corresponds to “free” water molecule. Pure PEGDA hydrogel (i) showed limited change of its original volume along with the gradual increase of the amount of “bound” water over time. In contrast, the PEGDA-PEI hydrogels (ii and iii) showed the rapid increase of “free” water volumes over time and, consequently, the drastic increase of the hydrogel volumes.

The degradation rates of the PEGDA-PEI hydrogels could also be tuned by extrinsic factors such as ionic strength of PBS. Interestingly, the rate of water uptake of the PEGDA-PEI hydrogel was decreased with increasing ionic strengths of the PBS (Table 7.3). Ultimately, increasing ionic strength of the PBS solution by 20 times induced the hydrogel’s swelling to reach an equilibrium state during the first 4 days of incubation. These results suggest that increasing the ionic strength of PBS inhibited swelling of the hydrogel, likely because of the screening of the charged groups in PEI, and the elevation of osmotic efflux of water from the hydrogels.
### Table 7.3 Swelling of PEGDA-PEI hydrogels in different ionic strengths.

<table>
<thead>
<tr>
<th>Incubation solution</th>
<th>Water uptake (mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.5 % PEI</td>
</tr>
<tr>
<td>1× PBS</td>
<td>0.081</td>
</tr>
<tr>
<td>5× PBS</td>
<td>0.035</td>
</tr>
<tr>
<td>10× PBS</td>
<td>0.029</td>
</tr>
<tr>
<td>20× PBS</td>
<td>0.003</td>
</tr>
</tbody>
</table>

3.5 % PEI and 10 % PEI hydrogels with fixed concentration of 20% PEGDA were immersed in solutions of different ionic strengths at 25 °C. Increasing ionic strength resulted in decreasing water uptake rates of the PEGDA-PEI hydrogels.

#### 7.4 In vitro drug release assay of PEGDA-PEI hydrogels

Next, the effects of PEI concentrations on drug release profiles were examined by encapsulating fluorescently labeled BSA into the hydrogel (Fig. 7.8). The encapsulation of the BSA into the PEGDA-PEI hydrogel decreased the degradation rate of the hydrogel. It was likely that electrostatic attraction between negatively charged BSA and amine groups of PEI slowed the degradation rate of the hydrogel. This could be attributed to the decrease in the effective number of protonated amine groups. However, the degradation rate of the hydrogel was still controlled by the concentration of PEI. Specifically, the BSA was released from the hydrogels following a lag period ($t_l$), which was extended from 1.0 to 6.5 days by decreasing the concentration of PEI (Table 7.4). The release rate of BSA ($k_2$), calculated in the chapter 3.29, was also increased with the concentration of the PEI. The dependency of $k_2$ on the degradation rate ($k_1$) of the hydrogels indicated that the drug release kinetics from the PEGDA-PEI hydrogel was controlled by the degradation behavior of the hydrogel. Together with the results of the MRI studies, it is likely that the degradation process of the hydrogel expanded the pores of the hydrogel and facilitated diffusion of BSA from the hydrogel.
Figure 7.8 The *in vitro* BSA release profiles from the PEGDA-PEI hydrogels were regulated by the concentrations of PEI. The PEI concentration was changed from 3.5 % (■) to 5 % (●), 10 % (▲) and 15 % (▽), while keeping the PEGDA concentration constant at 20 %.

<table>
<thead>
<tr>
<th>PEI (%)</th>
<th>20 % PEGDA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_1$ (days)</td>
</tr>
<tr>
<td>3.5</td>
<td>6.27</td>
</tr>
<tr>
<td>5</td>
<td>1.28</td>
</tr>
<tr>
<td>10</td>
<td>0.01</td>
</tr>
<tr>
<td>15</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 7.4 *In vitro* drug release constants of PEGDA-PEI hydrogels. The lag periods, quantified with time taken for 10% release of BSA ($t_1$), drug release constants ($k_2$), and release exponents ($m$) for different hydrogel formulations were determined from logarithmic plots of $M_t/M_\infty$ vs $t$ ($r_2 \geq 0.74$). $k_2$ and $m$ were regulated by the PEI concentrations at a given PEGDA concentration of 20 %.

7.5 Cytotoxicity assay of PEGDA-PEI hydrogels

Prior to the *in vivo* drug release experiments, the cytotoxicity and immunogenicity of the hydrogel fragments, resulting from the degradation process, were further examined by implanting hydrogel disks onto chicken chorioallantoic membranes (CAMs). Implantation of cytotoxic foreign substances on the CAMs would result in death of the chicken embryos while implantation of immunogenic foreign substances would result in extensive inflammatory responses.[154] The hydrogels implanted on CAMs degraded at a rate comparable to the hydrogels incubated in PBS. Following incubation for 6 days, it
was found that hydrogels containing less than 15 % PEI did not stimulate inflammation or present significant cytotoxicity (Fig. 7.9)

Figure 7.9 Cytotoxicity and inflammatory response analysis of PEGDA-PEI hydrogels. (a) Top view of the CAMs captured right after and 6 days after implantation of (i) 20 % PEGDA hydrogel formed from photo cross-linking reaction, (ii) 20 % PEGDA-3.5 % PEI hydrogel formed from Michael cross-linking reaction, and (iii) 20 % PEGDA-10 % PEI hydrogel formed from Michael cross-linking reaction. The photo cross-linked PEGDA hydrogel disk was still observed on day 6, whereas the PEGDA-PEI hydrogel disks completely disappeared. (b) Cross-sections of CAMs on which the hydrogels corresponding to the above conditions were implanted. After 6 days, the CAMs were fixed and stained with Hematoxylin and Eosin. Inflammatory cells were recruited to the site of implantation for all 3 conditions but more hyperplasia was observed on the CAM implanted with photo cross-linked PEGDA hydrogel as compared with the PEGDA-PEI hydrogels. The arrows in the photos represent the leukocytes recruited to the sites of implantation. (Scale bar corresponds to 100 µm) (c) Percentages of chicken embryos that survived 6 days after the implantation was significantly increased as the PEI concentration of the PEGDA-PEI hydrogels were decreased from 15 % to 10 %.
7.6 *In vivo* drug release assay of PEGDA-PEI hydrogels

In addition, the BSA-encapsulated PEGDA-PEI hydrogel was subcutaneously injected into mouse models to examine the role of the hydrogels in the displacement of BSA from the injection site *in vivo*. Unlike the *in vitro* studies, the initial elastic moduli of the hydrogels should also influence BSA’s displacement along with the degradation rate, because of the tissue pressures exerted on the hydrogels. In this study, hydrogels used consisted of 12.5 % PEGDA and 10 % PEI. These hydrogels had elastic moduli of approximately 4 MPa and degraded in less than a day in PBS solution. The fluorescent BSA administered via bolus injection dispersed rapidly, as confirmed with the rapid decrease of fluorescence at the injection site within three days ([Fig. 7.10](#)). In contrast, injection of the BSA-encapsulated PEGDA-PEI hydrogels slowed the spread of the BSA at the injection site as shown by the slower decrease of fluorescence over six days. Subsequently, the PEGDA-PEI hydrogels resulted in a smaller rate of decrease in circulating BSA, demonstrated by the slower change of fluorescence in the mice’s ears than bolus injection ([Fig. 7.11](#)). Coupled with the *in vitro* BSA release study, this result clearly demonstrated that the PEGDA-PEI hydrogel enabled the systemic and sustained delivery of BSA. The slower release of fluorescent BSA in the *in vivo* experiment could be attributed to the lower amounts of water present in the fat tissues.
Figure 7.10 In vivo release analysis of PEGDA-PEI hydrogels. (i) Bolus injection of the fluorescent BSA resulted in the rapid decrease of fluorescence at the injection site. (ii) In contrast, injection of the BSA-encapsulated hydrogel consisting of 12.5 % PEGDA and 10 % PEI led to the sustained fluorescence at the injection site over six days.

Figure 7.11 In vivo distribution of fluorescent BSA from PEGDA-PEI hydrogels. (a) Accumulation of fluorescently labeled BSA in the ears of mice administered with (i) bolus injection of fluorescent BSA and (ii) injection of BSA-encapsulated hydrogel formed from 12.5 % PEGDA and 10 % PEI. These images were captured at various time points over six days. (b) Normalized fluorescence intensities of BSA ($I/I_0$) in ears of mice administered with the bolus injection of the fluorescent BSA (■) and the injection of the fluorescent BSA-encapsulated hydrogel formed from 10 % PEI and 12.5 % PEGDA (●). The fluorescence intensities peaked one day after the injections and declined much faster with the bolus condition.
7.7 Stem cell mobilization with PEGDA-PEI hydrogels

Lastly, GCSF was encapsulated into hydrogels consisting of 20 % PEGDA and 3.5 % PEI, which presented an initial elastic modulus of 3 MPa and released BSA more slowly than the hydrogel containing 10 % PEI. As the GCSF with an isoelectric point between 5.8 and 6.6, would minimally interact with amine groups of PEI in a neutral condition, there would be insignificant decrease in the degradation rate of the hydrogel. The GCSF-encapsulated hydrogel was injected into the shoulder muscle of porcine test animals, where the load-bearing capacity of the hydrogels would also influence the release profiles of the GCSF along with the degradation rate. The mixtures of PEGDA and PEI formed a stiff hydrogel right after injection of the pre-gel solution, similar to the gel formed in vitro (Fig. 7.12). Injection of the GCSF-encapsulated hydrogels elevated the number of total number mononuclear cells in circulation one day after administration, and the number of cells was significantly greater than the injection of blank hydrogels without drug (Fig. 7.13). The elevated mononuclear cell number was retained for 48 hours followed by a gradual decrease over another 48 hours. In contrast, bolus injection of the same dose of GCSF elevated the cell number one day after injection, with drastic decrease thereafter. This rapid decrease could be attributed to the short half-life of GCSF which was reported to range from two to four hours.[155] The positive control which comprised of repeated daily injections of GCSF for four days maintained an elevated mononuclear cell number over the same time period with a rapid decrease after the final day of injection.

The mononuclear cells collected on Day 4 were further cultured in endothelial growth medium to isolate and expand endothelial progenitor cells (EPCs) which were
important in ischemia therapies and for treatment of myocardial infarction (Fig. 7.14). Interestingly, *in vitro* expansion of mononuclear cells mobilized with GCSF-encapsulating hydrogels produced a larger number of cells expressing CD34 and CD31 antigens, markers of EPCs, as compared with mononuclear cells mobilized via single or repeated bolus injections of GCSF.
Figure 7.12 Bolus injection of PEGDA-PEI hydrogel. The hydrogel formed was stained blue for better visualization. This demonstrated the localization of the encapsulated substance at the injection site. (Scale bar represents 1 cm)

Figure 7.13 Stem cell mobilization with GSCF-encapsulated PEGDA-PEI hydrogels. GCSF solutions or GCSF-encapsulated PEGDA-PEI hydrogels were injected intramuscularly into pigs, and the number of mononuclear cells in the peripheral blood was monitored for four days. The graph represents the number of mononuclear cells over baseline during the course of the experiment. The conditions tested were single injection of blank hydrogel (●), single bolus injection of 1.2 mg of GCSF dissolved in PBS (●), single injection of 1.2 mg of GCSF encapsulated within the hydrogel (★) and four daily bolus injections of 0.3mg of GCSF dissolved in PBS (●). Initial injections were given on day 0 and daily injections were continued to day 3. Injection of GCSF significantly increased the number of cells mobilized into circulation, but the time periods with sustained elevated cell number were mediated by the method of administering GCSF.
Figure 7.14 Mobilization cell population with GCSF-encapsulated PEGDA-PEI hydrogels. Circulating mononuclear cells were collected four days after initial injections and expanded in endothelial growth medium in vitro over three passages. Cells expressing CD31, CD34, or both were identified using flow cytometry. The conditions were (i) daily bolus injections of 0.3 mg of GCSF for four days, (ii) single injection of blank hydrogel, and (iii) single injection of 1.2 mg of GCSF encapsulated within the hydrogel. The condition with administration of GCSF-encapsulating hydrogel resulted in the largest fraction of culture-expanded cells expressing CD31 and CD34 as compared with other conditions.

7.8 Discussion on PEGDA-PEI hydrogels with tunable mechanical and drug release properties

In this chapter, we developed PEGDA-PEI hydrogel through the Michael reaction between the primary and secondary amine groups in the hyper-branched PEI and the acrylate groups in the PEGDA. The PEGDA-PEI hydrogel presented initial stiffness high enough to maintain its structural integrity, so as to overcome local tissue pressures and to sequester the GCSF at the injected site. In addition, the hydrogel was designed to degrade in a controlled manner, independent of its initial stiffness for sustained release of the encapsulated GCSF. These properties were distinct from conventional hydrogel systems which were often plagued by limited controllability of stiffness and an inverse dependency of degradation rate on the initial rigidity. Previously, certain efforts were made to develop structurally rigid hydrogel systems. These include double network hydrogels, topological gels, nanocomposite gels and cryogels, all of which designed to exhibit elastic moduli comparable to hard cartilage tissues.[156-160] However, these
hydrogels typically had limited degradation rates which precluded their use as drug-releasing carriers. The ease of degradability of the PEGDA-PEI hydrogel was attributed to the large number of amines on the PEI. Amines which did not take part in the Michael reaction were protonated and drove the non-equilibrium swelling of the hydrogel. By varying the mass fraction of PEGDA and PEI, the stiffness and degradation rate of the PEGDA-PEI hydrogel could be tuned independently.

Although higher concentrations of PEI resulted in cytotoxicity, PEI concentrations less than 15% presented limited cytotoxicity and immunogenicity. The stem cell mobilization experiment also demonstrated that the stiff and metastable PEGDA-PEI hydrogels improved the efficacy of GCSF for mobilizing stem and progenitor cells. GCSF released stem and progenitor cells residing in the bone marrow by limiting the expression of chemokines involved in cell adhesion (e.g. stromal-cell derived factor 1) and also by stimulating the monocytes’ expression of matrix metalloproteinase.[155] Because the GCSF administered into circulation had a half-life of two to four hours, the hydrogels could have a stabilizing effect on the GCSF, thus allowing for sustained mobilization as compared with the single bolus injection of GCSF. Furthermore, the largest yield of CD34+ and CD31+ EPCs obtained from mononuclear cells mobilized with the GCSF-encapsulating hydrogel implied that sustained GCSF delivery promoted stem and progenitor cell mobilization while minimally stimulating inflammatory cells. Conversely, bolus administration of GCSF significantly increased the number of inflammatory cells including neutrophils, monocytes, B cell, and dendritic cells in circulation.[161] However, the underlying
mechanism for the enhanced efficiency of stem and progenitor cell mobilization needs to be studied more thoroughly in future studies.

Although the PEGDA-PEI hydrogel had only been tested with BSA and GCSF, it has the potential to be combined with other drugs and cytokines due to its customizability. The PEGDA-PEI hydrogel could also be readily combined with the cell-instructive scaffolds to provide local regulation of both soluble and microenvironmental cues.

7.9 Conclusion on PEGDA-PEI hydrogels with tunable mechanical and drug release properties

Overall, the results of this study demonstrated that the stiff and metastable PEGDA-PEI hydrogels allowed the decoupled control of degradation rates and stiffness. This hydrogel system was successfully used as an injectable drug delivery system enabling sustained mobilization of stem and progenitor cells into circulation. The unique stiffness of the hydrogel was attained from the highly branched architecture of PEI and the decoupled controllability of degradation rate was achieved by tuning the number of protonated amine groups of the hydrogel. We therefore expect that the degradation rates of the hydrogels developed in this study may be further controlled over a broader range through chemical modification of PEI in the hydrogel so as to suit different delivery applications. Due to its injectability and ease of application, this hydrogel could also be readily combined with cell-instructive scaffolds to generate integrated tissue engineering systems.
8 Conclusions and future prospects

This chapter provides an overview of this work. In particular, the accomplishments in the development of cell-instructive microenvironmental scaffold cues and soluble factor cues will be covered. In addition, further studies contributing to the development of integrated tissue engineering approaches will also be proposed.
8.1 Development of 3D cell-instructive microenvironmental scaffold cues

In this work, we modified the microenvironmental scaffold cues of collagen-based scaffolds through various approaches. This approaches included (i) covalent cross-linking of collagen scaffold to increase bulk stiffness (chapter 4), (ii) MMP-1 degradation of covalently cross-linked collagen scaffold to decrease bulk stiffness (chapter 5), and (iii) regulation of collagen fiber stiffness and rigidity through control of thermodynamic driving force during collagen self-assembly (chapter 6). Following the various modifications, we further characterized the modified scaffolds in terms of the scaffolds’ mechanical properties, permeability, cytotoxicity and microstructures. Through these characterizations, we were able to ensure that the ranges of stiffness achieved through the various modification methods were physiologically relevant. We also checked that modified scaffolds possessed good permeability and were non-toxic to encapsulated cells.

With the covalently modified scaffolds which were further degradable by MMP-1, we successfully controlled the malignancy of encapsulated HCC, characterized by significant differences in the cells’ morphology, liver-specific activities and in vivo angiogenic capacity. The collagen scaffolds with varied fiber thickness and rigidity also allowed the regulation of fibroblast activation state, characterized by the differences in fibroblast morphology, actin levels and contractile activity of the cells.

While the effect of stiffness on cell phenotypes had been demonstrated in several previous studies, majority of the earlier studies were carried out in the 2D culture configuration.[6, 38, 46] As it had become apparent that cell-adhesion and signaling were significantly modified by the culture format, there is now general agreement that the 2D configuration might not be representative of in vivo conditions.[7] Although
emerging efforts in 3D control of stiffness had been described in several other studies, many of these works did not include such extensive characterizations of scaffold properties as presented in this work. These characterizations were essential to ensure that the scaffold modifications did not result in the unintended alteration of cell viabilities and cellular activities.

Furthermore, in the earlier studies, there was also no distinction between bulk and local stiffness when discussing scaffold material properties. Through our fabrication of scaffolds with different microstructures, we also demonstrated that apart from the overall stiffness of 3D scaffolds, the microstructures and local stiffness of the scaffolds played very important roles in regulating cell activities. This could be explained by the varied engagement of integrin with changes in gel microstructures. Hence, it is essential to consider these key factors when discussing 3D cell culture scaffolds in the future.

Overall, the different approaches for scaffold modification enabled us to generate distinct cell-instructive cues to bring about diverse phenotypic changes in various cell types. Although we only examined collagen-based scaffolds in this study, the approaches could also be readily adapted to other self-assembling materials such as fibrin. Such cell-instructive scaffolds will be highly useful as models to understand the emergent behavior of stem cells, cancer cells and other cell types. Owing to the promoting properties of the collagen-based scaffolds, they will also be very useful as cell-instructive and cell-supporting scaffolds when implanted or injected in injured tissues or organs.
8.2 Design of novel delivery vehicles to regulate soluble cell-instructive cues

For the second part of this work, we fabricated stiff and metastable PEGDA-PEI hydrogels for the release of cytokines and drugs \textit{in vivo}. The high stiffness of the material, attained from the highly branched architecture of PEI, allowed the hydrogel to release encapsulated substances independent of local tissue pressures. The decoupled control of degradation rate was achieved by tuning the number of protonated amine groups of the hydrogel.

Following synthesis, the hydrogel was extensively characterized in terms of its mechanical properties, degradation, cytotoxicity, \textit{in vitro} and \textit{in vivo} drug release. This hydrogel system was also successfully used as an injectable GCSF delivery system, enabling sustained mobilization of stem and progenitor cells into circulation. We expect that the degradation rates of the hydrogels developed in this study may be further controlled over a broader range by altering the precursor mass fractions and chemical modification of PEI. These modifications can be made to suit different delivery needs and applications. Due to its injectability and ease of application, we believe this hydrogel can also be readily combined with cell-instructive scaffolds to generate integrated tissue engineering systems.

8.3 Future developments

Having developed the cell-instructive scaffolds, the next phase of this study can be to modify these scaffolds to provide cell-instruction to other cell types. This should be achievable by tuning different parameters such as cross-linking density and fiber diameter to match the target tissues. The drug delivery vehicle formed from PEGDA and
PEI can also be modified to generate a variety of release windows for the release of other cytokines.

In addition, the integration of both scaffold and drug delivery vehicles for integrated tissue engineering approaches can also be explored. Due to the flexibility of the system, many possible applications can be devised. For example, the encapsulation and delivery of endothelial cells may be coupled with the provision of pro-angiogenic growth factors such as VEGF and platelet-derived growth factor to improve the vascularization at an ischemic area. Mesenchymal stem cells in suitable 3D cell-instructive scaffolds can also be provided with various differentiation factors to promote the differentiation of the mesenchymal stem cells *in situ*. Such a strategy will be useful for the endogenous repair of damaged tissues and the restoration of lost tissue function. As this study only provided a preliminary examination of how scaffold and delivery vehicles can be customized to provide cell-instruction, there needs to be further investigations to optimize scaffold properties and delivery windows for each intended application.
Bibliography


[22] University S. Chapter 4 Extracellular matrix, Cell junction and cell adhension.


